Incentive motivation and ultrasonic vocalizations in rats

Ultraschallvokalisation und Anreizmotivation bei Ratten

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Lic. Juan Carlos Brenes Sáenz

aus Costa Rica

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SUMMARY

After experiencing a reward, the positive affective reactions it induces can become associated with its sensory properties and related cues. However, the manner in which such affective reward representations are expressed in animals remains unclear. Juvenile and adult rats communicate through ultrasonic vocalizations (USVs), which also serve as situation-dependent affective signals. Since rats emit high frequency (i.e., 50-kHz) USVs in socially and non-socially rewarding situations, 50-kHz calls might prove to be a way incentive motivational state is signaled when training rats to anticipate food rewards under some predictable cues. In general, the results show that reward-cues become effective to elicit 50-kHz calls. Under certain conditions, however, the utterance of 50-kHz calls can be either suppressed during a highly motivational state, or more strikingly, can be elicited when food rewards were devalued by satiation. For rats, both a state of hunger and waiting for access to a daily meal can be negatively perceived if the food reward offered turns out to be less satisfying than expected. Learning to anticipate such a negative state seemed to suppress the otherwise positive affective reactions evoked by having access to a highly expected food. Such a frustration-like effect occurred only at the USVs level without being indicated behaviourally through changes in rats' learning and motivation to approach and consume the reward. In contrast, providing continued access to the reward prevented the suppression of USVs. Surprisingly, in spite of being sated and no longer interested in seeking and consuming the reward, rats nevertheless continued to emit appetitive USVs in the presence of cues predicting a previously desired food. Rats as a whole, just as with humans, seem to represent rewards affectively beyond basal appetite requirements. However, the ability to attribute incentive salience to reward cues has been shown to strongly differ among

individuals. The second study, therefore, focused on the analysis of individual differences in conditioned anticipatory activity elicited by reward-related cues as indicative of the predisposition of animals to attribute incentive salience to otherwise neutral stimuli. Across several experiments, individual rats prone to attribute incentive salience to reward cues –as indicated by high levels of either rearing activity, or sign-tracking behavior– showed heightened reward-induced affective responses, namely in the form of 50-kHz calls. When re-exposing rats to reward cues after a non-testing period, USVs were elicited even at higher rates than previously, especially in subjects prone to attributing incentive salience to reward cues. USVs appeared reliably expressed over time and persisted despite physiological needs have already been fulfilled. Interestingly, USVs were still elicited by reward cues even though reward-oriented behaviors and exploratory activity were drastically weakened by reward devaluation. Additionally, prone subjects seemed to undergo particular adaptations in their dopaminergic system related to incentive learning, as indicated by the attenuated response to the catecholamine agonist amphetamine and to the dopamine receptor antagonist flupenthixol. The investigation of the psychological and neurobiological factors underlying affective states as related to incentive motivation is of remarkable relevance in preclinical- and clinical-oriented research. The current findings may have translational potential, since for some individuals, excessive attribution of incentive salience to reward cues may lead to compulsive behavior disorders, such as overeating, pathological gambling, and drug addiction. Certain aspects of these disabling human conditions can be further investigated with the same animal models as implemented in the present studies.

ZUSAMMENFASSUNG

Bei Erfahrung einer Belohnung können die durch die Belohnung induzierten affektiven Reaktionen assoziiert werden mit den sensorischen Eigenschaften der Belohnung und dazu gehörigen Hinweisreizen. Jedoch ist die Art und Weise, in der solche affektiven Belohnungsrepräsentationen bei Tieren zum Ausdruck kommen, bislang weitgehend ungeklärt. Heranwachsende und ausgewachsene Ratten kommunizieren mithilfe von Ultraschallvokalisationen (USV), die auch als situationsabhängige affektive Signale dienen. Da Ratten hochfrequente USV (d.h. 50-kHz) in sozialen und nicht-sozialen Belohnungssituationen aussenden, könnten sich 50-kHz Rufe als Indikatoren für den anreiz-motivationalen Zustand von Ratten dienen, die darauf trainiert wurden, Nahrungsbelohnungen unter bestimmten Hinweisreizen zu antizipieren. Generell zeigen die vorliegenden Ergebnisse, dass Belohnungsreize wirksam werden, um 50-kHz Rufe auszulösen. Jedoch kann unter bestimmten Bedingungen die Aussendung von 50-kHz Rufen entweder durch einen hohen motivationalen Status unterdrückt werden, oder, noch bemerkenswerter, ausgelöst werden, obwohl Nahrungsbelohnungen durch einen Sättigungszustand abgewertet wurden. Ratten erleben eventuell sowohl einen Hungerzustand als auch das Warten auf eine tägliche Mahlzeit als negativ, falls sich die dargebotene Nahrungsbelohnung als weniger befriedigend als erwartet erweist. Das Erlernen der Antizipation solch eines negativen Zustandes schien die positiven affektiven Reaktionen zu unterdrücken, die ansonsten durch den Zugang zu erwartetem Futter ausgelöst worden wären. Solch ein frustrationsähnlicher Effekt trat nur auf der USV-Ebene auf, aber nicht auf der behavioralen, wie in Veränderungen im Lernen oder der Motivation, sich der Belohnung anzunähern und diese zu konsumieren. Im Gegensatz dazu verhinderte der

kontinuierliche Belohnungszugang die Unterdrückung von USV. Überraschenderweise äußerten die Ratten, trotz des Zustandes der Sättigung und fehlender Motivation die Belohnung aufzusuchen und zu konsumieren, weiterhin appetitive USV wenn Hinweisreize präsentiert wurden, die ein bislang begehrtes Futter vorhersagten. Ratten scheinen insgesamt, ähnlich wie Menschen, Belohnungen affektiv und über basale Nahrungsbedürfnisse hinaus zu repräsentieren. Allerdings ist auch bekannt, dass sich die Fähigkeit, Anreize auf Belohnungs-Hinweisreize zu attribuieren, interindividuell stark unterscheidet. Die zweite Studie befasste sich daher mit der Analyse von individuellen Unterschieden in konditionierter antizipatorischer Aktivität, die durch belohnungsassoziierte Hinweisreize ausgelöst wurde, quasi als Indiz für die ihre Prädisposition, motivationale Anreize auf eigentlich neutrale Stimuli zu attribuieren. In mehreren Experimenten zeigten diejenigen Ratten, die dazu neigten, Anreize auf Belohnungs-Hinweisreize zu attribuieren - angezeigt durch ein hohes Niveau an entweder Aufrichte- oder *sign-tracking*-Verhalten – auch erhöhte belohnungsinduzierte affektive Reaktionen, d.h. 50-kHz Rufe. Wenn Ratten nach einer testfreien Phase nochmals Belohnungs-Hinweisreizen ausgesetzt wurden, löste dies sogar noch mehr USV als zuvor, vor allem bei denjenigen Versuchstieren, die dazu neigten, Anreize auf Belohnungs-Hinweisreize zu attribuieren. Ultraschallrufe traten reliabel über die Zeit hinweg auf und auch trotz erfüllter physiologischer Bedürfnisse. Interessanterweise wurden USV auch dann noch ausgelöst, wenn belohnungsorientierte Verhaltensweisen und exploratorische Aktivität durch Belohnungsabwertung drastisch abgeschwächt wurden. Bei derartigen Individuen scheint es, assoziiert mit dem Anreizlernen, zu Adaptionen im dopaminergem System zu kommen, was durch eine abgeschwächte Verhaltensantwort auf den Katecholamin-Agonisten Amphetamin und den Dopaminrezeptor-Antagonisten Flupenthixol angezeigt wurde. Die Untersuchung der psychologischen und neurobiologischen Faktoren, die affektiven Zuständen, wie bei der Anreizmotivation, unterliegen, ist relevant für präklinische und klinisch-orientierte Forschung. Die vorliegenden Befunde könnten translationales Potential besitzen, da individuell die exzessive Attribution von Anreizen auf Belohnungs-Hinweisreize zu zwanghaften Verhaltensstörungen, wie beispielsweise übermäßigem Essen, Spielsucht, und Drogenabhängigkeit, führen könnte. Bestimmte Aspekte dieser menschlichen Störungen könnten mit Tiermodellen, wie sie in den vorliegenden Studien implementiert wurden, weitergehend untersucht werden.

1. INTRODUCTION

1.1. Sounds as communicational signals

Mammals emit different sounds to communicate information regarding social status and in anticipation of, during, and following intra- and interspecific social interactions, which are context and age dependent (Nyby & Whitney, 1978; Portfors, 2007). Increasing attention is currently being focused on the production and function of ultrasonic vocalizations (USVs) in rodents. Virtually all myomorph rodent species thus far examined both produce and hear sounds above 20-kHz, which is the upper limit of human sensitivity (Nyby & Whitney, 1978; Newman, 2010). USVs are produced by both infant and adult rodents and it appears likely that such USVs play an important role in intraspecific communication. In fact, the importance of ultrasonic communication in rodents has been established or implicated in such interrelated aspects of rodent sociality as parent-offspring interactions, aggression, courtship and mating behavior, territoriality, alarm behavior, social play, as well as social interaction and cooperation (Blanchard et al. 1991, 1992; Burgdorf et al. 2008; Knutson et al. 1998, 2002; Łopuch & Popik, 2011; Nyby & Whitney, 1978; Portfors, 2007; Sewell, 1970; White & Barfield, 1989). The analysis of USVs also has different practical applications in the area of laboratory animal science and veterinary medicine, such as in the monitoring of laboratory rodents' welfare (Burman et al. 2007; Portfors, 2007). Additionally, the relevance of analyzing USVs has been widely extended to preclinical research. Considering the affective nature of USVs, new approaches have been developed to model affective and motivational impairments, such as those observed in depression (Kroes et al. 2007; Mällo et al. 2007), anxiety (Bassi et al. 2007; Brenes et al. 2012), drug abuse (Meyer et al. 2012) and manic-related disorders (Pereira et al. 2014).

Aside from their affective character, USVs have a prominent communicative function (Brudzynski, 2005; Wöhr et al. 2008), which has motivated the use of USVs to model speech/voice impairment in Parkinson disease (Ciucci et al. 2007, 2009), or social communicational deficits in autism (Scattoni et al. 2009; Wöhr et al. 2011). In general, there is increasing evidence indicating that USVs provide unique information about affective and communicational states in rodents that might otherwise not be accessible through conventional behavioral approaches.

Various USVs categories exist with distinct acoustic features; some of them are present in both mice and rats, whereas others are solely emitted by one species (Wöhr & Schwarting, 2013). As the research studies further presented here dealt exclusively with 50-kHz calls in adult rats, descriptions of pup and 22-kHz calls, or USVs in other rodent species will be kept to a minimum.

1.2. Biomechanics of rat USVs production

USVs are produced physiologically by means of the larynx in which the constricted vocal folds are used as an ultrasonic whistling mechanism (Johnson et al. 2010; Riede, 2011). This larynx whistle is a well-regulated process dependent on a centrally-controlled activity of cricothyroid and thyroarytenoid laryngeal muscles (Johnson et al. 2010; Riede, 2011). The activity of these muscles is tonic in USVs with slow or no fundamental frequency changes (e.g. 22-kHz calls or flat 50 kHz calls), whereas during the production of frequency modulated (FM) 50-kHz calls, the muscular activity changes to high amplitude bursts (Johnson et al. 2010; Riede, 2011). In all vocal vertebrates, especially mammals and birds, the activity of these laryngeal muscles is regulated by central pattern generators for vocalization that are located in limbic midline cortex, the caudal hindbrain, and rostral spinal cord (Bass & Chagnaud, 2012;

Kelley & Bass, 2010; Newman, 2010). Different components of this system regulate both the wide functional types of vocalizations and the subtle fine-tuning variations according to the distinct motivational, social, and developmental status of the vocalizer (Newman, 2010). Utterance of social USVs is, evolutionarily speaking, an old activity of extraordinary biological relevance that has been shown to have developed over a long phylogenetic history in vertebrates (Brudzynski, 2013). In spite of the species-specific differences in calling and the wide acoustic repertoire animals possess, the brain areas and neural mechanisms controlling vocalizations are greatly conserved among species and over the course of evolution (Kelley & Bass, 2010; Newman, 2010).

1.3. Adult USVs subtypes in rats

With reference to sound frequency, call durations, and frequency modulation, different classes of USVs can be differentiated, classes that are highly dependent on the animal´s developmental stage as well as the appetitive or aversive salience of the situation (Knutson et al. 2002). Adolescent and adult rats emit two classes of USVs: 50-kHz USVs that reflect high levels of behavioral arousal and positively valence appetitive motivation, and 22-kHz USVs that reflect low levels of behavioral activation and the presence of an aversive motivational state (Brudzynski, 2007; Knutson et al. 2002; Wöhr & Schwarting, 2013). USVs convey information about the current affective state of the subject that is implicitly and eventually communicated when conspecifics receive such signals (Sewell, 1970; White & Barfield, 1989; Wöhr & Schwarting, 2007). For example, listening to conspecifics calls activates nearly the same brain regions as those involved in the production of such signals, but in this case, in the brains of the receivers (Beckett et al. 1997; Parsana et al. 2012; Sadananda et al. 2008; Wöhr & Schwarting, 2010). This suggests that there is a significant overlap between call production and the decoding of USVs messages. It is likely, therefore, that when listening to USVs, receivers are "experiencing" the message while it is decoded. The fact that USVs have evolved in many social situations clearly supports the prominent communicative function of such signals (Brudzynski, 2013). There is a wide body of evidence showing how the behavior of conspecifics (i.e., receivers) is readily affected by listening to the USVs message (Nobre & Brandão, 2004; Sadananda et al. 2008; Sewell, 1970; White & Barfield, 1989; Wöhr & Schwarting, 2007). The ability of USVs to affect behavior, however, varies according to the type of USVs presented to the animals (Brudzynski & Chiu, 1995; Sadananda et al. 2008; Wöhr & Schwarting, 2007). For example, the behavioral response to aversive calls (i.e., 22-kHz USVs) seems to be acquired through associative learning, which is facilitated by a biological predisposition to associate aversive events with such a 22-kHz peak frequency (for a detailed discussion see Wöhr & Schwarting, 2013); in contrast to the facilitator role of 50-kHz calls in mating behavior that appears to be unlearned (White & Barfield, 1987, 1989, 1990).

1.3.1. 22-kHz calls: Twenty-two kHz calls are considered to be part of the animal's defensive repertoire and appear in a number of socially and non-socially aversive situations in juvenile and adult rats (Brudzynski & Holland, 2005). Socially, 22-kHz calls are observed during confrontation with predators (Blanchard & Blanchard, 1989; Blanchard et al. 1991, 1992), submissive behavior during inter-male fighting (Kaltwasser, 1990), and in the refractory period of male rats after copulation (Barfield & Geyer, 1972). Aversive calls in such contexts have been thought to signal intention of cessation and/or withdrawal from ongoing social activities in dyadic interactions (Brudzynski, 2013; van der Poel & Miczek, 1991). In regards to the latter, playback studies have shown that natural 22-kHz USVs or 20 kHz sine wave tones are able to activate the defensive repertoire in the rat (i.e., fight-flight-freezing behaviors), but the extent of such activation is weak (for a detailed discussion see Wöhr & Schwarting, 2013). Non-socially aversive stimuli that elicit 22-kHz calls include: startling noises (Kaltwasser, 1991), handling and touch (Brudzynski & Ociepa, 1992; Brudzynski et al. 1993), air puffs (Knapp & Pohorecky, 1995), electric shocks (De Vry et al. 1993; Jelen et al. 2003; van der Poel & Miczek, 1991), social isolation (Francis, 1977), and withdrawal from drugs, such as alcohol, benzodiazepines, opiates and psychostimulants (Covington & Miczek, 2003; Vivian et al. 1994).

Since 22-kHz calls have also been elicited in contexts where rats do not interact directly with each other, it has been proposed that 22-kHz USVs occur as alarm calls, directed to members of the colony and other conspecifics (Blanchard et al. 1991, 1992). The alarm calls, under this proposition, would inform the colony about approaching or potential danger (e.g. a predator). The rat receivers in the colony need not be in close proximity to the caller but should at least be found within hearing range. This finding has been shown particularly evident during the presentation of a predator (cat) in an open surface of the visible burrow system (Blanchard & Blanchard, 1989). Exposure of a cat to individually kept rats elicited no calls, while a similar presentation of the predator was effective in inducing 22-kHz calls in an established colony, where other familiar conspecifics were present in the vicinity (Blanchard et al. 1991). One important aspect to note about this finding is that 22kHz calls are not only displayed during the actual aversive event (unconditioned stimulus, US), but may also occur in response to stimuli associated with that experiences such as the context, or specific conditioned stimuli (CS), as occurs in the fear conditioning paradigm. This paradigm is, by far, one of the easiest and most reliable methods of inducing 22-kHz calls (Antoniadis & McDonald, 1999; Borta et al. 2006; Cuomo et al. 1988; De Vry et al. 1993; Wöhr et al. 2005; Wöhr & Schwarting, 2008). Evidence coming from fear conditioning studies has confirmed that 22-kHz calls do not indicate pain, but instead represent distress signals. Such negative emotional USVs may convey an alarm message

(Blanchard et al. 1991), but their utterance does not rely upon the presence of other rats in close proximity, at least when induced by foot shocks (Wöhr & Schwarting, 2008).

1.3.2. 50-kHz calls: Juvenile and adult rats have a complex repertoire of 50-kHz calls that differ in their fundamental peak frequencies (with an average range of 35–90 kHz; Portfors, 2007), and in the contexts where they are usually emitted (for review see Burgdorf et al. 2011; Wöhr & Schwarting, 2013). Such affective and communicative signals are expressed in different social and non-social situations (for review see Knutson et al. 2002). Rats emit 50-kHz calls in anticipation of and/or during naturalistic rewarding situations such as rough-and-tumble play (Burgdorf et al. 2008), tickling (Burgdorf & Panksepp, 2001; Burgdorf et al. 2007; Schwarting et al. 2007) mating (Burgdorf et al. 2008; Thomas et al. 1982; White & Barfield 1990), as well as sexual and social contact (Burgdorf et al. 2008; White & Barfield 1987). Aside from calling before or during social interactions, rats also emit 50-kHz calls when they are transiently separated from cage mates. For instance, rats call when exposed individually to home or novel cages with bedding, to open field arenas, and to the elevated plus maze (EPM) (Brudzynski & Pniak, 2002; McGinnis & Vakulenko, 2003; Natusch & Schwarting, 2010; Schwarting et al. 2007; Wöhr et al. 2008). Additionally, in pharmacological studies, vehicle-injected animals have been shown to emit moderate and stable rates of 50-kHz calls simply by being placed in different testing chambers (Ahrens et al. 2013; Browning et al. 2011; Burgdorf & Panksepp, 2001; Knutson et al. 1999; Ma et al. 2010; Maier et al. 2010; Thompson et al. 2006; Wintink & Brudzynski, 2001; Wright et al. 2010, 2011, 2013). When recording USVs emitted by the rat that remains alone in the home cage, it calls even at higher rates than one transferred to another cage (Wöhr et al. 2008). In addition, USVs recorded consecutively during four days in a cage with bedding show no signs of habituation, suggesting that these USVs are not triggered by novelty alone (Schwarting et al. 2007). Instead, such isolation-induced USVs kHz calls are thought to serve as social signals aimed at (re)establishing positive social encounters (Wöhr & Schwarting, 2007; Wöhr et al. 2008). In support of the latter, there is broad evidence showing that rats emit 50-kHz calls during social ambiguous encounters, after a first social defeat experience, or faced with the threat of an attack (Blanchard et al. 1993; Haney & Miczek, 1994; Tornatzky & Miczek, 1994, 1995; Vivian & Miczek, 1993a, 1993b).

On the other hand, 50-kHz calls can also be triggered by non-naturalistic rewarding stimuli such as electrical stimulation of the mesolimbic dopamine pathways (Burgdorf et al. 2007), and the administration of psychostimulatory drugs (i.e., cocaine and amphetamine), either unconditionally or conditionally (Ahrens et al. 2013; Barker et al. 2010; Browning et al. 2011; Burgdorf et al. 2001; Knutson et al. 1999; Meyer et al. 2012; Mu et al. 2009; Natusch & Schwarting, 2010; Simola et al. 2014; Thompson et al. 2006; Williams & Undieh, 2010). Since 50-kHz calls are emitted in situations reflecting positive affect, and considering that social play and tickling have been shown to induce high rates of these USVs, it has been argued that such 50-kHz calls may constitute an ancient homolog of human laughter (Panksepp, 2005).

It has widely been reported that the production of spontaneous (Brudzynski & Pniak, 2002; Schwarting et al. 2007; Wöhr et al. 2008) and reward-induced USVs is highly dependent on individual differences (Ahrens et al. 2013; Browning et al. 2011; Mällo et al. 2007; Rygula et al. 2012; Wright et al. 2010). The inherently biological background of such inter-individual variability has been demonstrated through the selective breeding of rats for their levels of tickling-induced appetitive 50-kHz calls (Brudzynski et al. 2011; Burgdorf et al. 2005, 2009; Mu et al. 2009; Harmon et al. 2008). High callers of 50-kHz USVs seem to show greater reward sensitivity, as indicated by intra-accumbens and systemic amphetamine-increased calling (Ahrens et al. 2013; Brudzynski et al. 2011), higher sensitization to cocaine-induced 50-kHz calls (Mu et al. 2009), and higher electrical (Burgdorf et al. 2007) and cocaine self-administration rates (Browning et al. 2011).

2. RESEARCH FRAMEWORK

2.1 incentive motivation and 50-kHz calls

Possessing affective representations in terms of pleasures and desires is a fundamental part of humans' subjective experience. Rewards and reward-related stimuli can produce positive affective responses, and they can remind us not only how positively they affected us in the past, but also how good they would be if experienced again. Exposure to reward-related cues may also set a state of readiness for seeking and consuming that reward, even in instances where we have not experienced the reward for some time or when the ostensible physiological needs for the reward have already been fulfilled (Cornell et al. 1989; Nair et al. 2009; Petrovich et al. 2007; Weingarten, 1983). In humans, such mechanisms play a critical role in drug addiction and relapse, overeating in cases of obesity, and in binge disorders (Berridge, 2012; Schachter, 1968; Volkow et al. 2011, 2013). The incentive valence of such reward-related stimuli (like places, odors, sounds, and time periods) is primarily determined by the affective experience resulting from the preceding intake of that reward (Balleine, 2005; Dickinson & Balleine, 2002). Following Pavlovian learning, sensory reward properties and associated cues are transformed into attractive and desired incentives (Berridge, 2001; Bindra, 1978; Bolles, 1975; Toates, 1986; Weingarten, 1983). This motivational component of reward is normally referred to as incentive salience (Berridge, 2001, 2012). In classical and modern incentive motivation theories, the activation of a "central emotive state", "expectations about rewards", and "subjective wanting" have all been proposed as critical factors in the process of attributing incentive salience to reward cues (Berridge, 2001; Bindra, 1978; Bolles, 1975; Crespi, 1942; Dickinson & Balleine, 2002; Hull, 1952; Konorski, 1967; Mowrer, 1960; Rescorla & Solomon, 1967; Panksepp, 1992;

Toates, 1986). In non-human animals, especially rodents, incentive motivation has been extensively investigated using traditional behavioral parameters, such as nose-poking, lever-pressing, and approach behavior to cues and rewards in Pavlovian, instrumental, and Pavlovian-to-instrumental transfer paradigms (for review see Domjan, 2009; Robinson et al. 2014). The study of the emotional or affective conditioned responses underlying incentive motivation, however, has received less attention; this is due firstly, to the fact that the study of emotions was long disregarded in behavioristic tradition (for review see Berridge, 2001), and secondly, to the lack of direct and more precise measures of such states in animals. Currently, there is an increasing interest in studying rodent USVs in basic and clinically-oriented research, since USVs seem to provide a unique avenue toward studying the putative affective states of animals, which might otherwise remain inaccessible through conventional behavioral approaches. Whether 50-kHz calls may be indicative of incentive salience attributed to food cues, however, remains unclear. Previous studies have shown mixed results and have been inconclusive due to the lack of proper controls groups and concomitant behavioral confirmations of incentive learning (Browning et al. 2011; Burgdorf et al. 2000; Coffey et al. 2013; Willey & Spear, 2013). Encouraged by the translational potential of modeling subjective putative affective states in animals, we (from now on I will use the term “we” since this research was a teamwork) decided to perform a series of studies further exploring the hypothesis that 50-kHz calls can come to signal a state of incentive motivation in rats. These appetitive USVs may constitute an emotional reward representation, triggered by CS predicting reward or by some perceptual features of the food itself (unconditioned stimulus, UCS). One of the simplest conceivable tests to achieve this aim was training rats to anticipate their daily feeding, as taking place under certain predictable environmental cues. In experiments 1 to 4 of Study 1, a Pavlovian conditioning task was systematically modified to evaluate the effects of shifting the current physiological state of the subject (deprived vs. sated). The type of learning mechanism recruited (Pavlovian vs. instrumental) was assessed by training rats in a runway maze in experiment 2. Experiments 3 and 4 evaluated the effect of using food rewards (UCS) with different hedonic properties (low vs. high palatable food). Finally, the availability of the food reward (continued vs. discontinued) was manipulated in experiment 5. In all experiments approach and consummatory behaviors, USVs, and anticipatory activity were systematically measured.

2.2 Individual differences in incentive motivation and 50-kHz calls

In Pavlovian experimental preparations, a localizable visual stimulus usually evokes approach and consumption behaviors directed towards the reward cue itself (for review see Robinson et al. 2014), whereas diffuse or non-localizable stimuli such as a tone or a testing context would instead enhance behavioral exploration (Barbano & Cador, 2005, 2006; Bindra, 1978; Holland, 1984; Jenkins & Moore, 1973; Rescorla, 1988). Both types of non-contingent conditioned responses, although quite consistent, are nevertheless moderated by individual differences (Flagel et al. 2007, 2011; Robinson & Flagel, 2009; Robinson et al. 2014; Yager & Robinson, 2010). It has been widely demonstrated that variations in cue-induced conditioned behaviors indicate how animals attribute incentive salience to otherwise neutral stimuli (Bindra, 1978; Flagel et al. 2011; Meyer et al. 2012; Robinson & Flagel, 2009; Robinson et al. 2014). From these conditioned responses, anticipatory activity in the presence of reward-related cues has also traditionally been taken as evidence of incentive motivation (Bindra, 1978; Holland, 1984).

It has been consistently shown that reward-induced USVs exhibit great individual variability (Ahrens et al. 2013; Browning et al. 2011; Mällo et al. 2007; Wright et al. 2010), which may rely upon differences in the way mesolimbic dopaminergic and noradrenergic systems encode information about rewards and their predicting cues (Ahrens et al. 2013; Brudzynski et al. 2011; Burgdorf et al. 2007; Mu et al. 2009). Analysis of individual differences has focused on variations in the utterance of 50-kHz calls, specially using the tickling paradigm (i.e., rats are individually tickled by an experimenter through bouts of gentle, rapid finger movements, specially on their underside; Burgdorf et al. 2005, 2007, 2008; Webber et al. 2012). 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 (Burgdorf et al. 2005, 2007, 2008; Mällo et al. 2007; Rygula et al. 2012; Wöhr et al. 2009; for review see Burgdorf et al. 2011). 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 USVs variability by using screening tests of exploratory activity and unconditioned anxiety (Borta et al. 2006; Natusch & Schwarting, 2010; Schwarting et al. 2007; Wöhr et al. 2008), however not through the use of tests related to learning and motivation. In our Study 2, 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. 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 contextual 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 Plavlovian 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 dopaminergic (and noradrenergic) agonist amphetamine (experiment 6) or with the dopaminergic 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 dopaminergic-related drugs (Cosgrove et al. 2002; Lett et al. 2002; Smith et al. 2008). Secondly, we asked whether the effects of these dopaminergic 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 dopamine activity (Berridge et al. 2012; Flagel et al. 2007, 2011).

3. SUMMARY OF PUBLICATIONS

3.1. STUDY 1

3.1.1. Experiment 1

3.1.1.1. Rationale: The hypothesis that 50-kHz calls can come to signal a state of incentive motivation to food reward was investigated by training deprived rats to anticipate their daily feeding using a simple Pavlovian conditioning task.

3.1.1.2. Methods: Thirty experimentally naïve rats were used. One week before testing, animals were habituated to the experimental conditions and human contact. Before testing, animals were counterbalanced into two groups according to the number of spontaneous calls emitted while exploring a bedded cage. Afterwards, animals were put on a 22.5-h food deprivation (FD) schedule by being given free access to their maintenance diet for 1.5 h per day, starting one week before the appetitive cage test. From day 1 to 7, animals were food deprived (FD); thereafter (days 8–10), they obtained food ad libitum (FAL) in their own home cages. In this experiment the CS signaled the start of each feeding session (1.5 h access to food per day), which began in the ultrasonic lab (~2 min in the testing cage) and ended in the animal room. A reward-unpaired rat (i.e., controls) was tested simultaneously in an adjacent room, where it received the same pairing schedule as the matched reward rat, except that a hopper of chow pellets was never placed upon the cage grid.

3.1.1.3. Results: Reward rats showed typical motivational behavior, i.e. approach and food consumption, which decreased over days. The relative number of calls emitted during tone presentation did yield a higher percentage of tone-related calls in reward rats, which increased over days, indicating that the reward animals did learn the associations. After testing on day 7, animals received food in their home cages in order to devalue the food reward (i.e., on days 8 to 10). Surprisingly, we found that total call number increased in reward animals once they were sated, differing now from controls on all FAL days. Anticipatory conditioned activity (i.e., rearing behavior) increased in reward rats and remained consistently high until the end of the FAL phase. Remarkably, the increases in appetitive 50-kHz calls and rearing occurred even though approach and consummatory behaviors were completely abolished during all FAL days. Thus, the devalued feeding conditions dramatically increased both total call number and tone-induced USVs even after 72 h of experiencing the reward in a low motivational state.

3.1.1.4. Discussion: These data suggest that attribution of incentive salience to reward predictive cues (i.e., cage context and tone CS) may have occurred while animals were deprived, and surprisingly cues were able to trigger conditioned motivational reactions to reward, i.e. USVs, even though it was fully devalued. Since the conditioned response was learned under FD, expression but not acquisition of such a response is what seemed to be suppressed during this phase. Perhaps, approach/consummatory responses taking place in the same testing environment may have overlapped with the preparatory/emotional elements of the UCS producing a sort of inhibition in the expression of the latter.

3.1.2. Experiment 2

3.1.2.1. Rationale: Here, the procedure was modified so that USVs associated with anticipatory and consummatory acts could be measured in different testing compartments. A testing cage with bedding was also used here, but instead of training animals to passively wait for food reward to be delivered (i.e., Pavlovian schedule), they now learned to run down a runway maze connected to the cage, so that they could voluntarily enter it and access their daily food ration available there (i.e., instrumental component). With these modifications, animal´s motivation to attain the reward and cue-induced anticipatory 50-kHz calls in the runway could be assessed independently from consummatory responses in the goal cage. We sought to elucidate, therefore, whether the USVs effects observed in experiment 1 rely upon the type of associative process. The cage was the same as in Experiment 1, so that each animal had its own cage for testing throughout the whole experiment.

3.1.2.2. Methods: The same 30 rats used in Experiment 1 served as subjects. Exactly as there, all animals were put on a 22.5-h schedule of FD with free access to their maintenance diet (1.5 h per day) either immediately after testing (for reward animals in their own testing cages) or at least 3 h later (for controls once they were returned to their group cages). Rats were habituated to the runway maze, and in parallel, we performed reinstatement of tone/food pairing by repeating the cage test procedure of Experiment 1 during seven days. Afterwards, reward animals were trained to run through the runway maze to access food in the cage attached to the end of the runway goal arm. Rats were daily trained for 10 consecutive days 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 during approximately 4 min. Control rats followed the same procedure but food was never given in the cage. As in Experiment 1, animals were food deprived during days 1–7; thereafter (days 8–10), they received FAL in their own home cages.

3.1.2.3. Results: As expected, the latencies to eat declined while eating times increased over days in the reward group. During the FD, no differences in USVs were found either in the runway maze or in the cage attached to it. As in experiment 1, rats received FAL after testing on day 7. Again, the latencies to eat increased and eating times decreased in the reward group and consistent with experiment 1, reward cues associated to the runway maze now elicited high rates of 50-kHz calls. In the cage, calling increased in all rats during FAL without differing between groups.

3.1.2.4. Discussion: Again, no differences in total call number were observed under FD, but increased USVs occurred in reward animals once they became sated. The suppressive effect of FD probably did not depend on the type of learning recruited, the behavioral competition between approach/consummatory and anticipatory affective responses, or the interference of an opposite behavior such as digging since the maze had no bedding. As animals were free to shuttle between maze and cage, and most reward animals revisited the maze between eating bouts, maze cues were not just temporally predicting further access to reward, but also became imbued with incentive salience after animals re-experienced the UCS, facilitating CS representations to be re-updated within and across training days. As a consequence, runway maze cues, but not cage cues, triggered appetitive 50-kHz calls. Again, attribution of incentive salience to food cues seemed to take place during the FD period, whereas the expression of such an appetitive response occurred once animals became sated, that is, when the reward was devalued and when no other appetitive behaviors were emitted. Finally, the analysis of USVs in the cage revealed that satiation on its own increased 50-kHz calls irrespective of being food rewarded or not.

3.1.3. Experiment 3

3.1.3.1. Rationale: Here we asked whether increasing the incentive properties of the reward would enhance motivation for UCS during the FD period. To this end, a high palatable reward (i.e., sweetened condensed milk) was used. Since the reward delivered in the testing cage was different from normal rat chow, access to reward became independent from the daily feeding session. Thus, we expected that it could still be valuable when testing animals under satiation.

3.1.3.2. Methods: Twenty-four experimentally naïve rats were used. All experimental procedures were conducted as in Experiment 1, but now, the CS signaled access to a 30 min-drinking period: ~2 min in the cage and the remaining time in the animal room. The reward group had access to sweet condensed milk, whereas the control group had access to tap water. One week before testing, rats were habituated to sweetened condensed milk. Testing was performed with the former FD/FAL schedule.

3.1.3.3. Results: The latencies to drink diminished slightly once training began, with reward rats being faster than controls. The times spent drinking and daily milk intake were higher in the reward group. The percentage of tone-induced 50-kHz calls was higher in the reward group, but total call number did not differ significantly between groups. When tested FAL, there was a transitory increase in the latency to drink and a transitory reduction in the time spent drinking which fully recovered on the following FAL days. Milk intake, in contrast, declined drastically, almost reaching control levels on the first FAL day, but was higher than controls again thereafter. Similar to Experiments 2 and 3, the attenuation of approach and consummatory behaviors observed when shifting feeding conditions was not paralleled by a reduction in total call number and percentage of cue-induced calls. Instead, total call number was now significantly higher in the reward group, and tone cue-induced calls were also higher but returned towards control levels over days.

3.1.3.4. Discussion: Relative to previous experiments, USVs appeared slightly increased at the end of the FD period, although no overall effect on total call number was detected. However, the percentage of tone-induced calling increased during FD indicating that conditioning strengthened over days. Once again, reward devaluation decreased neither total call number nor tone-induced calling, as it had initially been expected. Instead, total call number increased while cue-induced USVs remained slightly high during FAL days. Regarding reward palatability, the higher incentive properties of UCS (i.e., milk) plus the likely invigorating effect of the CS seemed to maintain latencies to drink and times spent drinking in the cage while the UCS was degraded in agreement. However, the amount of milk intake, most of which was consumed in the animal room without the influence of the CS, appeared to be drastically reduced by satiety.

3.1.4. Experiment 4

3.1.4.1. Rationale: So far, the increased percentage of 50-kHz calls induced by the tone cue indicated that attribution of incentive salience to reward-related stimuli had successfully taken place during FD, even thought it was not clearly translated into an overall elevation of 50-kHz calls. To account for such an inhibition in USVs utterance, we assume that the ability of food CS cues to elicit appetitive 50-kHz calls was possibly suppressed by FD, an effect that occurred independently from learning acquisition. So far, the three preceding experiments showed that restoring FAL feeding conditions after FD increased spontaneous USVs in controls and potentiated total call number and food cues-induced appetitive 50-kHz calls in reward rats. This may suggest that FD itself was able to suppress USVs particularly at the time when animals were expecting the food reward (for evidence supporting this assumption see Study 1, Appendix A). This evidence raised the question of whether the same palatable reward (i.e., milk), now acquired in the absence of FD, would be sufficient to increase appetitive 50-kHz calls.

3.1.4.2. Methods: Twenty experimentally naïve rats were used. The experimental procedure was generally the same as in Experiment 3, with sweetened condensed milk also used as reward.

However, contrary to all previous experiments the acquisition phase (days 1–7) of UCS-CS pairing occurred first in the FAL phase and was followed by the FD phase (days 8–10).

3.1.4.3. Results: As expected, reward rats showed shorter latencies to drink and more time spent drinking than controls. Neither total call number nor the percentage of tone-induced calling differed between groups. Also, exploratory activity appeared unaffected by reward experience. When FD, latencies to drink and times spent drinking remained higher in the reward group compared to controls, whereas the amount of milk consumed increased substantially. Contrary to approach and consummatory behaviors, call rate dropped drastically on the first FD day in both groups. On the following FD days, total call number, and, to a lesser extent rearing behavior, progressively returned to FAL levels only in the reward rats. Similarly, the percentage of tone-induced USVs was significantly higher in the reward.

3.1.4.4. Discussion: This experiment shows first, that a highly palatable food reward was not sufficient to increase either total call number or tone cue-induced calling on its own and, second, that FD was able to suppress calling in both groups. Since the reward did not have a high hedonic value during the acquisition phase (i.e., when tested under FAL conditions), total call number and cue-induced 50-kHz calls recovered and differed from control levels only when the hedonic representation of that reward was updated while in the subsequent state of being hungry. In this as well as in previous experiments the motivational state of being FD seems to be required for reward cues to be imbued with incentive salience, even though it suppressed overall USVs utterance.

3.1.5. Experiment 5

3.1.5.1. Rationale: The likely aversive state provoked by long FD may have accounted for some suppressive effects in calling in our previous experiments. Experiment 3, however, total call number tended to increase while FD, an effect that according to Experiment 4, may not solely be attributed to palatability. Another factor might be critical: By replacing rat chow with milk as a reward we also inevitably altered the predictive association between access to reward in the cage and daily feeding session. If expectations about reward were controlled by the very first access to food or milk (2 min) –and not by the whole period of eating (1.5 h) or drinking (30 min)–, it is very likely that animals learned to anticipate the short access to reward instead of the long one. Thus, when animals encountered the reward a negative discrepancy between the reward expected and the one actually obtained may have been experienced, an effect probably energized by FD. We hypothesized, therefore, that providing continued access to reward in the testing environment would prevent such negative discrepancy to occur, 'releasing' the expression of reward-related appetitive USVs when FD. To test this idea, we adapted the procedure of Experiment 1 in which the higher suppression in calling was observed.

3.1.5.2. Methods: Twenty experimentally naïve rats served as subjects. All procedures were conducted largely as in Experiments 1 and 2: Food pellets served 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. Indeed, during habituation to FD, reward rats had access to the 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. During testing, rats were FD from days 1 to 7. Controls never accessed their daily food ration either in the cage or in the experimental room where testing took place.

3.1.5.3. Results: Animals approached the food and started eating without any noticeable change from the beginning to the end of testing. Contrary to our previous food experiments, reward cues did now increase total call number over FD days. Likewise, the percentage of tone-induced calling was significantly higher in reward rats than in controls. Unlike locomotion, rearing behavior in reward rats showed a progressive increase mirroring, to a lesser extent, the one observed for USVs.

3.1.5.4. Discussion: This experiment shows that providing continuous access to reward in the testing environment enables attribution and expression of incentive salience during the FD period, and contrary to previous experiments, this effect was now noted both on total call number and the percentage of cue-induced 50-kHz calls.

3.1.6. General discussion

In the previous experiments we sought to determine whether rat 50-kHz USVs may signal such a state of incentive motivation to natural, nutritional rewards. In general, we found that after Pavlovian incentive learning, reward-cues became able to elicit 50-kHz calls presumably signaling a state indicative of appetitive incentive motivation in the rat. Attribution and expression of incentive salience, however, do not seem to be a unified process, and were teased apart in two different ways: 1) under a high motivational state (i.e., hunger) the attribution of incentive salience to cues occurred without being expressed at the USVs level; and 2) under a low motivational state (i.e., food satiation), expression of appetitive USVs persisted despite physiological needs being fulfilled. In both cases, putative affective incentive responses were elicited independently from motivation to approach and consume the reward. While in a hungry state, short access to rewards may have led to a discrepancy between the reward expected and the one actually obtained that likely suppressed expression of USVs. When such a discrepancy between reward expectations and outcome was prevented by providing continued access to food, attribution and expression of incentive salience synchronized. Similarly, shifting feeding conditions from deprivation to satiation acted as a ‘releasing’ factor of the putative aversive state induced by both reward discrepancy and food deprivation.

3.2. STUDY 2

3.2.1. Experiments 1–3

3.2.1.1. Rationale: Here, we focused on investigating 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 Study 1, and since it seemed to be contingently and topographically related to the way food rewards were delivered.

3.2.1.2. Methods: Experimental subjects and other procedural details were already described in Study 1. Briefly, in experiment 1 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, 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). Experiment 3 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 control rats 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.

3.2.1.3. Results: In all experiments no differences in locomotor activity, latencies to drink, and times spent drinking were observed between LR and HR rats under FD. Regarding USVs, in experiment 1 LR rats showed less USVs than controls and HR rats, which did not differ from each other. When subsequently tested under FAL conditions, HR rats emitted more calls than the other groups, which did not differ from each. Interestingly, the effect on appetitive 50-kHz calls was detected even though the previous differences in rearing behavior between LR and HR groups vanished out once the salience of the UCS was devalued by FAL. In experiment 2, HR rats now showed more appetitive 50-kHz calls than LR and control rats during FD. Interestingly, in LR rats reward-related cues were ineffective to augment calling over control levels, despite being provided with a high palatable reward. When FAL, differences in calling between LR and HR rats were still observed even though they no longer differed in rearing. In experiment 3, calling was significantly higher in HR rats compared to LR and control groups, which did not differ from each other.

3.2.1.4. Discussion: In experiments 1 and 2 individual differences in conditioned anticipatory activity developed while rewards were still valued (FD) predicted levels of appetitive 50-kHz calls while 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, reward devaluation vanished out the differences in anticipatory rearing activity but not in the conditioned affective responses as indicated by the USVs levels. 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. The same occurred when 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.

3.2.2. Experiment 4

3.2.2.1. Rationale: 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” (Flagel et al. 2007, 2011) (for review see Robinson et al. 2014).

3.2.2.2. Methods: In the current experiment, reward animals were trained to run through the runway maze to access their daily food ration in a cage attached to the end of the goal arm (for details see experiment 2, Study 1). Although in the 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. In GT and controls, in contrast, the number of revisits rather decreased over time. 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).

3.2.2.3. Results: Latencies to eat and times spent eating were about the same in the ST and GT groups. In fact, ST rats entered the cage faster than GT and controls, but they did not engage in eating faster than the GT rats, 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. Contrary to experiments 1 to 3, rearing behavior did not become conditioned in the runway maze and therefore did not differ among groups. Regarding USVs, none of the subgroups differed from controls while FD. 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 differing from GT and controls, which vocalized at similar rates.

3.2.2.4. 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. When food was not provided from above as occurred in the runway maze, reward animals neither developed conditioned rearing, nor showed individual differences in such a parameter. However, certain individuals developed there a sort of somehow counterintuitive, unreinforced behavior towards the contextual 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 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. The latter finding provides evidence for within-subjects stability in attributing incentive salience to reward cues.

3.2.3. Experiment 5

3.2.3.1. Rationale: Here, we asked whether contextual 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.

3.2.3.2. Methods: The same 24 rats used in experiment 2 served as experimental subjects, with sweetened condensed milk used as reward. Before reinstatement animals underwent a 7-days training period on FD, and a 3-days 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. 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. 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.

3.2.3.3. Results: On day 17 reward animals approached the milk bottles as fast as they did on days 7 and 10, and spent as much as the same time drinking as they did before. The amount of milk consumed, however, was lower than that on the last FD day but higher than that on the last FAL day, one week before reinstatement. FD and the re-exposition to testing cues increased appetitive 50-kHz calls both compared to that in control rats and to their own previous FAL and FD levels. The analysis of individual differences in rearing behavior on day 17 revealed that reward cues elicited more 50-kHz calls in HR rats than in LR and control conspecifics, which did not differ from each other. Locomotor activity, rearing, the latencies to approach the milk bottles, and the total amount of milk consumed did not differ between groups.

3.2.3.4. 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 trails, 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 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 predicted rates of 50-kHz calls now when re-exposed to testing cues, in agreement with findings of experiments 1 and 4.

3.2.4. Experiments 6–7:

3.2.4.1. Rationale: Cross-tolerance refers to the expression of a lessened response to a treatment, even though subjects have never experienced it before (for evidence about cross tolerance see Study 2, Appendix B). In the current experiment, animals were challenged with the euphorigenic drug amphetamine. Based on previous studies (see Study 2, Appendix B), we anticipated that previous reward experience lead to lessened responses to the psychostimulatory and affective effects of amphetamine. In order to provide additional evidence of the involvement of the dopaminergic system in food cue-induced appetitive 50-kHz calls, the effects of flupenthixol, an antagonist of dopamine D1/D2 receptors, were also evaluated. Behavioral cross-tolerance between drugs and food reward was expected to be more pronounced in rats with higher levels of anticipatory activity displayed during the acquisition phase.

3.2.4.2. Methods: Before drug administration, animals (control=12, reward=12) had already learned to anticipate the delivery of their daily food ration (1.5 h access) in the testing cage from days 1 to 7. On the following two days, animals were handled and habituated to an injection needle while they continued to being tested. On the third day, all animals were injected with vehicle, which served as a baseline measure. On the next day, d-amphetamine (Sigma St. Louis, MO, USA) was administered ip at a dose of 2.5 mg/kg 10 min before testing, which was conducted exactly as in previous training days. In experiment 7, the same 24 rats used in experiment 2 served as experimental subjects. After the reinstatement on day 17, animals continued to being tested during 2 consecutive days while they were handled and habituated to the injection needle. On the third day, all animals were injected with vehicle (0.9% NaCl) and this measure was used as a baseline. In the subsequent days, animals randomly received either flupenthixol (Sigma St. Louis, MO, USA) or vehicle following a Latin square design in which drug-vehicle days were separated by one drug-free testing day. Flupenthixol was dissolved in vehicle and injected ip at a dose of 0.8mg/kg 30 min before testing.

3.2.4.3. Results: saline levels of locomotion and rearing were about the same between reward and control groups. When given amphetamine, locomotion and rearing increased in both groups. These increases, however, were less pronounced in reward rats. Regarding reward consumption under amphetamine, none of the rats even approached the cage grid where the food was delivered. In saline-treated animals cue-induced 50-kHz calls in reward rats were significantly higher than spontaneous calling in controls. Under amphetamine, calling increased in both groups, and again, previous reward experience attenuated amphetamine effects, now on 50-kHz calls. In addition to total call number, we further analyzed different subtypes of 50-kHz calls. Under saline, such the analysis revealed that both groups emitted more flat than step-calls, and trills, which did not differ from one another. Under amphetamine, the relative amount of FM calls increased in both groups, this increase being less pronounced in reward rats, especially regarding the percentage of trills. 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. Psychomotor hyperactivity was about the same between LR and HR groups. In the case of USVs, HR rats showed descriptively less, but not significant levels of amphetamine-induced 50-kHz calls than LR rats. Regarding call subtypes, we found that the percentage of trills calls was significantly lower in HR rats compared to controls rats.

As expected, the latencies to drink, which were significantly lower in reward rats, were increased after flupenthixol administration there. Flupenthixol also led to an inhibition of locomotion, rearing, and USVs as compared to vehicle. Relative to the saline levels, however, locomotion and rearing appeared equally reduced in both groups, whereas the reduction in total call number was less pronounced in reward rats. On the other hand, the analysis of 50-kHz calls subtypes under flupenthixol revealed the relative amount of flat calls increased in both groups, this increase being slightly more pronounced in controls than in reward rats. In controls, both step-calls and trills were reduced, whereas in reward rats the trill subtype was unaffected by the dopamine antagonist. Finally, the analysis of individual differences revealed that the effect of flupenthixol on the percentage of trills calls was significantly less pronounced in HR rats as compared with LR and control counterparts, which did not differ from each other. This effect was not observed for the total call number.

3.2.4.4. Discussion: The findings from the pharmacological experiments suggested that repeated activation of dopaminergic reward system by Pavlovian incentive learning may have decreased the rewarding impact of amphetamine, and the inhibitory effect of flupenthixol, being this effect greater for USVs than for psychomotor activity. Regarding individual differences, HR rats showed less activation and less inhibition of trills calls induced by amphetamine and flupenthixol, respectively, supporting the notion that animals prone to attribute incentive salience to reward cues undergo particular adaptations in the mesolimbic dopaminergic/noradrenergic system.

3.2.5. General discussion

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 physiological needs being 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 dopaminergic system related with incentive learning as indicated by the effects of dopaminergic 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.

4. DISCUSSION

4.1. Implications for incentive motivation theories

The evidence that rats use USVs for signalling a state of incentive motivation for food may be interesting to areas as diverse as bioacoustics, ethology, and behavioral neuroscience. Particularly, our findings are relevant for the study of the biological basis of learning and motivation. The fact that appetitive USVs could be either suppressed or elicited quite independently of classical learning parameters and current physiological states challenges the traditional view of how rewarding affective states might be represented and expressed in animals, specifically rats. The specific import of these significant contributions regarding the role of USVs will be elaborated upon in what is to follow.

4.1.1 Two-process theory (TPT), reward expectancies, and 50-kHz calls: One of the most important learning theories into which our data can be framed is the two-process theory (TPT) (Rescorla & Solomon, 1967; for a review see, Berridge, 2001). This theory stipulates that during the course of instrumental conditioning, the stimuli (S) –in the presence of which the instrumental response is reinforced– become associated with the response (R) outcome (O) through Pavlovian conditioning, resulting in an S-O association. Such an S-O association activates an emotional state (also called “expectancies”) which motivates the instrumental response (Domjan, 2009; Toates, 1986). In the case of food, the emotional state is assumed to be positive. As a result, when a previously conditioned stimulus (CS) for food (i.e., following Pavlovian conditioning) is presented during performance of instrumental response, the rate of such a response increases. It is assumed that the positive emotional state elicited by the CS for food summates with the appetitive motivation that is involved in acquiring the food (Bindra, 1978; Domjan, 2009). One of our contributions to the TPT has been providing genuine and direct evidence regarding the occurrence of emotional states activated by S-O associations through the analysis of USVs in non-human animals. Our data have been shown to illustrate that the unconditioned internal representations that natural food rewards produce can be transferred to otherwise neutral stimuli, which are then able to elicit appetitive 50-kHz USVs. Intriguingly, our data suggests that such emotional states can be either triggered or suppressed without necessarily being followed by a similar pattern of Pavlovian and instrumental responses. For instance, in experiments where deprived rats had short access to food in the presence of CS+ stimuli (2 min in the testing cage and circa 1.5 h in the animal room), no increases in conditioned 50-kHz calls were observed, although latencies to eat/drink and times spent eating/drinking indicated that learning had strengthened over a period of days. Providing animals with long access to the same rewards while presenting them with the exact same CS+ stimuli (1.5 h in the testing cage) led to a gradual increase in appetitive 50-kHz calls, with latencies to eat/drink and times spent eating/drinking indicating that learning had strengthened over the training period, as in the previous experiments. Therefore, allowing animals to complete their daily feeding in the testing cage ensured that the positive affective effects associated with the food, which may have been experienced only when animals started to become sated, strengthened the associations between the CS+ and the affective components of the UCS. Without analyzing USVs, therefore, we would have concluded that both experimental preparations led to the exact same outcome. This evidence suggests, on the other hand, that the induction of an underlying positive emotional state is critical for incentive motivation reactions to be expressed; this is especially the case when they are directly measured instead of being inferred from changes in instrumental or Pavlovian responses, as traditional approaches have done.

4.1.2. Incentive learning theory (ILT), reward valuation, and 50-kHz calls: In studies based on ILT (Balleine, 2005; Dickinson & Balleine, 2002), an initial phase of instrumental conditioning is followed by a phase in which the reward is devalued by pairing it with aversive proprioceptive stimuli (i.e., lithium chloride) or by satiating the subject so that it no longer has an appetite for that reward (Domjan, 2009). The rate of the instrumental behavior is then measured in the absence of the reward. Only if the subject has had the opportunity to learn what the new incentive value of the reinforcer is will the frequency of its instrumental behavior be reduced (for review see Dickinson & Balleine, 2002). Our data, however, contrasts with this traditional view of reward-related behaviors as being controlled by R-O or CS-US associations based upon current biological drives. In all of our experiments, shifting feeding conditions from food deprivation to food ad libitum allowed rats to update the hedonic representations of food rewards when re-experiencing them in a new state of satiety. As predicted by the ILT, approach and consummatory responses were drastically reduced, but surprisingly, anticipatory appetitive USVs were not. To give an account of these findings we argued that approaching and consuming palatable rewards constitute normal unconditioned feeding behaviors that are expected to be rapidly suppressed or activated according to physiological demands, whereas cue-induced USVs are acquired conditioned responses controlled more by environmental stimuli rather than by the current appetitive state. From this point of view, it seems quite reasonable that cues that had signaled food availability while in a state of need were still able to guide behavior in a state of satiation, since food requirements may fluctuate and change within a matter of hours. Therefore, places where food was consistently available should be well remembered. This differential expression of incentive motivation responses may explain why immediately after satiation, 50-kHz calls appeared detached from food seeking and consumption behaviors.

4.1.3. Incentive salience theory (IST), unlearned physiological fluctuations, and 50-kHz calls: The fact that cues-induced 50-kHz calls appeared suddenly augmented once rats were fed ad libitum might be better understood from the perspective of the incentive salience theory (IST) (Toates, 1986; Berridge 2001, 2012), which stipulates that physiological state changes can produce unlearned fluctuations or even independent reversals in the ability of a previously learned reward cue to trigger motivation. Such fluctuations in cue-triggered motivation can depart from all previously learned values about the associated reward outcome; however, under some physiological states cue-triggered motivation can decouple unexpectedly from previously learned values. For instance, it has been recently shown that a learned cue for unpleasantness can become suddenly desired if the US was made physiologically necessary (Robinson & Berridge, 2013). There, rats learned repulsion toward a Pavlovian lever cue that always predicted an unpleasant saltiness, a sensation produced through oral infusions of 9% NaCl, delivered via implanted cannula. Upon first reencounter with the already learned cue in a novel sodium-depletion state, rats suddenly jumped and gnawed on a now attractive Pavlovian lever cue, despite never having been exposed to the cue while in a state of sodium depletion. Thus, unlearned physiological fluctuations can instantly transform an aversive cue into an appetitive one (Robinson & Berridge, 2013). In our present studies, such fluctuations occurred when deprived animals were fed ad libitum. In this case, incentive attribution to reward-predicting cues may have occurred while animals were food deprived, and in the absence of the suppressing effect induced by food deprivation, incentive salience became suddenly expressed (i.e., USVs and to a lesser extent, rearing behavior), as soon as the appetite physiological state shifted.

4.2. Implications for the research in ultrasonic vocalizations

One of the most important conclusions derived from our studies is that 50-kHz USVs may signal a state of incentive motivation toward natural, nutritional rewards. These data are in agreement with previous findings indicating that different kind of rewards such as rough-and-tumble-play (Burgdorf et al. 2008), tickling (Burgdorf & Panksepp, 2001; Burgdorf et al. 2007; Schwarting et al. 2007) mating (Burgdorf et al. 2008; Thomas et al. 1982; White & Barfield 1990), sexual and social contact (Burgdorf et al. 2008; White & Barfield 1987), electrical brain stimulation (Burgdorf et al. 2007), and psychostimulants (i.e., cocaine and amphetamine) either unconditionally or conditionally elicit 50-kHz calls (Ahrens et al. 2013; Barker et al. 2010; Browning et al. 2011; Burgdorf et al. 2001; Knutson et al. 1999; Meyer et al. 2012; Mu et al. 2009; Natusch & Schwarting, 2010; Simola et al. 2014; Thompson et al. 2006; Williams & Undieh, 2010). From an affective perspective, 50-kHz calls have been thought to occur as a function of rats' anticipation of rewards (Burgdorf et al. 2000; Knutson et al. 1998). Indeed, in some cases, prior emission of USVs in response to presentation of a stimulus predicts approach behavior directed toward that stimulus (Burgdorf & Panksepp, 2001; Burgdorf et al. 2007; Knutson et al. 1998).

4.2.1. Putative biological relevance of food-related 50-kHz calls: As water, sex, and social contact, food rewards are thought to produce hedonic states in order to signal organisms which stimuli are relevant for survival, encouraging learning and memory of all related cues that have been able to predict such states. Production of social USVs is, evolutionarily speaking, an old activity, and it is regulated by well-preserved brain circuitries (for a review see, Brudzynski, 2013). USVs have been detected in several naturalistic contexts such as during mating, rough-and-tumble play, social contact, and social exploration (Burgdorf & Panksepp, 2001; Burgdorf et al. 2008; Schwarting et al. 2007; Thomas et al. 1982). This communication is an adaptation of high biological significance that is understood to have developed over a long phylogenetic history of vertebrates (Brudzynski, 2013). In this regard, USVs might support behaviors aimed at the involvement in socially rewarding situations: signaling the associated affective state to others might serve to strengthen the social contact that is about to occur or that has been initiated. Panksepp et al. (2002) have found that rats spend more time with conspecifics that emit high levels of 50-kHz USVs than with others that produce fewer 50-kHz USVs. Playback studies have also repeatedly shown that 50-kHz USVs elicit social approach behavior in the recipients (Wöhr & Schwarting, 2007, 2012; for review see Wöhr & Schwarting, 2010). Significantly, deafening or devocalizing of rats has been found to disrupt reciprocal social interaction in juveniles (Siviy & Panksepp, 1987), during which high rates of 50-kHz USVs occur under normal conditions (Knutson et al.1998; Webber et al. 2012). Signaling an affective state also announces or advises of a state of receptiveness to engage in social interaction, which ultimately increases the likelihood of a successful social encounter or may eventually reduce the likelihood of intraspecific aggression. The occurrence of this social signaling may be relevant for social competence and fitness as well. For instance, the emission of 50-kHz USVs constitutes a prominent aspect of mating behavior in establishing and maintaining close male-female contact (Sales, 1972; Thomas & Barfield 1985; Thomas et al. 1981, 1982; White & Barfield 1987, 1989, 1990).

On the other hand, and in contrast with social-related stimuli, non-social stimuli such as palatable and nutritional foods appear incapable of inducing spontaneous calling in rats. According to our own experiments, and based on previous reports (Browning et al. 2011; Burgdorf et al. 2000; Coffey et al. 2013; Mateus-Pinheiro et al. 2014; Willey & Spear 2013), it is clear that first encounters with even highly palatable food do not trigger unconditioned USVs.

Certainly, rats normally avoid unknown food even when hungry, an effect referred to as food neophobia (for review see Corey, 1978). In our experiments food rewards were presented days before in the home cages to prevent this effect to occur when testing. When young rats start eating solid foods for the first time, they use adult rats as guides: they forage where the adults were foraging or where adults have previously scent-marked (Galef, 1971; Galef & Clark 1971a, 1971b; Galef & Heiber 1976, Laland & Plotkin 1991, 1993). When rats forage on their own, their food choices are influenced by social interactions: rats strongly prefer foods that other conspecifics have previously eaten after smelling food odor on their fur, whiskers, and especially breath (Galef, 1986; Galef & Beck, 1990; Galef & Wigmore 1983). The latter suggests that sensory, contextual, and situational stimuli related to food are necessary but insufficient for choosing food: those food cues have to be learned in the presence of or directly from other rats. It is in this context that USVs might be biologically relevant: food-related 50-kHz calls emitted by the sender (the food guide rat) would encourage behaviors in the receiver such as foraging, eating, collecting, or storing that particular food. From such a social and ecological perspective, the question of whether food reward-induced USVs might convey pro-social value, thereby promoting food-related behaviors in the listeners (i.e., audience effect), has not yet been fully investigated. It has been found, however, that 50-kHz USVs are emitted while rats cooperate (i.e., simultaneous nose-poking) to receive a sucrose reward (Łopuch & Popik, 2011). In this case, USVs were not only positively associated with nose-poking, but also seemed to be necessary for nose-poking to synchronize between the two rats (Łopuch & Popik, 2011). Such evidence suggests that 50-kHz calls can be used by rats to signal the availability of food. From the perspective of the sender, signaling the presence of food during first food encounters may be socially irrelevant, until rats acquires information about the palatability and nutritional value of that food. With repeated exposure to food and food associated cues, learning mechanisms are recruited, thereby allowing for the transfer of incentive properties to otherwise neutral stimuli. Once a prediction has been established, food rewards and their related cues become capable of inducing calling, likely by acting upon the same reward system that supports social rewards. Therefore, food cue-induced appetitive 50-kHz calls might initially be unintended social signals. Over time, however, such signals would influence food choices and foraging behavior in the listeners (e.g., pups, unexperienced young rats, or adults foraging outside the burrow or confronted with unknown foods). Further research on this topic is warranted.

4.2.2. Reward, incentive motivation, and 50-kHz calls subtypes: USVs are complex signals, which can be classified into different subtypes; often, they are clustered into two general categories: flat and frequency-modulated (FM) calls (Burgdorf et al. 2008; Wright et al. 2010). Of these call types, flat calls may serve a socially communicative function (Schwarting et al. 2007), while FM calls, including the call subtypes step-calls and trills (Wöhr et al. 2008), seem to signal a dopamine/norepinephrine-dependent affective state in the rat (Burgdorf et al. 2008; Wright et al. 2010). Another important issue raised by the results of our experiments is the fact that food-related cues were shown to primarily induce flat calls, rather than the FM calls that would have been expected. In the field of USVs, the categorization of call subtypes, especially those of 50-kHz calls, is in a nascent state (however for a detailed categorization see Wright et al. 2010) Therefore, data within and among labs are sometimes not fully consistent, especially as regards quantification and classification criteria (Brudzynski et al. 2011, 2012; Burgdorf et al. 2008), as well as the interpretation of the functional meaning of call subtypes (Ciucci et al. 2007, 2009; Wright et al. 2010). In some studies where USVs and food rewards were analyzed, call subtypes were occasionally not scored or reported (Browning et al. 2011; Brudzynski et al.

2011; Burgdorf et al. 2000; Coffey et al. 2013; Ma et al. 2010; Maier et al. 2010). Thus far, there is insufficient evidence regarding the particular USVs syntax (e.g., call shape, peak frequency, sequence of call elements, etc.) that should be observed in situations related to arousal, appetitive motivation, or positive affect.

Although socially rewarding stimuli such as rough-and-tumble play and tickling (i.e., hetero-specific play) have been found to induce primarily FM calls (Burgdorf & Panksepp, 2001; Burgdorf et al. 2005, 2007; Mällo et al. 2007), most of the evidence about the reward-related role of some particular USVs subtypes comes from studies in which the mesolimbic dopaminergic system was pharmacologically manipulated, especially through the administration of psychostimulatory drugs (Browning et al. 2011; Burgdorf et al. 2001; Meyer et al. 2012; Mu et al. 2009; Natusch & Schwarting 2010; Simola et al. 2014; Thompson et al. 2006; Williams & Undieh, 2010). For instance, cocaine and amphetamine have been shown to increase both total 50-kHz calls and FM calls (Barker et al. 2010; Pereira et al. 2014; Wright et al. 2010, 2011, 2013), of which the trills are the most consistent and recurrent FM call types induced by these drugs (Burgdorf et al.2008; Wright et al. 2010, 2011). However, there is still controversy regarding how this data might be generalized to other rewarding situations. For example, morphine, which is known to have strong rewarding and addictive properties, has been shown to neither increase calling nor alter the USVs subtypes produced (Simola et al. 2012, 2014; Wright et al. 2012). Other substances such as caffeine, nicotine, and 3,4-methylenedioxymethamphetamine (MDMA) have also failed to unconditionally elicit 50-kHz calls (Simola et al. 2009, 2012, 2014; Wright et al. 2012). Therefore, the attribute of being rewarding, psychostimulatory, or an agonist of opioids, acetylcholine, and monoamines is not the *sine qua non* condition in order for drugs to induce 50-kHz calls and/or to increase FM USVs. In a recent study, rats emitted more FM calls under the effects of amphetamine, but not during the anticipatory period predicting drug administration. That is, the high number of calls elicited by the testing chamber cues during the 10 min prior to the injection distributed equally between FM and non-FM subtypes (Ahrens et al. 2013). This finding suggests that calls produced while the rewarding drug is experienced and those induced by incentive cues cannot be understood as having the same functional meaning or as being controlled by the exact same mechanisms. Our data are in agreement with this last study, since we have also found that anticipatory 50-kHz calls did not consistently differ between the control and reward rat groups, despite the total call rate being higher in reward rats. In the experiment 6 of our Study 2 we observed that the administration of amphetamine did increase the number of FM calls (i.e., trills and step-calls) both in control and reward groups. Interestingly, this effect was less pronounced in rewarded rats, although no differences in call subtype were detected when administered with saline or during undrugged training. Thus, the repeated reward experience (Pavlovian learning) seemed to induce cross tolerance-like effects by desensitizing brain mechanisms underlying the expression of USVs, especially trills. Again, these findings support the notion of FM calls being triggered by rewarding, euphorigenic experiences rather than by anticipatory, appetitive emotional states developed during incentive learning.

One of the most interesting findings consistently seen across experiments is that food deprivation suppresses both spontaneous and reward-induced 50-kHz calls. Returning the animals to feeding on an ad libitum diet reverses such suppressive effect on USVs. We assumed, therefore, that food deprivation on its own induced a putative negative state that affected USVs likelihood. This assumption is also supported by previous findings from our lab in which food deprivation consistently suppressed spontaneous calling over a period of four consecutive days (Schwarting et al. 2007). In adult rats, reductions in 50-kHz calls or increases in distress USVs (i.e., 22-kHz calls) have also been reported following timeout, withdrawal, or devaluation of different rewards (Burgdorf et al. 2000; Coffey et al. 2013; Covington & Miczek, 2003). Since food deprivation is aversive enough to strongly motivate escape and avoidance responses (Baron & Galizio, 2005; Daly, 1974; D'Andrea, 1971), it is not surprising that food deprivation exerted a suppressive effect on the utterance of 50-kHz calls in similar fashion to that produced by other aversive stimuli (Burgdorf et al. 2008; for a review see Wöhr & Schwarting, 2013).

It has been suggested that only FM calls reflect appetitive behavior, reward, and positive affect in rats (for a review see Burgdorf et al. 2011). In our experiments, however, starvation equally reduced flat and FM calls, indicating that 50-kHz calls in general may gauge the wellbeing of the rat. When rats were fed ad libitum, USVs increased suddenly both in control and reward groups, with the latter showing rather huge rates of 50-kHz calls. Considering that flat calls were the most prominent call subtype emitted, it can be argued that these calls also denote appetitive, rewarding affective states. Further investigation on USVs categorization and on identifying their putative functional role according to the different social and non-social triggering stimuli is warranted.

4.2.3. Incentive motivation, individual differences, and 50-kHz calls: The production of spontaneous (Brudzynski & Pniak, 2002; Schwarting et al. 2007; Wöhr et al. 2008), and reward-induced USVs is highly dependent on individual differences (Ahrens et al. 2013; Browning et al. 2011; Mällo et al. 2007; Rygula et al. 2012). 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 (Brudzynski et al. 2011; Burgdorf et al. 2005, 2009; Mu et al. 2009; Harmon et al. 2008). The use of screening tests to identify possible behavioral measures useful in explaining variability in USVs utterance has garnered great attention (Borta et al. 2006; Natusch & Schwarting, 2010; Schwarting et al. 2007; Wöhr et al. 2008). For instance, individual differences in unconditioned anxiety assessed in the elevated plus-maze test have been found to predict fear conditioning-induced aversive USVs (i.e., 22-kHz calls) (Borta et al. 2006). In the case of 50-kHz calls, moderate and positive associations have been reported between 50-kHz calls and ongoing exploratory activity in different behavioral paradigms (Natusch & Schwarting, 2010; Schwarting et al. 2007; Wöhr et al. 2008). In our Study 2, evidence was provided for the substantial inter-individual variability and intra-individual stability in the predisposition to call across different experiments with independent cohorts of rats. In this case, the analysis of exploratory activity during the course of incentive conditioning revealed strong individual differences in certain behaviors displayed prior to the delivery of food rewards. In general, we found that individuals prone to attribute incentive salience to reward cues, as indicated by high levels of rearing activity or sign-tracking behavior (i.e., number of returns in an instrumental runway maze task), showed rather high rates of reward-induced 50-kHz calls. Put another way, reward cues were effective in augmenting 50-kHz calling only if the animals had previously developed conditioned activity in anticipation of food. Reducing the salience of the UCS by satiation abolished individual differences in conditioned activity but not in cue-induced appetitive 50-kHz calls, a finding which supports our assumption that affective conditioned responses, such as USVs, can outlast appetitive behaviors. This data indicates that USVs carry an underlying significance, in this case regarding the affective dimension of incentive learning, which would not otherwise be accessible through the analysis of other behavioral dimensions.

On the other hand, high callers of 50-kHz USVs have been found to have greater reward sensitivity, as indicated by intra-accumbens and systemic amphetamine-increased calling (Ahrens et al. 2013; Brudzynski et al. 2011), higher sensitization to cocaine-induced 50-kHz calls (Mu et al. 2009), and higher electrical (Burgdorf et al. 2007), and cocaine self-administration rates (Browning et al. 2011). In our experiments, rats that had displayed higher anticipatory activity showed less amphetamine-induced 50-kHz calls and a reduced percentage of trills as compared to controls. When administering the dopamine receptor antagonist flupenthixol, high activity rats showed less inhibition both in total call number and in the percentage of trills than the other groups. These results support the evidence that animals prone to attribute incentive salience to reward cues undergo differential adaptations in the mesolimbic dopaminergic system (Flagel et al. 2011, for a review see Robinson et al. 2014). Such adaptations would not have been the same between mechanisms controlling conditioned affective reward responses (i.e., USVs) and those controlling psychomotor activity, since the interaction between reward experience and dopaminergic drugs was much more prominent for calling than for locomotion or rearing behavior.

4.2.4. Exploratory activity and rates of spontaneous 50-kHz calls: The observation that rats emit 50-kHz calls spontaneously when suddenly placed individually in a novel environment is probably the most unintentionally replicated finding in the USVs field (Ahrens et al. 2009; Browning et al. 2011; Brudzynski & Pniak, 2002; Burgdorf & Panksepp, 2001; Knutson et al. 1999; Ma et al. 2010; Maier et al. 2010; McGinnis & Vakulenko, 2003; Natusch & Schwarting, 2010; Schwarting et al. 2007; Thompson et al. 2006; Wintink & Brudzynski, 2001; Wöhr et al. 2008; Wright et al. 2010; Wright et al. 2011). In our studies, we systematically observed spontaneous calling occurring in a very consistent way during periods of more than 10 consecutive days across different experiments. Such USVs rate did not habituate with repeated testing. In contrast, calling tended to increase during the first three of four testing days, likely once rats became familiar with the experimental manipulations and settings; from this point onwards, call rate remained quite stable. USVs emission and exploratory activity were both triggered by the same procedure, namely transient separation from conspecifics and exposure to a different environment. During the first testing days, both measures followed a very similar trend; however, once rats habituated after repeated exposure to the same experimental context, exploratory activity and rates of 50-kHz calls dissociated.

It has been convincingly ruled out that USVs are not a mere by-product of locomotion (for review see Knutson & Panksepp, 2002); nevertheless, this finding does not preclude the fact that USVs have been shown functionally related to the ongoing behavioral activity taking place during exploration under certain conditions. Indeed, exploratory behavior in the cage test (i.e., a normal housing cage with bedding), measured in terms of locomotion and rearing, has been shown positively related to call rate (Schwarting et al. 2007), suggesting that the same level of salience motivating exploration also induces 50-kHz calls. This phenomenon was also observed in the open field test, where the time spent calling also correlated positively with the distance traveled in this setting (Wöhr et al. 2008). This ruled out that 50-kHz calls were exclusively triggered by specific features of the cage, such as bedding. Interestingly, and contrary to intuition, animals exploring in the EPM emitted 50-kHz calls and almost no 22-kHz calls (Schwarting et al. 2007; Wöhr et al. 2008). When the EPM was used as a screening test, it revealed that animals with more risk-assessment behavior in the EPM were more likely to emit 50-kHz calls during the cage test (Schwarting et al. 2007). In addition, the level of 50-kHz calls in the open field test correlated positively with those observed 24 hours later in the EPM (Wöhr et al. 2008), suggesting that the tendency to call in such challenging environments is a stable trait among rats. Taken together, this evidence supports our assumption that exploratory activity and 50-kHz calls might be integrated into a broader behavioral system, subserving the assessment of potential risks (i.e., predators or dominant conspecifics) or opportunities (a prey or a receptive female), as emanating from intra- or interspecific individuals.

Since rats are colonial in the wild, they likely experience repeated encounters occurring after brief periods of separation from the colony, while foraging or exploring the surroundings. In this context, 50-kHz calls could have emerged as a preventive signal aimed at reducing intra-specific aggression in colonial life (Blanchard et al. 1993; Sales, 1979). Therefore, they would be expected to occur in every situation where an uncertain social contact is more likely to occur; that is, after being transiently isolated either in a novel or in a familiar environment with no other rats nearby or present. Reinforcing this assumption, there is broad evidence showing that rats emit 50-kHz calls during socially ambiguous encounters, after a first experience of social defeat, or in the face of an impending attack (Blanchard et al. 1993; Haney & Miczek 1994; Tornatzky & Miczek 1994, 1995; Vivian & Miczek, 1993a, 1993b). In a series of experiments conducted with independent groups of animals, rats were found to emit 50-kHz calls when visiting a cage with bedding (Brudzynski & Pniak, 2002). The call rate of individual rats exploring this cage increased proportionally to the number of rats that had previously visited the cage, leaving their odor marks therein. This proportional increase occurred in spite of a lack of social contact preceding or following the cage visit, as rats were housed in social isolation throughout the entire experiment (i.e., 7 to 10 days before testing) (Brudzynski & Pniak, 2002). The latter suggests that calling is driven by potential social contact, yet not as a consequence of being suddenly separated from cage mates. In this case, an unexpected exposure to even a familiar environment, rather than the transient social isolation itself, nevertheless triggered calling. We argue, therefore, that the emission of spontaneous 50-kHz calls illustrates a type of modal action pattern (MAP, for review see Domjan, 2009) that is reactivated any time an animal is individually confronted with a different context. USVs rates are not expected to habituate over repeated testing, and instead similar or even higher rates are likely to occur even after having been exposed to the same experimental setting days before. From a narrow perspective, one might say that what reward-related cues or amphetamine administration were shown to precipitate was the increased likelihood of this MAP being displayed by subjects that had already exhibited certain levels of spontaneous calling when confronted with the experimental settings. Further investigation is needed to carefully examine these assumptions.

4.3. Translational implications

In evolutionary terms, the ability to experience strong pleasure from palatable foods used to be advantageous because it ensured that food was overeaten when available, enabling energy to be stored in the body as fat for future needs in environments where food sources were scarce and/or unreliable (Volkow et al. 2011, 2013). However, in modern societies, where food is widely available and highly caloric and humans are increasingly becoming less active, this adaptation has become a liability, especially in the case of certain vulnerable individuals (Volkow et al. 2011). According to the World Health Organization (WHO, 2008), 30-70% of adults in European Union countries are overweight, and 10-30% suffer from obesity. In spite of the fact that this is a problem that continues to escalate at alarming rates, it is important to note that only a minority of those living in similar societal conditions is at risk for developing eating disorders (e.g., obesity, binge or bulimia disorders). Likewise, for those who use narcotic substances, only 15% of users will progress from recreational use to a substance-related disorder (Anthony et al. 1994; Nutt et al. 2007). This point highlights the enormous importance of individual differences in reward-related disorders. Aside from genetic and metabolic factors of susceptibility (Volkow et al. 2011, 2013), individual variations in the propensity to attribute incentive salience to reward cues and the manner in which such cues acquire motivational control over behavior constitute one of the most important vulnerabilities (Nair et al. 2009; Robinson et al. 2014).

While many people attempt to control their food intake through dieting, most have been shown to relapse to unhealthy eating habits within a few months (McGuire, 1999). This relapse to unhealthy eating habits often occurs after acute exposure to palatable foods, food-associated cues, or stress (Hunt et al. 1971; McGuire, 1999; Nair et al. 2009; Torres & Nowson, 2007; Volkow et al. 2013). Apart from obesity, compulsive eating in binge and bulimia disorders may also be triggered by the same factors associated with attribution of incentive salience to food-associated cues. Likewise, a principle problem in the treatment of drug addiction is relapse to drug use after periods of abstinence (Hunt et al. 1971; Nair et al. 2009; O'Brien 1992). In drug addicts, drug relapse and craving during abstinence are often triggered exactly as in the case of food; that is, by acute re-exposure to the self-administered drug, drug associated cues and contexts, or certain stressors (Hunt et al. 1971; Nair et al. 2009; Torres & Nowson, 2007; O'Brien 1992). The incentive valence of reward-related stimuli (like places, odor, sounds, and time periods) is primarily determined by the affective experience resulting from the preceding intake of that reward (Balleine, 2005; Dickinson & Balleine, 2002; Petrovich et al. 2007). The ability of reward-related cues to induce expectancy about rewards is one of the most important factors implicated in food and drugs (over)consumption (Berridge, 2012; Cornell et al. 1989; Nair et al. 2009; Schachter, 1968; Volkow et al. 2013). This induced emotional state or expectancy is thought to precipitate the pursuit of the reward by reminding the user or consumer how positive they would feel were the reward to be experienced again; this, in spite of a potential absence of hunger or withdrawal symptoms (Schachter, 1968; Volkow et al. 2013). Therefore, the investigation of the psychological and neurobiological factors underlying affective states as related to incentive motivation and the compulsive pursuit of rewards is of remarkable relevance and importance. In preclinical-oriented research the aforementioned factors can be investigated with the same animal models as implemented in our studies. The results presented here would also be, to a certain extent, translatable to human conditions such as eating disorders and other forms of persistent motivation as observed in drug addiction and pathological gambling.

4.4. Achievements and shortcomings

We acknowledge that some of the most interesting findings coming out of the two studies were not initially anticipated. In the study 1, experiments 1 and 2 were modified on the fly to account for the unexpected effect of not seeing differences in call rate between reward and control groups while tested under food deprivation (i.e., days 1 to 7). The following experiments (3 to 5), consequently, were designed to replicate the former ones without changing the previous testing schedule. Since feeding conditions were shifted during the last 3 days of testing in all experiments, the effect of such an experimental manipulation was inseparable from the likely effect of consecutive training, especially on testing days 8 to 10. Counterbalancing the order of feeding conditions during training, that is, testing some animals first ad lib, others first deprived was technically possible but experimentally useless; this holds true at least for the experiments using normal rat chow, since none of the rats would have eaten the reward while fed ad libitum with the same food. In the experiments using a different reward, feeding conditions were reversed (i.e., experiment 4, Study 1). In cases where shifting the feeding conditions clearly affected behavioral parameters of conditioning (e.g. USVs, approach to and consumption of rewards, and anticipatory activity), there were no noticeable indications to suspect that such changes (i.e., on days 8 to 10) resulted only from the ongoing tendency the data already had. Thus, we concluded that those changes were alone the consequence of shifting the feeding conditions.

We recognize that our experiments were not totally orthogonal in the way independent variables were evaluated, and that the experimental design and the conformation of groups may not have allowed us to answer all questions arising from the data; this is something that could be seen as a potential, significant limitation. Nevertheless, it is important to point out that while running the first experiments, we did not yet have the luxury of a clear picture of the study as a whole ; therefore, we kept constant the testing schedule while manipulating other variables such as the incentive properties of food rewards, the appetite physiological state, and the order of testing phases. At that time, all these factors were judged as being more meaningful than changing the testing schedule. Additionally, most of the decisions we made were based on certain behavioral parameters and on preliminary estimations about the likely effect obtained on calling, due also to the fact that USVs analysis took months for completion. All of these variables forced the adoption of a very particular experimental strategy while also restricting our ability to assess other methodological approaches to the data. We determined, however, that none of these limitations compromised the veracity of our findings. In spite of the fact that the initial data appeared unusual at the beginning of testing, successive replications, new experiments, and detailed behavioral and USVs analyses have shown the data to be consistent.

In study 2, we used data from four experiments of study 1 to analyze appetitive USVs and reward-oriented behaviors according to individual differences in anticipatory activity developed while testing rats in a state of food deprivation. As already detailed for the experiments of study 1, the experimental design and the testing schedule imposed some limitations. In spite of this fact, individual differences in rearing behavior across experiments proved consistent in predicting cue-induced USVs. Likewise, animals ranked higher according to manifestations of sign-tracking behavior as displayed in the runway maze (experiment 4, study 2) showed more 50-kHz calls while sated, consistent with the results of the appetitive cage test experiments (experiments 1 to 3). We acknowledge that in the runway maze, there was no localizable sign-stimulus specifically paired with the UCS at which attention and behavior could be directed in order to properly analyze sign-tracking. Nevertheless, the behavior that emerged naturally during the runway training shared some important features with sign-tracking behavior reported elsewhere (Robinson et al. 2014). For example, the frequency of returns to the runway maze gradually increased over testing days, even though such behavior was unreinforced and opposed to approaching and consuming the food reward, which was available only in the cage attached to maze.

Regarding prior works on this topic, it is worth noting that when we designed our first experiment the only paper published was the pioneering work of Burgdorf and colleagues (Burgdorf et al. 2000). In their study, an increase in 50-kHz calls over a period of days in anticipation of daily feeding session was observed in food deprived rats. During the course of the dissertation, five papers related to our research were published. In the first paper, lever-pressing for sucrose in ad lib rats led to an increase in 50-kHz calls (Browning et al. 2011). In a second study, differences in 50-kHz calls between adolescent and adult rats when offered chocolate chips in a one-trial test were described (Willey & Spear 2013). In a third study, no differences were found in calling following positive and negative reward contrasts with different probabilities to get a sucrose reinforcement (Coffey et al. 2013). In the last study, 50-kHz calls were used to assess anhedonia (i.e., a core symptom of depression) in a new animal model of depression, in which anhedonia was defined as a reduction in the preference for sweet pellets. In this latter study, animals chronically stressed showed a reduction both in the preference for sweet pellets and in the rate of 50-kHz calls elicited during the test as compared to unstressed controls (Mateus-Pinheiro et al. 2014). Finally, a paper measuring cocaine-induced 50-kHz calls in individuals prone to attribute incentive salience to food cues was published in 2012 (Meyer et al. 2012). In this study, sign-tracker rats (i.e., animals that approached and engaged a food cue) displayed a higher conditioned place preference for cocaine and showed more 50-kHz calls both when injected with cocaine and when exposed to its related cues. This paper was particularly helpful in addressing the analyses and discussion of our Study 2. To our knowledge, it was also the first paper published on this particular topic.

In spite of the interesting findings presented in the abovementioned studies, we determined that our experiments continue to provide valuable data about the role of 50-kHz calls in incentive motivation, findings which are not overshadowed by the more recent papers. Firstly, one of the primary differences proffered by our studies is that we specifically designed them to evaluate the particular conditions by which food rewards and their related cues were able to elicit appetitive 50-kHz calls. Secondly, in our experiments, we included matched control groups, which allowed us to differentiate changes in USVs induced by food cues from those merely produced by spontaneous calling. It has been repeatedly observed that rats call at moderate rates merely by the fact of being placed in different testing environments (Natusch & Schwarting 2010; Schwarting et al. 2007; Wöhr et al. 2008). For example, in the studies of Browning et al. (2011) and Burgdorf et al. (2000), for which no controls were included, call rate increased over a period of days in a similar fashion as it did in our control rats in a number of our experiments (i.e., see study 1, experiments 1 and 4). Thirdly, and in contrast to the studies of our colleagues (Browning et al. 2011; Burgdorf et al. 2000; Willey & Spear 2013), we measured reward seeking, consumption, and anticipatory activity in order to provide evidence that USVs were actually elicited by rewards and their related cues. Fourthly, we manipulated other significant variables such as the current physiological state of the subject (deprived vs. sated), the type of learning mechanism recruited (more Pavlovian vs. more instrumental), the hedonic properties of the UCS (low vs. high palatable food), and the reward availability (continued vs.

discontinued). Regarding individual differences in anticipatory activity and appetitive 50-kHz calls, we complemented and extended some of the findings presented by Meyer and colleges (Meyer et al. 2012). For instance, in our experiments, individual differences in anticipatory activity (i.e., rearing behavior) emerged naturally during conditioning and predicted the utterance of 50-kHz calls. In Meyer´s experiment, animals underwent a long, intricate autoshaping conditioning procedure which allowed for the differentiation of rats that approached the lever while the CS was presented (sign-trackers) from those that approached the food magazine during the same period (goal-trackers). Although this conditioning protocol produced strong individual differences, the behavior analyzed seems not to have been as natural as exploring, rearing, and sniffing around, all behaviors that may be considered part of the foraging repertoire of the rat triggered by food cues. In a similar vein, in our experiments, animals were classified based on the frequency of such an anticipatory activity emitted, whereas in Meyer´s experiment the differences were instead qualitative: animals varied in the type of behavior displayed (sign-tracking vs. goal tracking), even from the beginning of the autoshaping procedure. Finally, and in contrast to our experiments, in the Meyer´s study (2012), 50-kHz calls were not an object of analysis during the course of conditioning; therefore, there was no information provided on whether the autoshaping procedure induced calling and whether sign- and goal-trackers already differed in the call rate during such a testing phase.

4.5. Future investigations

Further experiments employing different conditioning paradigms with longer and more diverse testing schedules are required to elucidate the phenomena showed in our studies, especially regarding USVs. One interesting line of research would be linking the affective role of food-related 50-kHz calls with their putative communicational function. As previously mentioned, rats strongly prefer foods that other conspecifics have previously eaten after smelling food scents especially from breath (Galef, 1986; Galef & Beck, 1990; Galef & Heiber 1976; Galef & Wigmore 1983; Mason et al. 1989). Other experiments have shown that volatile cues present in the breath of demonstrators (Galef & Stein 1985) determine food choices when combined with food scents. The most important components of the breath are carbon disulfide (CS2) and carbonyl sulfide, which are present in relatively high concentrations (1-2 ppm) (Galef et al. 1988; Mason et al. 1989). When 1-ppm CS2 was associated with diet on a surrogate rat (cotton batting), it elicited transfer of diet preference similar to that produced by exposure to a live demonstrator (Galef et al. 1988; Mason et al. 1989). When applying 0.1-10.0-ppm CS2 to food, consumption increased significantly, and bait stations containing CS2-scented food were entered more frequently and for longer periods that bait stations containing unscented food (Galef et al. 1988; Mason et al. 1989). Since CS2 is a safe social signal for food to be eaten, and is only present in the breath of the congeners, its presentation shall have almost the same effects as if were emanated from a real rat. To investigate whether social factors (i.e., other subjects signaling food, which can be mimicked by presenting CS2 scents) would influence associative learning and the utterance of appetitive 50-kHz calls, the following experiments would be implemented: 1) CS2 can be presented alone or paired with discrete, non-food related cues both in food deprived and ad libitum rats, to evaluate whether CS2 can facilitate attribution of incentive value to otherwise neutral cues, and whether it is able to induce 50-kHz calls conditionally and/or unconditionally. In these experiments foraging behavior (i.e., digging) can be easily assessed by hiding food pellets in a thick layer of bedding. Approach behavior to the location of cues, and exploratory activity can also be measured. 2) In the next phase, previous experiments should be replicated but with food as UCS either alone or paired with CS2. 3) Alternatively, conditioned place preference test for food (e.g., food alone vs. food marked with

CS2) could be implemented to have distinct dependent variables to be associated with 50-kHz calls. 4) Since CS2 is required for the acquisition of socially transmitted food preferences (Galef et al. 1988; Mason et al. 1989), CS2 can make food to be perceived as more attractive and palatable, so that in a progressive-ratio schedule rats would work harder for a food marked by CS2. Also, to assess whether CS2 would invigorate instrumental responding for food, a Pavlovian-to-instrumental transfer paradigm would be appropriate. Here, CS2 can be presented during acquisition and test phases or only in the test phase to assess the strength of CS2 to affect instrumental responses both conditionally and unconditionally. In all these experiments 50-kHz calls should be recorder before and during conditioning sessions. Additionally, in the early phases of these experiments other social olfactory stimuli that have been shown to elicit 50-kHz calls (e.g., soiled cage bedding from other rats, Brudzynski & Pniak, 2002) should be evaluated to contrast the effects of CS2 against olfactory signals that are not likely to be related with food.

Once the best protocols for assessing the role 50-kHz calls in food-related social learning (using CS2 as olfactory social signal) have been defined, a second phase will start. There, the question of whether food reward-induced USVs might convey pro-social value, thereby promoting food-related behaviors in the listeners (i.e., audience effect), would be approached by conducting playback studies with different combinations of CS2 and food stimuli. For example, in the playback settings normally used in the lab (Wöhr & Schwarting, 2007) the effect of CS2 on approach behavior to playback of 50-kHz calls (with odor well distributed either in the 8 arms of the maze and/or in the testing room) would be evaluated. In addition, the effect of 50-kHz calls on how rats make food choices, and on approach and consummatory behaviors to already chosen foods, would be evaluated in Pavlovian and instrumental paradigms, where rats would listen to playback of 50-kHz calls while food and food related cues, either alone or marked with CS2, are presented. Finally, a third phase would be the initiation of a psychopharmacological program to further evaluate the motivational and communicational role of 50-kHz calls in food-related social learning.

On the other hand, the analysis individual differences in incentive motivation and appetitive 50-kHz calls deserves to be continued by using different methods to gauge variability in relation to positive affect and motivation. For instance, Pavlovian autoshaping procedures, by which sign-tracker and goal tracker rats are normally obtained (Flagel et al. 2007, 2011), have been extensively validated both at behavioral and neurobiological level on diverse parameters relevant for incentive motivation and preclinical research of addiction (for review see Robinson et al. 2014). In a new set of experiments, sign-trackers and goal trackers would be compared on different USVs tests, such as spontaneous calling in the cage test, tickling- and amphetamine-induced calling, and playback of 50-kHz calls. Here can be estimated whether animals prone to attribute incentive salience to reward cues (i.e., sign-trackers) showed higher rates of 50-kHz calls. If the predisposition to call is reward-dependent, high rates of USVs should be observed after tickling or amphetamine administration, but not in the cage test. If these animals experience others' 50-kHz calls as being more appetitive, enhanced approach behavior toward playback of 50-kHz calls should be observed. If the latter experiments with sign-trackers and goal trackers hold true, further psychopharmacological studies are warranted.

In my knowledge, individual differences in the utterance of 50-kHz calls have been predominantly explored using high responders to tickling (Brudzynski et al. 2011; Burgdorf et al. 2005, 2009; Mällo et al. 2007; Mu et al. 2009; Harmon et al. 2008); whereas the analysis of such individual variability in response to other social and non-social stimuli is scarce. The question of whether rates of 50-kHz calls induced by 1) exploration (i.e., cage test or open field with bedding), 2) tickling, 3) mating, and 4) amphetamine administration are particularly related with positive affect, pro-social behaviors, incentive motivation, or learning in general has not been fully investigated. Experiments in this direction would help to further enhance our understanding of the distinct biological functions 50-kHz calls may have, based on the likely association they would exhibit with behavioral parameters relevant for emotionality, sociability, and motivation. For instance, a simple test like the conditioned place preference would be useful to assess the motivational effects of rewarding stimuli such as food (different palatable foods under food deprivation or satiation), novelty (i.e., salient objects), psychoactive drugs (i.e., amphetamine, alcohol, nicotine, morphine) and social stimuli (i.e., male or female scents, CS2, and social interaction with a congener) in rats previously selected according to their rates of 50-kHz calls on a given USVs test (i.e., cage test, amphetamine-induced calling, or tickling).

5. References

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Appendix

Appendix A:	Study 1	Brenes, J.C. & Schwarting, R.K.W. Attribution and expression of incentive salience are differentially signaled by ultrasonic vocalizations in rats.
Appendix B:	Study 2	Brenes, J.C. & Schwarting, R.K.W. Individual differences in anticipatory activity to food rewards predict cue-induced appetitive 50-kHz calls in rats
Appendix C:		Curriculum Vitae
Appendix D:		Disclosure of authorship contributions
Appendix E:		Eidesstattliche Erklärung *[declaration of academic honesty]*

Appendix A: Study 1

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Attribution and Expression of Incentive Salience Are Differentially Signaled by Ultrasonic Vocalizations in Rats

Juan C. Brenes[1,2,3]*, **Rainer K. W. Schwarting**[3]

1 Institute for Psychological Research, University of Costa Rica, Rodrigo Facio Campus, San Pedro, Costa Rica, **2** Neuroscience Research Center, University of Costa Rica, Rodrigo Facio Campus, San Pedro, Costa Rica, **3** Behavioral Neuroscience, Experimental and Biological Psychology, Philipps-University of Marburg, Marburg, Germany

Abstract

During Pavlovian incentive learning, the affective properties of rewards are thought to be transferred to their predicting cues. However, how rewards are represented emotionally in animals is widely unknown. This study sought to determine whether 50-kHz ultrasonic vocalizations (USVs) in rats may signal such a state of incentive motivation to natural, nutritional rewards. To this end, rats learned to anticipate food rewards and, across experiments, the current physiological state (deprived vs. sated), the type of learning mechanism recruited (Pavlovian vs. instrumental), the hedonic properties of UCS (low vs. high palatable food), and the availability of food reward (continued vs. discontinued) were manipulated. Overall, we found that reward-cues elicited 50-kHz calls as they were signaling a putative affective state indicative of incentive motivation in the rat. Attribution and expression of incentive salience, however, seemed not to be an unified process, and could be teased apart in two different ways: 1) under high motivational state (i.e., hunger), the attribution of incentive salience to cues occurred without being expressed at the USVs level, if reward expectations were higher than the outcome; 2) in all experiments when food rewards were devalued by satiation, reward cues were still able to elicit USVs and conditioned anticipatory activity although reward seeking and consumption were drastically weakened. Our results suggest that rats are capable of representing rewards emotionally beyond apparent, immediate physiological demands. These findings may have translational potential in uncovering mechanisms underlying aberrant and persistent motivation as observed in drug addiction, gambling, and eating disorders.

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* Email: brenesaenz@gmail.com

Introduction

Having affective representations in terms of pleasures and desires is a fundamental part of our subjective experience. Rewards and reward-related stimuli can make us feel good, and remind us how good they were in the past, but also how good they would be if experienced again. Being exposed to reward-related cues may also set a state of readiness for seeking and consuming that reward, even though we have not experienced it for a while or when physiological needs for it have been fulfilled [1–4]. In humans, such mechanisms play a critical role in drug addiction and relapse, overeating in obesity, and binge disorders [5–7]. The incentive valence of such reward-related stimuli (like places, odors, sounds, and time periods) is mainly determined by the affective experience resulting from preceding intake of that reward [8,9]. Following Pavlovian learning, sensory reward properties and associated cues are transformed into attractive and desired incentives [4,10–13]. This motivational component of reward is normally referred to as incentive salience [5,10]. In classical and modern incentive motivation theories, either activation of a "central emotive state", "expectations about rewards", or "subjective wanting" have been proposed as critical factors in the process of attributing incentive salience to reward cues [9–19]. In non-human animals, especially rodents, incentive motivation has been extensively investigated using traditional behavioral parameters, like nose-poking, lever-pressing, and approach behavior to cues and rewards in Pavlovian, instrumental, and Pavlovian-to-instrumental transfer paradigms [5,9,20]. The study of the emotional or affective conditioned responses underlying incentive motivation, however, has received less attention, first, because the study of emotions was disregarded in behavioristic tradition (for review see [10]) and second, due to the lack of direct and more precise measures of such states in animals.

Currently, there is an increasing interest in studying rodent ultrasonic vocalizations (USVs) in basic and clinically-oriented research, since USVs seem to provide a unique avenue to study the putative affective state of an animal which might not be accessible by conventional behavioral approaches. Rat USVs are complex affective and communicative signals expressed in different social and non-social situations, which vary according to age and context [21,22]. Out of these, high-frequency calls (i.e., 50-kHz calls) are normally emitted in naturalistic rewarding situations such as mating, and rough-and-tumble play, or triggered by non-naturalistic stimuli such as electrical stimulation of the mesolimbic dopamine pathways, or by psychostimulant drugs like amphetamine and cocaine [23–28]. For instance, individual differences in incentive salience attribution to food cues predicted conditioned place preference for cocaine and 50-kHz calls induced by cocaine related cues [29]. Whether 50-kHz calls may be indicative of incentive salience attributed to food cues, however, is still unclear: previous studies showed mixed results and were not conclusive due to the lack of proper controls groups and concomitant behavioral confirmations of incentive learning [23,30–32].

Encouraged by the translational potential of modeling subjective putative affective states in animals we decided to perform a series of studies to explore further the hypothesis that 50-kHz calls can come to signal a state of incentive motivation in the rat, which may constitute an emotional reward representation triggered by conditioned stimuli (CS) predicting reward or by some perceptual features of the food itself (unconditioned stimulus, UCS). One of the simplest conceivable tests to achieve this aim was training rats to anticipate their daily feeding taking place under certain predictable environmental cues. Within or across experiments the conditioning task was systematically modified so that the current physiological state of the subject (deprived vs. sated), the type of learning mechanism recruited (Pavlovian vs. instrumental), the hedonic properties of UCS (low vs. high palatable food), and the availability of the food reward (continued vs. discontinued) were manipulated.

General Methods

Ethics statement

All experimental procedures were approved by the appropriate governmental agency (Regierungspräsidium, Giessen, Germany) and complied with the EU directive 86/609/EEC. Every effort was made to minimize the number of animals used and their suffering.

Subjects

Adult male Wistar rats (Harlan-Winkelmann, Netherlands) served as subjects. They were housed 4–5 per polycarbonate cage (595×380×200 mm) in a climate-controlled room with a 12:12 h light–dark schedule (light on at 07:00 h). Food (Altromin, Lage, Germany) and water (0.0004% HCL-solution) were freely available unless otherwise specified. In all experiments, animal order of testing was counterbalanced within and across days and experiments to the fullest extent possible.

Screening cage test

Since rats show substantial and rather stable inter-individual variability in 50-kHz calls [33], we applied a screening test in which rats are tested for their levels of spontaneous USVs before being assigned in a counterbalanced order to further tests or treatments [27,33]. Briefly, at the beginning of each experiment all animals' cages were removed from the housing-rack and placed on a desk in the same room. Afterwards, a given rat was individually placed into a clean polycarbonate cage (425×266×185 mm) with fresh bedding (Tapvei) and then transported to an adjacent ultrasonic lab, where a recording session immediately started. The cage was placed on a desk under a microphone positioned at 35 cm above the center of cage floor. It was illuminated by red light (about 7 lx inside the cage) and visually separated from the data acquisition area by a curtain. The cage test was conducted on two consecutive days (5 min each). Testing took place from 8:00 to 17:00 h in a counterbalanced order between days and subjects. Based on the average number of spontaneous 50-kHz calls on both days, animals were equally assigned either to the control or the reward group.

Appetitive cage test

All test settings and the general procedure were the same as described in the screening cage test. Briefly, 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% concentrated milk 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 did, except that food or milk were 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 both group cages 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 milk experiments (3 and 4), all animals were first habituated to the sweetened condensed milk for a week. During this period, controls rats had milk in the evening together with their daily food, whereas reward rats had milk in the light period, coinciding exactly with the daily time in which they would be further tested.

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 was 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 maze was thoroughly cleaned between trials and subjects with a 0.1% acetic acid solution. The testing area was illuminated by red light (about 10 lx inside the maze) and surrounded by curtains.

Behavioral analysis

Behavior was recorded with a video camera positioned at a longitudinal side of the cage. In Experiment 2, an additional camera recorded behavioral activity in the runway maze. 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), digging (moving cage bedding with forepaws and snout, in seconds), eating or drinking time (seconds), and latency to consume the reward (i.e., time difference between the presentation of food or milk and the first eating or drinking bout, in seconds) were manually counted from videotapes using the EthoLog 2.25 software (University of São Paulo, Institute of Psychology SP, Brazil). Fluid intake (experiments 3 and 4) was determined by weighing bottles before and after testing.

Ultrasonic recording and analysis

As previously reported [27], 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: 0.488 kHz, time resolution: 0.512 ms) were obtained after fast Fourier transformation (512 FFT-length, 100% frame, Hamming window, 75% time window overlap), by using the Avisoft SASLabPro 4.38 software. Experienced observers manually counted USVs off-line from the spectrograms. All USVs emitted over 33 kHz were considered as 50-kHz calls. If two call elements were at least 0.048 s apart, two independent calls were counted. USVs occurring during the tasks were expressed as the number of calls emitted per time (calls/min), except otherwise specified. In the cage tests, the proportion of calls emitted during the tone presentation was expressed as: [(call number during tone/total call number) ×100]. The analysis of 50-kHz calls subtypes (e.g., flat, step-calls, trills) provided no relevant information as groups showed rather similar distributions of such USVs subtypes (data not shown). Therefore they were not included in the analyses and only total call number is presented. Since 22-kHz calls were only rarely and non-systematically observed, they were also omitted from the study.

Statistical analysis

Results were expressed as means ± SEM. The main effect of groups (G, control vs. reward), training days (D), and their interaction (DxG) was assessed by means of independent mixed ANOVA analyses. In one of the analyses, the repeated-measures factor was the food deprivation days, and in the other, the food ad libitum days. When only one group provided data (i.e., latency to eat and time spent eating in Experiments 1, 2, and 5), a repeated-measures ANOVA within the reward group was computed. All multiple comparisons among days were adjusted with the Bonferroni *post hoc* test. In all repeated-measures analyses that did not meet the sphericity assumption, the Greenhouse-Geisser correction was used. When appropriate, *t*-tests for related samples were used to compare feeding phases within groups. Statistical significance was defined as $p<.05$.

Results

Experiment 1

Introduction. The hypothesis that 50-kHz calls can come to signal a state of incentive motivation to food reward was investigated by training deprived rats to anticipate their daily feeding. In this experiment the CS signaled the start of each feeding session (1.5 h access to food per day), which began in the ultrasonic lab (~2 min in the testing cage) and ended in the animal room (see general materials and methods for details). A reward-unpaired rat (i.e., controls) was tested simultaneously in an adjacent room, where it received the same pairing schedule as the matched reward rat, except that a hopper of chow pellets was never placed upon the cage grid.

Methods. Thirty experimentally naïve rats weighing 277–351 g on arrival were used. One week before testing, animals were habituated to the experimental conditions and were handled during four days (5 min each). Afterwards, two consecutive screening cage tests were conducted (see screening cage test). Subsequently, animals were counterbalanced into two groups and put on a 22.5-h food deprivation (FD) schedule by being given free access to their maintenance diet for 1.5 h per day, starting one week before the appetitive cage test. During this period, rats were handled and weighed every other day. From day 1 to 7, animals were food deprived (FD); thereafter (days 8–10), they obtained food ad libitum (FAL) in their own home cages.

Results. *FD phase*: Reward rats (Fig. 1; days 1–7) showed typical motivational behavior, i.e. approach and food consumption. The latencies to approach the reward decreased over days (D: $F_{3,57} = 9.57$, $p = .0001$). Locomotor activity was lower in reward rats than in controls (G: $F_{1,28} = 9.24$, $p = .005$), whereas rearing activity did not differ between groups (Figure S1). Total call number increased over days (D: $F_{3,84} = 12.79$, $p = .0001$), but contrary to our expectations this effect was observed in both groups (G: $p>.05$) (Figure 1C). The relative number of calls emitted during tone presentation (Figure 1D) did yield a higher percentage of tone-related calls in reward rats (G: $F_{1,28} = 17.08$, $p = .0001$), which increased over days (D: $F_{3,57} = 9.84$, $p = .0001$), indicating that the reward animals did learn the associations. *FAL phase:* After testing on day 7, animals received food in their home cages in order to devalue the food reward. If the previous lack of group differences seen on total call number was unrelated to incentive learning, no changes in USVs would be expected, but if part of the USVs in reward rats was emitted in anticipation of food, then satiation should decrease them. Surprisingly, we found that total call number increased in reward animals once they were sated (FD vs. FAL: $t_{19} = -13.10$, $p = .0001$), differing now from controls on all FAL days (G: $F_{1,28} = 13.09$, $p = .001$). For instance, call rate in the reward group on day 8 exceeded both, their own levels of day 7 (160%), and controls levels (86%) on the same day (Figure 1C). After 48 h of FAL (i.e. on day 9), total 50-kHz calls reached a maximum, that is, an elevation of ~310% and ~166% over FD and control levels, respectively. In contrast, the proportion of calls emitted during tone presentations dropped, especially in the reward group, but was still significantly higher in reward rats (G: $F_{1,28} = 10.51$, $p = .003$). Rearing behavior (Figure S1B), which occurred mostly at the cage side where the food reward was delivered (details not shown), increased in reward rats (FD vs. FAL: $t_{19} = -4.89$, $p = .0001$) and remained consistently high until the end of the FAL phase (G: $F_{1,28} = 11.04$, $p = .002$). Remarkably, the increases in appetitive 50-kHz calls and rearing occurred even though approach and consummatory behaviors were completely abolished during all FAL days (Figure 1A and B). Thus, the devalued feeding conditions dramatically increased both total call number and tone-induced USVs even after 72 h of experiencing the reward in a low motivational state.

Discussion. These data suggest that attribution of incentive salience to reward predictive cues (i.e., cage context and tone CS) may have occurred while animals were deprived, and surprisingly cues were able to trigger conditioned motivational reactions to reward, i.e. USVs, even though it was fully devalued. Since the conditioned response was learned under FD, expression but not acquisition of such a response is what seemed to be suppressed during this phase. Perhaps, approach/consummatory responses taking place in the same testing environment may have overlapped with the preparatory/emotional elements of the UCS producing a sort of inhibition in the expression of the latter [34]. For example, species-specific foraging behavior such as digging/snout-down locomotion, which was also observed here (Table S1, supporting information) is known to increase during FD even when animals

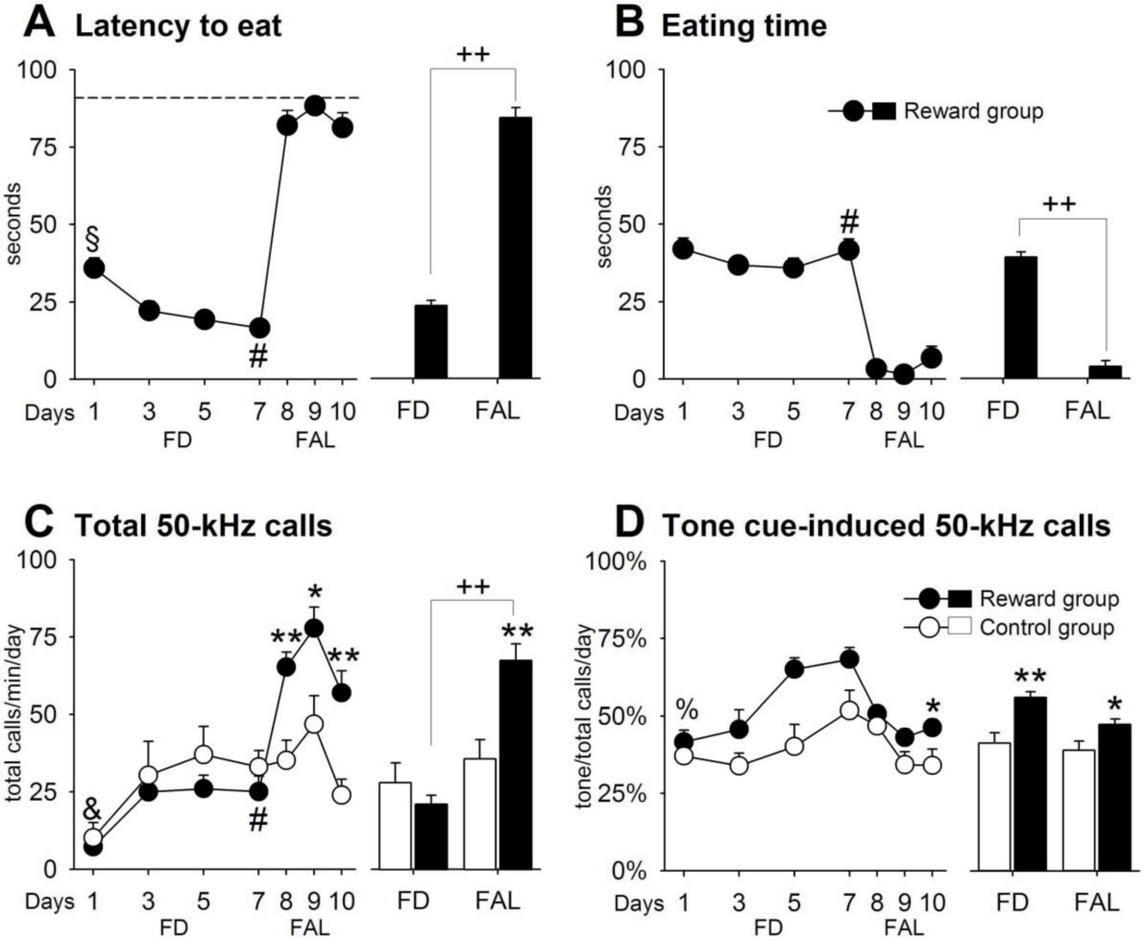

Figure 1. Experiment 1. Animals learned to associate incentive Pavlovian cues with access to daily feeding as reward. A. Latency to eat. B. Eating time. C. Total 50-kHz calls. D. Tone cue-induced 50-kHz calls. The dashed line indicates that a maximal latency time was set at 90 s. Control vs. reward: * $p<.05$, ** $p<.01$. FD vs. FAL: ++ $p<.01$. Day 1 differed from days 5 and 7 in reward rats: § $p<.05$. Day 7 differed from days 8 to 10 in reward rats: # all $p<.05$. Day 1 differed from days 3 to 7 in both groups: & $p<.05$. Data are expressed as mean+SEM (control = 10, reward = 20).
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never obtained food by these means [35,36]. Since these and other approach/consummatory behaviors were reduced drastically when tested under FAL conditions, we consider the idea of competition between different behavioral systems.

Experiment 2

Introduction. Here, the procedure was modified so that USVs associated with anticipatory and consummatory acts could be measured in different testing compartments. A testing cage with bedding was also used here, but instead of training animals to passively wait for food reward to be delivered (i.e., Pavlovian schedule), they now learned to run down a runway maze connected to the cage, so that they could voluntarily enter it and access their daily food ration available there (i.e., instrumental component). With these modifications, animals motivation to attain the reward and cue-induced anticipatory 50-kHz calls in the runway could be assessed independently from consummatory responses in the goal cage. As anticipating and earning a reward appear to be distinct processes [8] we sought to elucidate whether the USV effects observed in experiment 1 rely upon the type of associative process. The cage was the same as in Experiment 1, so that each animal had its own cage for testing throughout the whole experiment.

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 apart from the first experiment. Exactly as there, all animals were put on a 22.5-h schedule of FD with free access to their maintenance diet (1.5 h per day) either immediately after testing (for reward animals in their own testing cages) or at least 3 h later (for controls once they were returned to their group cages). After 3 handling days, 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. In parallel, we performed reinstatement of tone/food pairing by repeating the cage test procedure of Experiment 1. During seven days, starting from the second day of the runway habituation, animals were given a maze habituation session followed by a 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). When they entered the cage, a 3-kHz tone was played as in Experiment 1. During habituation, animals were weighed and handled every other day. Afterwards, reward animals were trained to run through the runway maze to access food in the cage attached to the end of the runway goal arm. Rats received daily training sessions for 10 consecutive days 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 during approximately 4 min. Control rats followed the same procedure but food was never given in the cage. As in Experiment 1, animals were food deprived during days 1–7; thereafter (days 8–10) they received FAL in their own home cages. USVs were recorded during the entire testing period, since animals used to shuttle between runway and cage.

Results. *FD phase:* As shown in Figure 2, the latencies to eat declined (D: $F_{3,57} = 8.43$, $p = .0001$) and eating times increased over days in the reward group (D: $F_{3,57} = 5.03$, $p = .004$). In the runway maze, locomotion (D: $F_{3,84} = 26.79$, $p = .0001$) and rearing (D: $F_{3,84} = 90.17$, $p = .0001$) but not USV (D: $p > .05$) decreased over days (Figure S2A, S2B, and 2C, respectively). For all these variables, no group differences were observed (G: $p > .05$). In the cage, all animals emitted more 50-kHz calls per time than in the maze (Figure 2D). There, USVs diminished over days (D: $F_{3,84} = 9.67$, $p = .0001$) without differing between groups (G: $p > .05$). Cage locomotion (G: $F_{1,28} = 44.93$, $p = .0001$) and rearing (G: $F_{1,28} = 18.89$, $p = .0001$) were higher in controls (Figure S2C and S2D), perhaps since reward rats were now engaged in eating while controls still explored the cage. *FAL phase:* As in experiment 1, rats received FAL after testing on day 7. Again, the latencies to eat increased (FD vs. FAL: $t_{19} = -29.64$, $p = .0001$) and eating times decreased in the reward group (FD vs. FAL: $t_{19} = 10.31$, $p = .0001$). In contrast, and consistent with experiment 1, reward cues associated to the runway maze now elicited enhanced 50-kHz calls (Figure 2C). For instance, total call number in reward rats was now ~210% higher compared with their own USVs levels while FD (FD vs. FAL: $t_{19} = -13.10$, $p = .0001$), and ~195% higher than that in controls on all FAL days (G: $F_{1,28} = 7.07$, $p = .01$). In the cage (Figure 2D), 50-kHz calls increased in both groups (D: $F_{3,84} = 4.72$, $p = .004$), but did not differ from each other (G: $p > .05$). There were no group differences in locomotion or rearing (G: $p > .05$) (Figure S2).

Discussion. This experiment replicated the results from Experiment 1 in which no differences in total call number were observed under FD, but increased USVs occurred in reward animals once they became sated. The suppressive effect of FD probably did not depend on the type of learning recruited, the behavioral competition between approach/consummatory and anticipatory affective responses, or the interference of an opposite behavior such as digging since the maze had no bedding. Since animals were free to shuttle between maze and cage and most reward animals revisited the maze between eating bouts (details not shown), maze cues were not just temporally predicting further access to reward, but also became imbued with incentive salience after animals re-experienced the UCS, facilitating CS representations to be re-updated within and across training days. As a consequence, runway maze cues, but not cage cues, triggered appetitive 50-kHz calls suggesting that USVs were specifically sensitive to the type of learning mechanism recruited. Again, attribution of incentive salience to food cues seemed to take place during the FD period, whereas the expression of such an appetitive response occurred once animals became sated, that is, when the reward was devalued and when no other appetitive behaviors were emitted. Finally, the analysis of USVs in the cage revealed that satiation on its own increased 50-kHz calls irrespective of being food rewarded or not.

Experiment 3

Introduction. As just shown, the current physiological state of the rat produces a biphasic expression of 50-kHz calls in reward rats, which according to Experiment 2 is not dependent on the interference between preparatory and consummatory responses [16] or the competition between species-specific responses activated by the UCS (e.g., foraging inhibiting calling, for review see [37]). To simplify data collection and analysis we went back to the test settings of Experiment 1. Now, we asked whether increasing the incentive properties of the reward would enhance motivation for UCS during the FD period. To this end, a high palatable reward (i.e., sweetened condensed milk) was used. Since the reward delivered in the testing cage was different from normal rat chow, access to reward became independent from the daily feeding session. Thus, we expected that it could still be valuable when testing animals under satiation.

Methods. Twenty-four experimentally naïve rats weighing 231–256 g on arrival were used. Habituation to the animal facilities, handling, the screening cage test, and FD schedule were conducted as in Experiment 1, but now, the CS signaled access to a 30 min-drinking period: ~2 min in the cage and the remaining time in the animal room. The reward group had access to sweet condensed milk, whereas the control group had access to tap water. One week before testing, rats were habituated to sweetened condensed milk. During this period, all rats were handled and weighed every other day. Testing was performed with the former FD/FAL schedule: FD from day 1 to 7, and FAL thereafter (days 8–10).

Results. *FD phase:* The latencies to drink (Figure 3) diminished slightly once training began, with reward rats being ~6 times faster than controls, which were given a bottle with tap water (G: $F_{1,22} = 85.05$, $p = .0001$; DxG: $F_{3,66} = 16.34$, $p = .002$). The times spent drinking (G: $F_{1,22} = 538.80$, $p = .0001$; DxG: $F_{3,66} = 15.78$, $p = .0001$) and daily milk consumption (G: $F_{1,22} = 582.71$, $p = .0001$; DxG: $F_{3,66} = 4.19$, $p = .009$) were higher in the reward group where they increased also over days. Total call number augmented over days in all animals (D: $F_{3,66} = 12.05$, $p = .0001$), especially after the third day (Figure 3D), but did not differ significantly between groups (G: $p > .05$). In contrast, the percentage of tone-induced 50-kHz calls was higher in the reward group (G: $F_{1,22} = 19.05$, $p = .0001$) (Figure 3E). *FAL phase:* When tested without deprivation, there was a transitory increase in the latency to drink and a transitory reduction in the time spent drinking which fully recovered on the following FAL days. Despite such small variations when feeding conditions changed, reward rats still differed from controls on latencies to drink (G: $F_{1,22} = 76.23$, $p = .0001$) and times spent drinking ($F_{1,22} = 76.23$, $p = .0001$). Milk intake declined drastically (FD vs. FAL: $t_{11} = -11.41$, $p = .0001$), almost reaching control levels on the first FAL day, but was higher than controls again thereafter. On the following FAL days, milk intake partially recovered between about 25% to 43% of the maximal intake achieved under FD (G: $F_{1,22} = 17.08$, $p = .0001$; DxG: $F_{3,66} = 10.03$, $p = .0001$). In addition, locomotion (FD vs. FAL: $t_{11} = -8.45$, $p = .0001$) and rearing (FD vs. FAL: $t_{11} = -9.01$, $p = .0001$) increased in the reward group and exceeded those of controls (locomotion, G, $F_{1,22} = 758$, $p = .01$; rearing, G: $F_{1,22} = 18.06$, $p = .0001$; Figure S3A and S3B). Similar to Experiments 2 and 3, call rate was potentiated by shifting the feeding conditions, an effect which now occurred in both groups (D: $F_{3,66} = 3.14$, $p = .03$). Interestingly, the attenuation

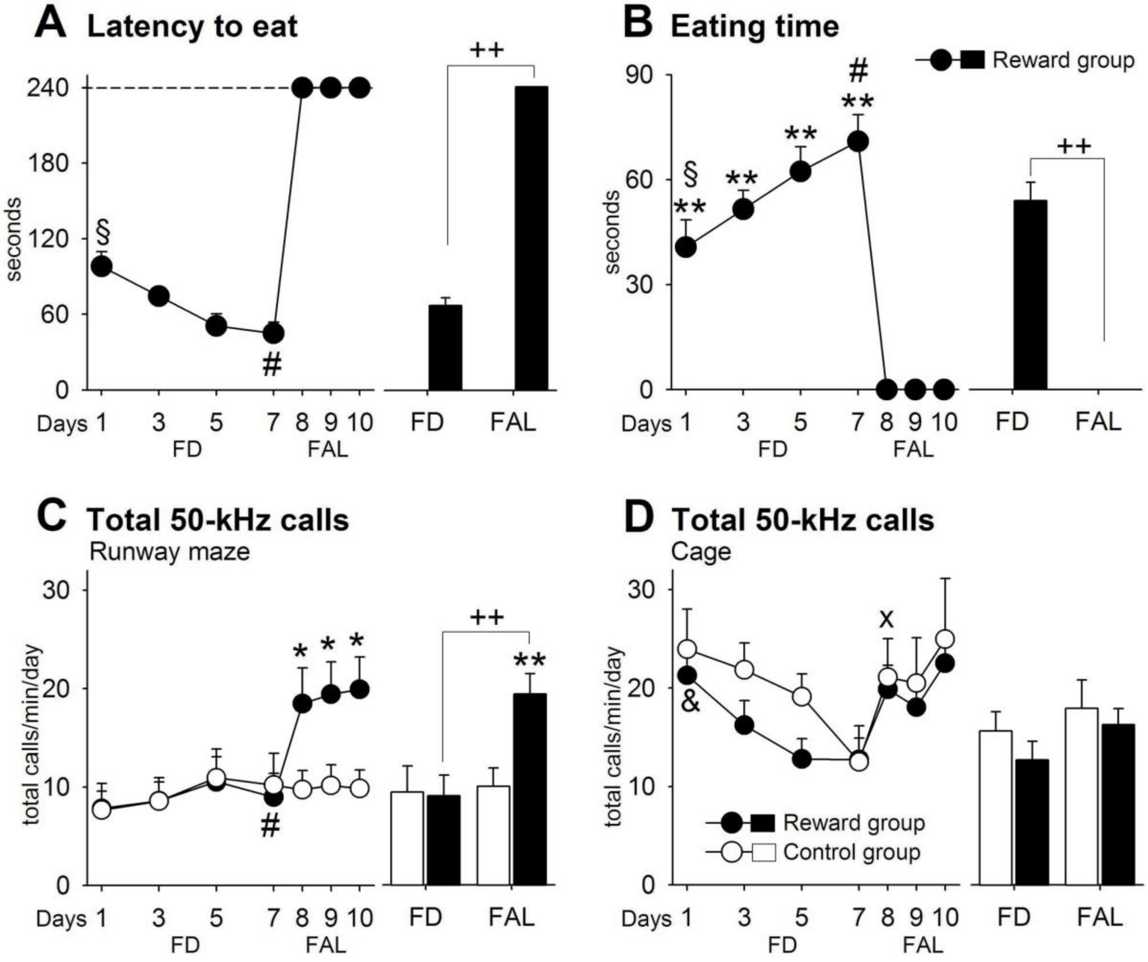

Figure 2. Experiment 2. Animals learned to access their daily feeding in a cage by running through a runway maze attached to it. A. Latency to eat. B. Eating time. C. Total 50-kHz calls in the runway maze. D. Total 50-kHz calls in the cage. The dashed line indicates that a maximal latency time was set at 240 s. Control vs. reward: * $p<.05$, ** $p<.01$. FD vs. FAL: ++ $p<.01$. Day 1 differed from days 5 and 7 in reward rats: § $p<.05$. % Day 1 differed from days 3 to 7 in reward rats: § $p<.05$. Days 1 and 3 differed from day 7 in both groups: & $p<.05$. Day 7 differed from days 8 to 10 in reward rats: # all $p<.05$. Day 7 differed from days 8 and 10 in both groups: x all $p<.05$. Data are expressed as mean+SEM (control = 10, reward = 20).
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of approach and consummatory behaviors observed when shifting feeding conditions was not paralleled by a reduction in total call number and percentage of cue-induced calls. Instead, total call number (Figure 3D) was now significantly higher in the reward group (G: $F_{1,22} = 6.60$, $p = .02$), and tone cue-induced calls were also higher (G: $F_{1,22} = 5.18$, $p = .03$) but returned towards control levels over days (Figure 3E).

Discussion. Relative to previous experiments, USVs appeared slightly increased at the end of the FD period, although no overall effect on total call number was detected. However, the percentage of tone-induced calling increased during FD indicating that conditioning strengthened over days. Once again, reward devaluation decreased neither total call number nor tone-induced calling, as it had initially been expected. Instead, total call number increased while cue-induced USVs remained slightly high during FAL days. Regarding reward palatability, the higher incentive properties of the UCS (i.e., milk) plus the likely invigorating effect of the CS seemed to maintain latencies to drink and times spent drinking in the cage while the UCS was degraded in agreement with previous reports [1,3,4]. However, the amount of milk intake, most of which was consumed in the animal room without the influence of the CS, appeared to be drastically reduced by satiety.

Experiment 4

Introduction. So far, the increased percentage of 50-kHz calls induced by the tone cue indicated that attribution of incentive salience to reward-related stimuli had successfully taken place during FD, even thought it was not clearly translated into an overall elevation of 50-kHz calls. To account for such an inhibition in USVs utterance, we assume that the ability of food CS cues to elicit appetitive 50-kHz calls was possibly suppressed by FD, an effect that occurred independently from learning acquisition. So far, the three preceding experiments showed that restoring FAL feeding conditions after FD increased spontaneous USVs in controls and potentiated total call number and food cues-induced appetitive 50-kHz calls in reward rats. This may suggest that FD

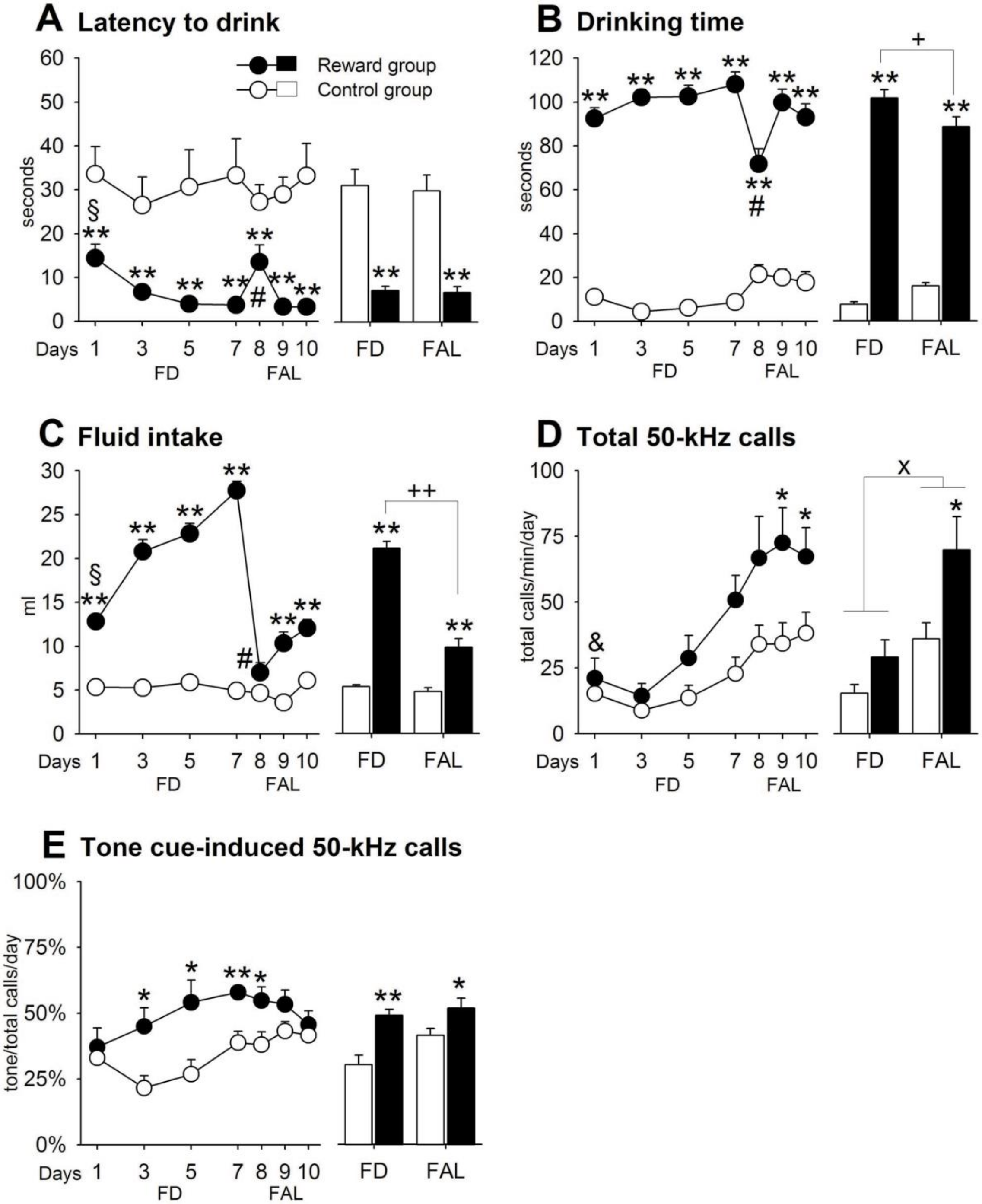

Figure 3. Experiment 3. Animals learned to associate incentive Pavlovian cues with access to sweetened condensed milk as reward. A. Latency to drink. B. Drinking time. C. Fluid intake. D. Total 50-kHz calls. E. Tone cue-induced 50-kHz calls. Control vs. reward: * $p<.05$, ** $p<.01$. FD vs. FAL: + $p<.05$, ++ $p<.01$. FD vs. FAL in both groups: x all $p<.05$. Day 1 differed from days 3 to 7 in reward rats: § $p<.05$. Day 1 to 5 differed from day 7 in both groups: & $p<.05$. Day 8 differed from days 7, 9, and 10 in reward rats: # all $p<.05$. Data are expressed as mean+SEM (control = 12, reward = 12).
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itself was able to suppress USVs particularly at the time when animals were expecting the food reward. Having access to food after a long FD period recruits not only positive but also negative reinforcement mechanisms, and may be described as a transition from distress to pleasure [38]. FD can induce an aversive state so that animals will work to prevent starvation by either prolonging the period of food availability [39] or escaping from a CS signaling the omission of an expected food reward [40]. This evidence raised the question of whether the same palatable reward (i.e., milk), now acquired in the absence of FD, would be sufficient to increase appetitive 50-kHz calls.

Methods. Twenty experimentally naïve rats weighing 259–279 g on arrival were used. The experimental procedure was generally the same as in Experiment 3, with sweetened condensed milk also used as reward. However, contrary to all previous experiments, the acquisition phase (days 1–7) of UCS-CS pairing occurred first in the FAL phase and was followed by the FD phase (days 8–10).

Results. *FAL phase:* As expected, reward rats showed shorter latencies to drink (G: $F_{1,18} = 1252.46$, $p = .0001$) and more time spent drinking (G: $F_{1,18} = 172.56$, $p = .0001$) than controls which consumed almost none of the tap water (Figure 4A and B). As soon as reward animals had learned that milk was available, latencies and drinking times did not change over the FAL days (D: $p > .05$). However, the amount of milk (Figure 4C) consumed augmented with repeated testing (G: $F_{1,18} = 8.02$, $p = .01$; DxG: $F_{3,54} = 4.05$, $p = .01$). Total call number increased over days (D: $F_{3,54} = 24.86$, $p = .0001$), with no significant differences between groups (G: $p > .05$) (Figure 4D). Likewise, no differences were observed for the percentage of tone-induced calling (Figure 4E). Also, exploratory activity appeared unaffected by both, repeated testing and reward experience (Figure S4A and S4B). *FD phase:* Latencies to drink (G: $F_{1,18} = 147.51$, $p = .0001$) and times spent drinking (G: $F_{1,18} = 2039.35$, $p = .0001$) remained higher in the reward group compared to controls (Figure 4A–B). The more noticeable effect of FD occurred on the amount of milk consumed, which scaled up between 34% to 78% over preceding FAL levels (FD vs. FAL: $t_9 = -11.47$, $p = .0001$; Figure 4C), whereas water intake remained unaffected (G: $F_{1,18} = 190.58$, $p = .0001$). Contrary to approach and consummatory behaviors, call rate dropped drastically on the first FD day in both groups (Figure 4D) (D: $F_{3,54} = 16.58$, $p = .0001$). On the following FD days, total call number progressively returned to FAL levels only in the reward rats (DxG: $F_{3,54} = 3.74$, $p = .03$). Similarly, the percentage of tone-induced USVs was significantly higher in the reward group (G: $F_{1,18} = 11.82$, $p = .003$) (Figure 4E). Locomotion (FD vs. FAL: $t_9 = 3.31$, $p = .009$) and rearing behavior (FD vs. FAL: $t_9 = 5.96$, $p = .0001$) were reduced in controls (Figure S4), whereas in reward rats only locomotor activity was reduced by FD (FD vs. FAL: $t_9 = 2.96$, $p = .02$).

Discussion. This experiment shows first, that a highly palatable food reward was not sufficient to increase either total call number or tone cue-induced calling on its own and, second, that FD was able to suppress calling in both groups. Since the reward did not have a high hedonic value during the acquisition phase (i.e., when tested under FAL conditions), total call number and cue-induced 50-kHz calls recovered and differed from control levels only when the hedonic representation of that reward was updated while in the subsequent state of being hungry. This is consistent with studies showing that the instrumental response guided by previous reward expectations changes only when the new hedonic value of the incentive is experienced [8,9]. In this as well as in previous experiments, the motivational state of being FD seems to be required for reward cues to be imbued with incentive salience, even though it suppressed overall USVs utterance.

Experiment 5

Introduction. The likely aversive state provoked by long FD may have accounted for some suppressive effects in calling in our previous experiments. In experiment 3, however, total call number tended to increase while FD, an effect that, according to Experiment 4, may not solely be attributed to palatability. Another factor might be critical: By replacing rat chow with milk as a reward we also inevitably altered the predictive association between access to reward in the cage and daily feeding session. If expectations about reward were controlled by the very first access to food or milk (2 min) –and not by the whole period of eating (1.5 h) or drinking (30 min)–, it is very likely that animals learned to anticipate the short access to reward instead of the long one. Thus, when animals encountered the reward, a negative discrepancy between the reward expected and the one actually obtained may have been experienced, an effect probably energized by FD. We hypothesized, therefore, that providing continued access to reward in the testing environment would prevent such negative discrepancy to occur, 'releasing' the expression of reward-related appetitive USVs when FD. To test this idea, we adapted the procedure of Experiment 1 in which the higher suppression in calling was observed.

Methods. Twenty experimentally naïve rats weighing 240–265 g on arrival served as subjects. Habituation to the animal facilities, handling, and the screening cage test were conducted largely as in Experiments 1 and 2: Food pellets (normal rat chow) served 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. Indeed, during habituation to FD, reward rats had access to the 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. During testing, rats were FD from days 1 to 7. Controls never accessed their daily food ration either in the cage or in the experimental room where testing took place.

Results. Animals approached the food and started eating without any noticeable change from the beginning to the end of testing (D: $p > .05$) (Figure 5A and B). Contrary to our previous food experiments, reward cues did now increase total call number over FD days (DxG: $F_{3,54} = 8.19$, $p = .0001$) (Figure 5C), and calls were approximately 120% higher than that in controls (G: $F_{1,18} = 7.54$, $p = .01$), which showed rather stable call rates over days. Likewise, the percentage of tone-induced calling was significantly higher in reward rats than in controls (G: $F_{1,18} = 17.90$, $p = .001$) (Figure 5D). Unlike locomotion (Figure S5A), rearing behavior in reward rats showed a progressive increase mirroring the one observed for USVs (G: $F_{1,18} = 11.07$, $p = .004$; DxG: $F_{3,54} = 9.81$, $p = .0001$), yet to a lesser extent (Figure S5B).

Discussion. This experiment shows that providing continuous access to reward in the testing environment enables attribution and expression of incentive salience during the FD period, and contrary to previous experiments, this effect was now noted both in terms of total call number and the percentage of cue-induced 50-kHz calls.

General Discussion

Following Pavlovian incentive learning, a CS for reward comes to produce expectations and potentiates motivation about the UCS. Here, we sought to determine whether rat 50-kHz USVs may signal such a state of incentive motivation to a natural,

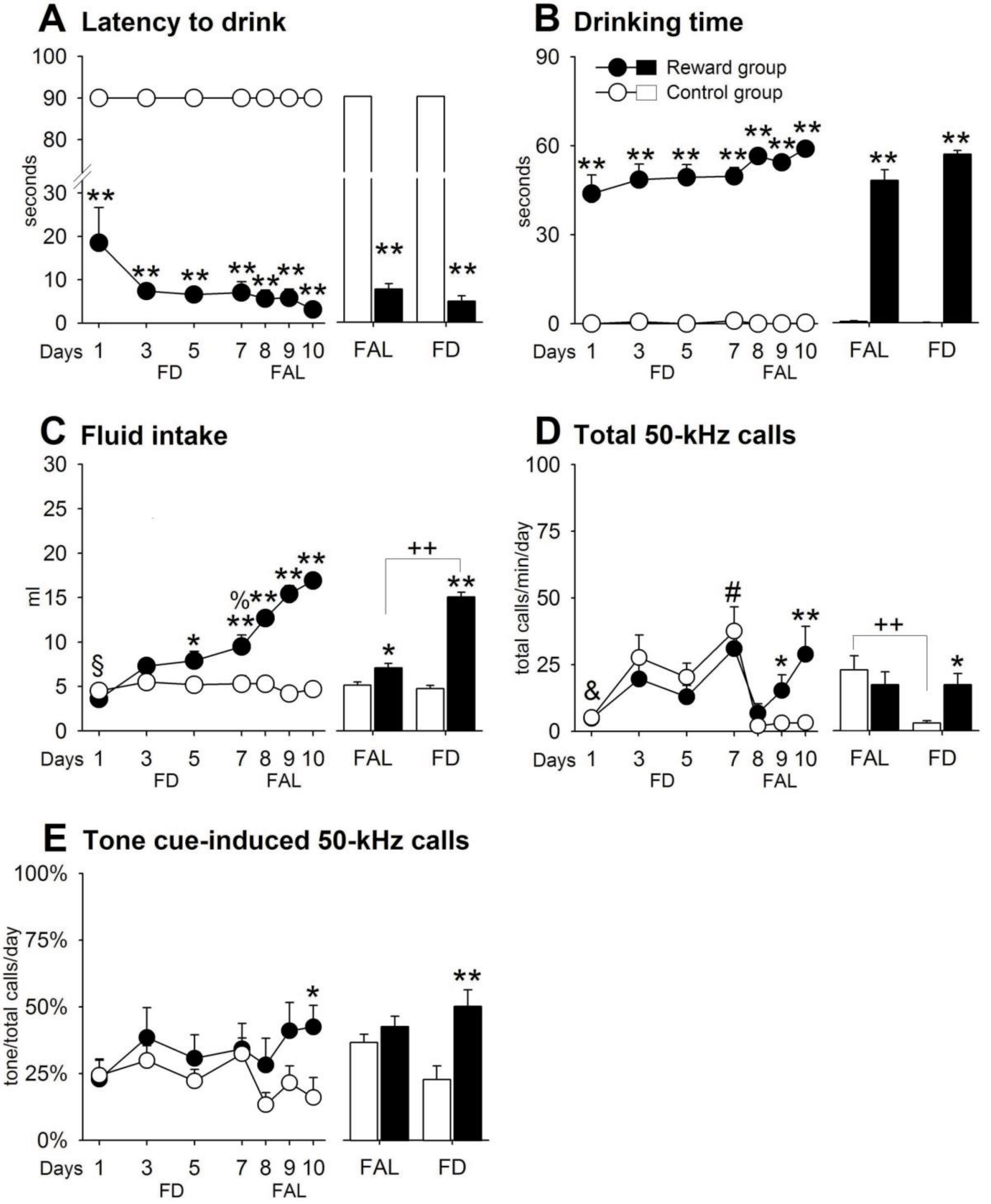

Figure 4. Experiment 4. Animals learned to associate incentive Pavlovian cues with access to sweetened condensed milk as reward. A. Latency to drink. B. Drinking time. C. Fluid intake. D. Total 50-kHz calls. E. Tone cue-induced 50-kHz calls. Control vs. reward: * $p<.05$, ** $p<.01$. FAL vs. FD: ++ $p<.01$. Day 1 differed from days 5 and 7 in reward rats: § $p<.05$. Day 1 differed from days 3 to 7, and day 5 differed from day 7 in both groups: & $p<.05$. Day 7 differed from days 8 to 10: % $p<.05$. Day 7 differed from all FD days in controls, and from day 8 in reward rats: # all $p<.05$. Data are expressed as mean+SEM (control = 10, reward = 10).

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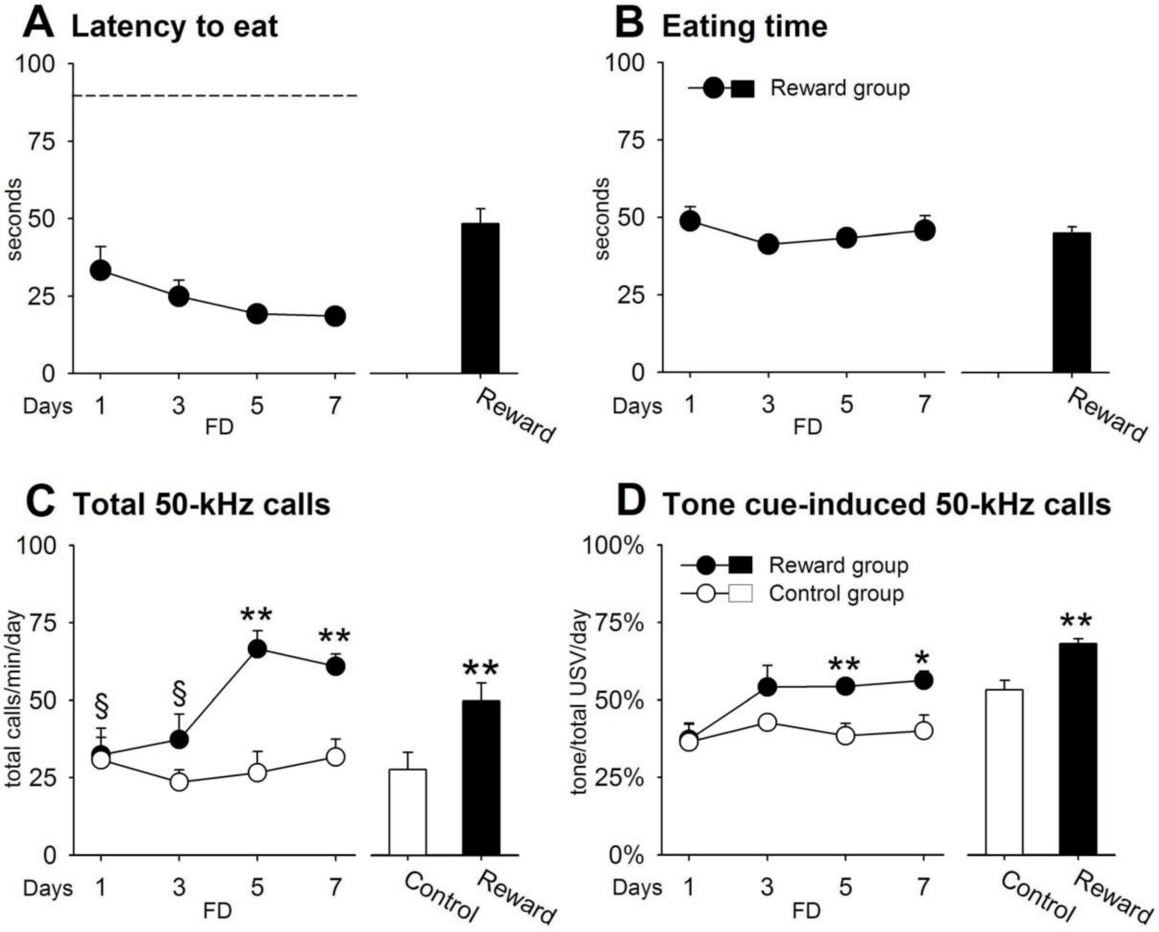

Figure 5. Experiment 5. Animals learned to associate incentive Pavlovian cues with access to daily feeding as reward. A. Latency to eat. B. Eating time. C. Total 50-kHz calls. D. Tone cue-induced 50-kHz calls. The dashed line indicated that a maximal latency time was set at 90 s. Control vs. reward: * $p<.05$, ** $p<.01$. Days 1 and 3 differed from days 5 and 7 in reward rats: § $p<.05$. Data are expressed as mean+SEM (control = 10, reward = 10).
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nutritional reward. In general, we found that reward-cues become effective to elicit 50-kHz calls. Under certain conditions, however, utterance of 50-kHz calls can be either suppressed during a highly motivational state, or more strikingly, can be elicited when food rewards were devalued by satiation. In both cases, changes in calling occurred independently from motivation to approach and consume the reward. Interestingly, this phasic character of USVs was consistently seen across independent experiments.

We acknowledge that the question of whether 50-kHz calls can be elicited by food rewards has already been addressed. The pioneer study [23] showed an increase in anticipatory 50-kHz calls in FD rats (i.e., before daily feeding) at the end of a 6-days period of training. In a sucrose self-administration paradigm conducted with non-FD rats, 50-kHz calls increased over time when comparing different time points throughout a testing period of 4–5 weeks [30]. Others described differences in 50-kHz calls between adolescent and adult rats when offered chocolate chips in a one-trial test [32], and one study yielded differences in calling following positive and negative reward contrasts with different probabilities to obtain sucrose reinforcement [31]. In all these studies, however, it was unclear whether call rate in food-paired rats was different from spontaneous calling mainly because no control rats were included [23,30–32]. It has repeatedly been observed that rats call at moderate rates simply by the fact of placing them in certain testing environments [27,33,41]. For example, in the only two studies showing positive results [23,30] calling increased over days in a similar way as it did in our control rats in Experiments 1 and 4, in which no groups differences were found until feeding conditions were changed. Also, previous studies did not test whether increases in 50-kHz calls were accompanied by changes in other relevant learning or motivational parameters because no concomitant behavioral measures were described [23,30,32]. In general, we went beyond those studies showing that USVs in reward rats as compared to matched controls were differentially sensitive to the current physiological state of the subject (FD vs. FAL), the type of learning mechanism recruited (more Pavlovian vs. more instrumental), the hedonic properties of the UCS (low vs. high palatable food), its availability (continued vs. discontinued), and the relation between 50-kHz calls and other behavioral dimensions indicative of incentive learning and conditioning.

Our results also make us believe that FD on its own induced a putative negative state that affected USVs likelihood. This assumption is also supported by our previous findings in which FD consistently suppressed spontaneous calling over four consecutive days [33]. Since FD is aversive enough to strongly motivate escape and avoidance responses [38–40], it is not surprising that FD exerted a suppressive effect on 50-kHz calls utterance similar to those produced by other aversive stimuli [21,22,25]. Still the question remains, how FD particularly suppressed the expression of the emotional conditioned response during acquisition while being FD. In all experiments in which reward intake was discontinued (Experiments 1–4), animals seemed to learn about the association between testing and the 2-min access to reward permitted while they remained in the testing room. Figure 6 depicts a model that summarizes our assumptions in terms of a suppression-release hypothesis, in which we propose that FD played a critical role in the suppression of cue-induced calling by increasing the negative contrast between reward expectation and outcome. As training progressed, the 2-min access to food became gradually insufficient for experiencing the positive effects of consuming the food and therefore the expectation of being fed and becoming sated did not match each other. For example, in the rat chow experiment access to the reward in the cage predicted the 1.5-h feeding session, which was the only feeding opportunity animals had. In the milk experiment, conversely, access to reward in the cage predicted only a 30-min drinking time, which was independent of feeding. Although the duration of daily feeding was adjusted to keep body weight at 90% of baseline based on the caloric income of milk, animals still had two unrelated feeding opportunities as compared to only one in rat chow experiments. Thus, reward expectation should have been proportional to the magnitude and density of reward, being therefore greater when giving chow pellets than when giving milk. To better illustrate this point, calling was compared between Experiments 1 and 3: Figure 7 shows call rate as relative to control levels to normalize the fluctuations in calling due to the FD itself. As can be seen in FD rats receiving normal rat chow, the score for cue-induced calling is negative, namely, 25% lower than spontaneous calling in controls. Once sated, calling increased by 90% over control levels. In rats receiving milk reward, in contrast, the relative increase in cue-induced calling was about 90% over control levels while FD but also in the FAL phase. This indicates that suppression in cue-induced calling depended upon the predictive strength of the reward in relation to feeding: the larger the discrepancy between expectation and UCS outcome, the greater the suppression. Once sated, the magnitude of suppression released was inversed to the strength of suppression that preceded it. We acknowledge that both rewards differed in their incentive properties, but as shown in Experiment 4, palatability on its own is unlikely to account for all differences observed between experiments using chow pellets vs. milk. In addition, we plotted calling results of Experiments 1 and 5 to highlight the contribution of reward expectations on USVs suppression (Figure 7B). When providing continued access to reward while all other factors remained equal, reward expectation matched outcome and thus, suppression was no longer observed. Then, attribution and expression of cue-induced calling occurred simultaneously over FD days. Based on this assumption one might think, even counter-intuitively, that what FAL did was removing the negative expectation of the outcome given by the difference between being hungry and having access to an insufficient reward. Without the urge for food, the outcome matched the expectation of being rewarded. In other words, restoring the normal feeding conditions acted as a 'releasing' factor of the negative difference between reward expectation and outcome, which did not require updating and occurred afresh as soon as the current physiological state shifted (for an example of instant transformation of an aversive cue into a desired one see [42]). The suppression in the conditioned response cannot be explained in terms of conditioned inhibition, because the UCS was always presented and therefore no CS signaled its absence. A negative difference between reward expectations and the actual outcome has previously been described in terms of a frustration effect [40,43] (for review see [44]). Such a reward discrepancy or devaluation can induce a putative, negative affective state able to elicit escape responses [39,40], intra- and hetero-specific aggression [45], high corticosterone levels [46], and distress USVs. For example, 11-day-old rat pups that had learned to approach an anesthetized dam with dry suckling as a reward showed distress USVs when reward was denied [47]. In adult rats, reductions in 50-kHz calls or increases in distress USVs (i.e., 22-kHz calls) have also been reported following timeout, withdrawal, or devaluation of different rewards [26,31,48]. Here, it should be noted that distress calls were only rarely observed in our present experiments.

It is still surprising however, that the updating of hedonic value of the UCS under a state of satiation did not reduce the ability of the CS to induce appetitive 50-kHz calls across experiments. If animals had not experienced the reward during the FAL phase we can perhaps assume that rats were behaving based on previous expectations built up when the reward was still valued [8,9]. However, some animals in the rat chow experiments approached the food, sniffed it or gnawed it; something that may have been enough to re-update the new hedonic value of that reward, even though none actually ate a piece of food in the cage (it was freely available in home cages). In contrast, in the milk experiment (3) all animals re-experienced the reward during satiation. At least from day 8 to day 9, and from there to day 10, a re-update of the current hedonic value of the food reward should have happened. Nevertheless, it did not impede incentive motivation reactions to occur (i.e., 50-kHz calls and, to a lesser extent, rearing behavior). We acknowledge that the FAL period measured may not have been long enough for a back-to-baseline drop in USVs rate, however, it was apparently sufficient for consummatory behavior to be completely abolished (rat chow experiments) or drastically reduced (milk experiment). We have provided a putative explanation for this effect consistent with the suppression-release hypothesis developed here. According to the Konorskian opponent-process model [16] (e.g., see [9]), we propose that the release of a motivational system (i.e., appetitive) from inhibition by the opponent system (i.e., aversive state induced by negative reward expectations maximized by FD) produced a rebound appetitive excitation, which may have lasted longer and decayed slower for preparatory/emotional responses than for consummatory/sensorial ones [16,34]. The latter responses are normal unconditioned feeding behaviors that are expected to be rapidly suppressed or activated according to physiological demands. Cue-induced USVs and exploratory activity, in contrast, are acquired conditioned responses controlled more by environmental stimuli rather than by the current appetitive state. It seems quite reasonable that cues that had signaled food availability while in a state of need were still able to guide behavior despite physiological needs were transiently fulfilled, since requirements of food may change as a matter of hours and, therefore, places where it was consistently available should be well remembered. This differential expression of incentive motivation responses may explain why right after satiation 50-kHz calls appeared detached from food seeking and consumption. To our knowledge, there is no such evidence as the one presented here, and as recently reviewed elsewhere [5], persistent incentive motivation has only been described when the

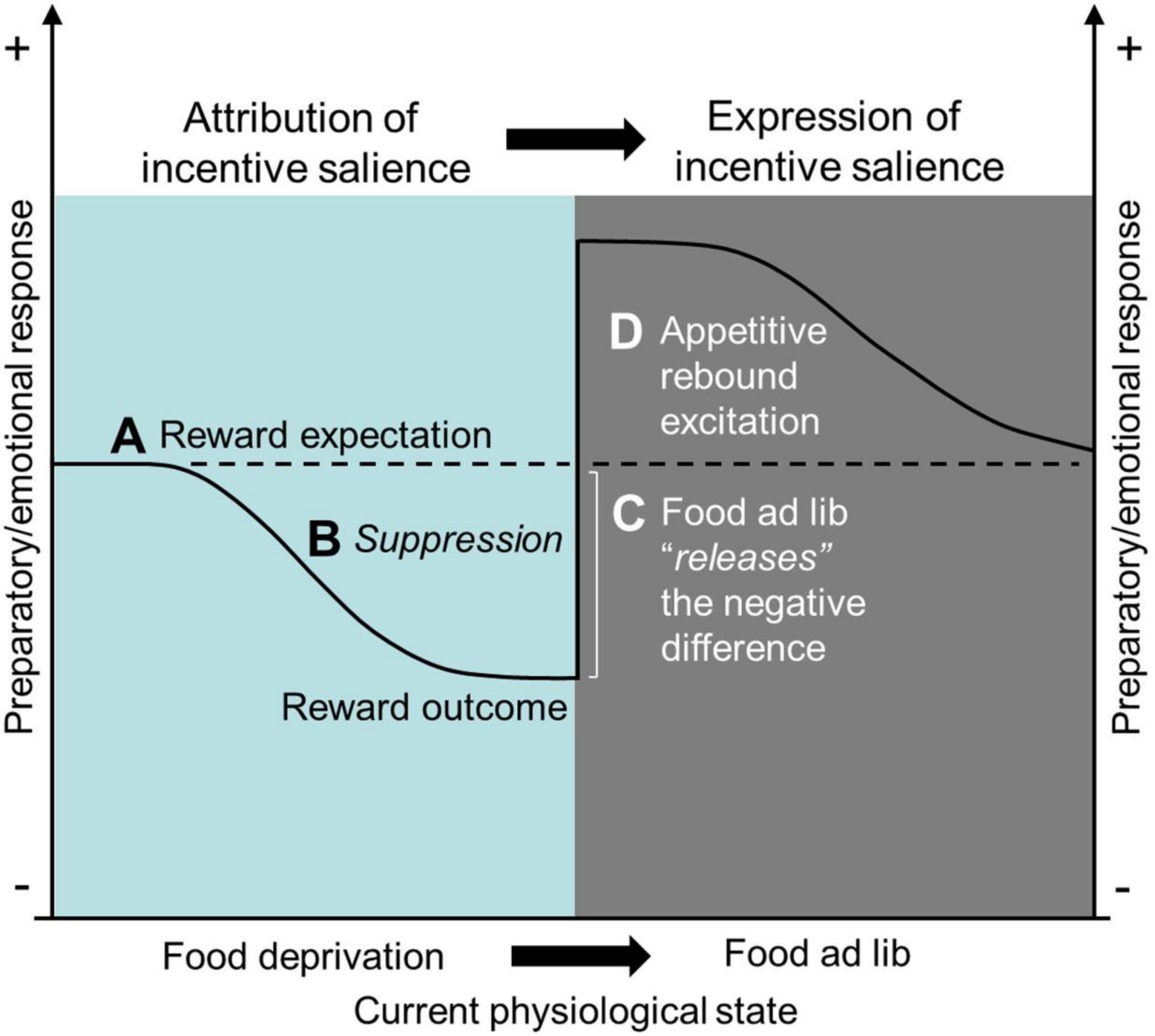

Figure 6. Suppression-release hypothesis. This hypothesis integrates statements modified from previous models [16,34]. A) If the strength (positive magnitude) of the actual UCS is less than the strength of the subject's expectation (maximized by food FD), the result will be suppression in the expression of the conditioned response. B) The larger the discrepancy between the strength of the expectation and the strength of the UCS outcome, the greater the suppression. C) If the strength of the actual UCS equals the strength of the subject's expectation, no suppression will be observed. Then, the magnitude of the suppression released would be proportionally inversed to the strength of the suppression that precedes it. Restoring the normal feeding conditions acts as a ''releasing'' factor of the negative difference between reward expectation and outcome, which does not require updating and which occurs afresh as soon as the current physiological state shifts. In the present experiments, the suppression in the expression of the conditioned response cannot be explained in terms of conditioned inhibition, because the UCS was always presented and therefore no CS- signaled its absence. D) The release of a motivational system (i.e., appetitive) from inhibition by the opponent system (i.e., aversive state induced by negative reward expectations) produces a rebound appetitive excitation that may last longer and decay slower for preparatory/emotional conditioned responses than for consummatory/sensorial ones.
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UCS was devalued in the absence of the previous CS or following over-training, which was not our issue. Further experiments employing different conditioning paradigms with longer and more diverse testing schedules are required to elucidate the phenomena shown here, especially regarding USVs. However, it seems clear so far that independent of Pavlovian or instrumental task demands, reward palatability, reward accessibility and availability, devaluation of reward did not reduce cue-induced appetitive 50-kHz and rearing behavior, and more strikingly, shifting the current physiological state of the animals, and probably their subjective emotional state too, elicited appetitive 50-kHz calls by the pure incentive salience attributed to the environmental cues of the testing situation.

Conclusions

After Pavlovian incentive learning, reward-cues became able to elicit 50-kHz calls presumably signaling a state indicative of

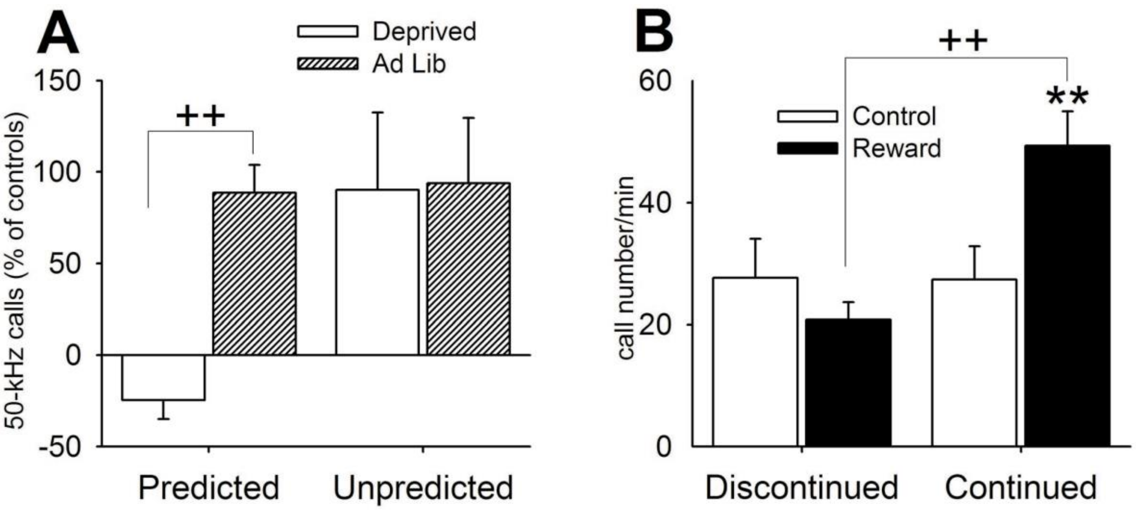

Figure 7. Comparisons between Experiments 1 and 3 (A) and 1 and 5 (B). Access to daily feeding (1.5-h) was predicted by reward in the cage in Experiment 1 (A, left) but not in Experiment 3 (A, right), in which animals received a reward independent of feeding. Access to reward was discontinued (B, left) in Experiment 1 (∼2 min in the cage and the remaining time of 1.5-h period in the animal room). Providing continued access to reward in the testing cage (B, right) prevented the suppression in calling observed in Experiment 3. Control vs. reward: ** $p<.01$. FD vs. FAL interaction: ++ $p<.01$. Data are expressed as mean+SEM.
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appetitive incentive motivation in the rat. Attribution and expression of incentive salience, however, do not seem to be a unified process, and were teased apart in two different ways: 1) under a high motivational state (i.e., hunger) the attribution of incentive salience to cues occurred without being expressed at the USVs level; and 2) under a low motivational state (i.e., food satiation), expression of appetitive USVs persisted despite physiological needs being fulfilled. In both cases, putative affective incentive responses were elicited independently from motivation to approach and consume the reward. While in a hungry state, short access to rewards may have led to a discrepancy between the reward expected and the one actually obtained that likely suppressed expression of USVs. When such a discrepancy between reward expectations and outcome was prevented by providing continued access to food, attribution and expression of incentive salience synchronized. Similarly, shifting feeding conditions from deprivation to satiation acted as a 'releasing' factor of the putative aversive state induced by both reward discrepancy and food deprivation. Such a release of a motivational system from inhibition led to a rebound appetitive excitation that lasted longer and decayed slower for preparatory/emotional responses than for consummatory/sensorial ones. The latter may explain why appetitive 50-kHz calls increased while sated but detached from reward seeking and consumption. Finally, the fact that rats seem to represent rewards emotionally (for review see, [21]) and beyond apparent, immediate physiological demands, provides an unparalleled translational tool to model motivational mechanisms underlying eating disorders, and may even be extendable to other forms of aberrant or persistent motivation such as in drug addiction, or gambling disorders.

Supporting Information

Figure S1 Experiment 1. Anticipatory activity. Locomotion (A) and rearing behavior (B). Control vs. reward: * $p<.05$, ** $p<.01$. FD vs. FAL: ++ $p<.01$. Day 7 differed from days 8 to 10 in reward rats: # all $p<.05$. Data are expressed as mean+SEM (control = 10, reward = 20).
(TIF)

Figure S2 Experiment 2. Anticipatory activity. Locomotion and rearing in the runway maze (A–B). A. Locomotion and rearing in the cage (C–D). Control vs. reward: ** $p<.01$. FD vs. FAL: + $p<.05$, ++ $p<.01$. FD vs. FAL in both groups: xx all $p<.01$. Day 1 differed from days 3 to 7 in both groups: § $p<.05$. Data are expressed as mean+SEM (control = 10, reward = 20).
(TIF)

Figure S3 Experiment 3. Anticipatory activity. Locomotion (A) and rearing behavior (B). Control vs. reward: ** $p<.01$. FD vs. FAL in both groups: xx all $p<.01$. Data are expressed as mean+SEM (control = 12, reward = 12).
(TIF)

Figure S4 Experiment 4. Anticipatory activity. Locomotion (A) and rearing behavior (B). Control vs. reward: * $p<.05$, ** $p<.01$. FD vs. FAL in both groups: xx all $p<.01$. FD vs. FAL: ++ $p<.01$. Day 7 differed from all FD days in controls, and from day 8 in reward rats: & all $p<.05$. Day 8 differed from day 10 in reward rats: # $p<.05$. Data are expressed as mean+SEM (control = 10, reward = 10).
(TIF)

Figure S5 Experiment 5. Anticipatory activity. Locomotion (A) and rearing behavior (B). Control vs. reward: ** $p<.01$. Day 1 differed from days 3 to 7 in reward rats: § $p<.05$. Data are expressed as mean+SEM (control = 10, reward = 10).
(TIF)

Table S1 (DOC)

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Author Contributions

Conceived and designed the experiments: JCB RKWS. Performed the experiments: JCB. Analyzed the data: JCB. Contributed reagents/materials/analysis tools: RKWS. Wrote the paper: JCB RKWS.

Appendix B: Study 2

Elsevier Editorial System(tm) for Physiology & Behavior
Manuscript Draft

Manuscript Number:

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

Article Type: Review Article

Keywords: incentive motivation; pavlovian conditioning; learning; ultrasonic vocalizations; dopamine; behavior

Corresponding Author: Juan C. Brenes,

Corresponding Author's Institution: University of Costa Rica

First Author: Juan C. Brenes,

Order of Authors: Juan C. Brenes, Rainer K.W. Schwarting, PhD

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 contextual 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 modelling compulsive disorders, which may be due to excessive attribution of incentive salience to reward cues, such as overeating, pathological gambling, and drug addiction.

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

Running title: Individual differences in attributing incentive salience to food cues

Juan C. Brenes [1,2,3] & Rainer K.W. Schwarting [3]

[1] Institute for Psychological Research, [2] Neuroscience Research Center, University of Costa Rica, Rodrigo Facio Campus, 2060 San Pedro, Costa Rica

[3]Behavioral Neuroscience, Experimental and Biological Psychology, Philipps-University of Marburg, Gutenbergstr. 18, 35032 Marburg, Germany

Research Article

Correspondence should be addressed to:

Juan Carlos Brenes

Centro de Investigación en Neurociencias, Universidad de Costa Rica, Post-Office Box ZIP code 2060, San Pedro, Costa Rica

Tel.: +506 2511 49 03

Fax: +506 2511 58 27

E-mail address: juan.brenessaenz@ucr.ac.cr (J.C. Brenes)

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 contextual 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 modelling compulsive disorders, which may be due to excessive attribution of incentive salience to reward cues, such as overeating, pathological gambling, and drug addiction.

Keywords: incentive motivation; Pavlovian conditioning; associative learning; ultrasonic vocalizations; dopamine; amphetamine.

1. Introduction

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: [1]). Out of these, high-frequency calls (i.e., 50-kHz calls) are normally emitted in naturalistic rewarding situations such as mating, and rough-and-tumble play, or triggered by non-naturalistic stimuli such as hetero-specific play simulated by tickling [2–5], electrical stimulation of the mesolimbic dopamine (DA) pathways [6], or by psychostimulant drugs like amphetamine and cocaine [7–16]. The production of spontaneous [4,17,18], and reward-induced USVs is highly dependent of individual differences [4,15,19–24], and the inherent biological background of such inter-individual variability has been demonstrated by breeding rats selectively for their levels of tickling-induced appetitive 50-kHz calls [21,23,25,26]. High callers of 50-kHz USVs seem to show greater reward sensitivity indicated by intra-accumbens and systemic amphetamine-increased calling [19,25], higher sensitization to cocaine-induced 50-kHz calls [23], and higher electrical, and cocaine self-administration rates [6,20].

Sensory, contextual, and situational stimuli related with previous reward experience come to acquire incentive valence through Pavlovian learning [27,28]. Such reward associated cues are not only predicting access to a given reward, but also become transformed into attractive and desired incentives capable of controlling reward seeking and consumption [29–33]. This motivational component of reward is normally referred to as incentive salience [29,34]. Following associative learning, 50-kHz calls can be elicited by contextual and discrete cues previously paired with unconditioned rewarding stimuli such as drugs (i.e., amphetamine, cocaine, or morphine: [10,19,35–38]), or food [35,39]. In the case of food the process of attributing incentive salience to cues and their ability to induce appetitive 50-kHz calls seem to vary as a result of the complex interaction among the physiological state of the rat, and palatability and availability of reward [39]. One remarkable aspect of cue-induced appetitive 50-kHz calls is that their expression can persist despite physiological needs being fulfilled, which therefore detach them from other reward-oriented behaviors that are rather controlled by actual appetitive drives [39]. Although non-human animals may not experience emotions in the same way as humans do, these appetitive 50-kHz calls induced by reward cues resemble the way rewarding affective representations are experienced in humans when discrete cues, contexts, and situations work as reminders of how good that reward had been in the past, but also of how good it will be if experienced again, despite having no actual need for it [30,40–42].

Anticipatory activity in the presence of reward-related cues has traditionally been also taken as evidence of incentive motivation [43,44]. In Pavlovian experimental preparations a localizable visual stimulus usually evokes approach and consummatory behaviors directed towards the reward cue itself (for review see: [45]), whereas diffuse or non-localizable stimulus such as a tone or a testing context would rather enhance behavioral activation [43,46–49]. Both types of non-contingent conditioned responses, although quite consistent, are moderated by individual differences [45,50–52]. It has been widely demonstrated that variations in cue-induced conditioned behaviors indicate how animals attribute incentive salience to otherwise neutral stimuli [10,43,45,53]. In a recent study [39] we noticed that –during training of rats while food deprived– some animals readily displayed high levels of exploratory activity, especially rearing behavior, as soon as they encountered the testing cues even during the first training trails. Such rearing activity seemed to be contingently and topographically related to reward delivery since food was provided from above upon the testing cages. Once fed ad libitum, high rearing levels were still observed in some subjects in anticipation of food, even though they were sated and thus no longer motivated to eat. In the current study, therefore, individual differences in rearing behavior as well as in other forms of anticipatory activity elicited by food related cues were analyzed. In experiments 1 to 3, food deprived rats were trained to anticipate food rewards (normal rat chow vs. sweetened condensed milk) under certain contextual cues. In a fourth experiment, rats were instrumentally conditioned to access their daily feeding ration by running down a runway maze. In both types of experiments we asked whether animals that develop high levels of conditioned anticipatory activity show higher rates of 50-kHz calls, especially when food rewards were devalued. Afterwards, previously trained rats were re-exposed to food cues after a free-training week (experiment 5) to evaluate first the ability of reward cues to induce appetitive 50-kHz calls, and second to determine whether preceding individual differences in anticipatory activity still affect rates of USVs. Finally, reward-experienced rats were challenged with the DAergic (and noradrenergic) agonist amphetamine (experiment 6) or with the DA receptor antagonist flupenthixol (experiment 7). There, reward-experienced rats were expected to show a diminished response to the particular effect of each drug, and such an effect would indicate the occurrence of behavioral cross-tolerance between Pavlovian incentive learning and DAergic-related drugs [54–57]. Second, 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 of mesolimbic DA activity [34,50,53].

2. General Materials and Methods

2.1. Subjects

Adult male Wistar rats (Harlan-Winkelmann, Netherlands) served as subjects. Upon arrival they 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 [16]. 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 [4,11,18]. 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 [39], 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 were 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 milk experiments (3 and 4) all animals were first habituated to the sweetened condensed milk for one week. During this period, controls 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.

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 x 20 cm W x 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 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 during 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 used to shuttle 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 [39]. Fluid intake was determined by weighing bottles before and after testing.

2.6. General procedure

For all experiments, rats were handled during four days (5 min each); afterwards, two consecutive screening cage tests were conducted (see 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 [11,16,18], 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. Experienced observers manually counted the USVs off-line from the spectrograms. Exactly as recently described [16], 50-kHz calls were further classified into flat, step-calls, and trills according to their shape and peak frequency (for exemplary sonograms see Figures 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 [58,59]. 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 (sing-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-groups 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 calls 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. Statistical significance was defined as $p<.05$.

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

3.1. Introduction

Here, we analyzed individual differences in anticipatory activity in animals trained to associate cues with food rewards through Pavlovian conditioning (reward rats) compared to matched-unpaired rats (controls), which underwent the same procedure but food rewards were never provided to them in the testing setting. Animals were FD on days 1 to 7 and afterwards they obtained FAL in their home cages (days 8 to 10). The comparisons between control and reward groups within feeding schedules and across experiments have already been published elsewhere [39], and are therefore beyond the scope of the current analyses. Here, we focused on investigating 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 [39], and since it seemed to be contingently and topographically related to the way that food rewards were delivered (data not shown). In experiment 1, rats were provided with a low palatable food reward (i.e., 90 min access to their daily food ration of normal rat chow), the consumption of which started in the presence of the experimental cues but lasted only for a short time (~2 min). In experiment 2, access to reward was exactly as in experiment 1, but to a higher palatable reward (sweetened condensed milk). In experiment 3, animals were provided with the same reward as in experiment 1, but access to their daily food ration took place exclusively under the testing cues. The magnitude of food rewards was expected to be differentially perceived in experiments 2 and 3 as compared to that in experiment 1, leading to proportional differences in anticipatory activity (despite reward density being almost the same among experiments). For all experiments, latencies to approach the rewards, the times spent consuming them, locomotor activity, and USVs were analyzed.

3.2. Methods and results - experiment 1

Thirty experimentally naïve rats weighing 277–351 g on arrival were used. Here, the tone CS signaled the start of each feeding session, which began in the ultrasonic lab and ended in the animal room (see 2. Materials and Methods for details). A control rat was tested simultaneously in an adjacent room, where it received the same pairing schedule as the matched reward rat, except that a hopper of chow pellets was never placed upon the cage grid. As shown in Fig. 1A, 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. The decreased rearing activity of LR seemed to develop with time, since it was not observable on the first day of testing. Latencies to eat and times spent eating were unaffected by individual differences in rearing behavior (Fig. 1B) (G: p>.05). Similarly, locomotor activity did not differ between LR (11.76±.55, mean±SEM) and HR (13.18±.73) rats (G: p>.05). As observed for rearing behavior, LR rats showed less USVs than controls and HR rats (G: $F_{2,27}$=4.66, p=.02), which did not differ from each other (Fig. 1C). When subsequently tested under FAL conditions, HR rats emitted more calls than the other groups (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).

3.3. Methods and results - experiment 2

Twenty-four experimentally naïve rats weighing 231–256 g on arrival were used. Contrary to experiment 1, the CS now signaled access to a 30 min-drinking period: ~2 min in the cage and the remaining time in the animal room. The reward group had access to sweet condensed milk, whereas the control group had access to tap water. As shown in Fig. 2A, 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). Like in experiment 1, no differences in locomotor activity (LR: 16.15±1.69 and HR: 16.10±.27; G: p>.05), latencies to drink, and times spent drinking were observed between LR and HR rats (G: p>.05) (Fig. 2B). Regarding USVs (Fig. 2C), HR rats showed more appetitive 50-kHz calls than LR and control rats during FD (G: $F_{2,21}$=8.27 p=.002) and FAL phases (G: $F_{2,21}$=5.94 p=.009). Interestingly, in LR rats reward-related cues were ineffective to augment calling over control levels (Fig. 2C). While FAL, differences in calling between LR and HR rats were still observed even though they no longer differed in rearing (LR: 14.05±1.49, HR: 14.45±.64; G: $p>.05$).

3.4. Methods and results - experiment 3

Twenty experimentally naïve rats weighing 240–265 g on arrival served as subjects. The experimental procedure was generally the same as in 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. Thus, during habituation to FD, reward rats had access to the 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. Controls never accessed their daily food ration either in the cage or in the experimental room where testing took place. During testing, rats were FD from days 1 to 7. Rearing increased over days in both LR and HR subgroups (Fig. 3A) with higher increases in HR rats (DxG: $F_{6,51}=6.58$, $p=.0001$), which consequently showed higher cumulative rearing levels than LR rats and controls (G: $F_{2,17}=6.51$, $p=.008$), which, in turn, did not differ from one another. As in the previous experiments, LR and HR rats showed about the same levels of locomotor activity (LR: 10.15±.66 and HR: 11.75±.91; G: $p>.05$), eating times and latencies to eat (G: $p>.05$) (Fig 3B). Call rate (Fig. 3C) was significantly higher in HR rats compared to LR and control groups, which did not differ from each other (G: $F_{2,17}=8.07$, $p=.003$).

3.5. Discussion

In the first experiment, short access to a low palatable reward did no lead to differences in anticipatory activity during the FD phase. There, controls and HR rats showed about the same levels of activity, which were higher than in LR rats. Appetitive USVs emitted in anticipation of food reward mirrored the activity patterns of rearing suggesting consistency between both behavioral indicators of incentive motivation. In the second experiment, providing animals with a highly palatable reward while FD enhanced differences in conditioned anticipatory activity between LR and HR rats, which translated into higher rates of cue-induced appetitive 50-kHz calls in HR rats. The same occurred when the perceived magnitude 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. The manipulations of reward palatability and accessibility during FD led to a very similar perceived magnitude between the rewards, as indicated by the differences in calling between the HR group and the other groups in experiments 2 (281%) and 3 (224%). The most interesting findings, however, were obtained from experiments 1 and 2 in which individual differences in conditioned anticipatory activity developed while rewards were still valued (FD) predicted levels of appetitive 50-kHz calls while FAL. Remarkably, reward devaluation vanished out the differences in anticipatory rearing activity but not in the conditioned affective responses as indicated by the USVs levels. 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, in agreement with previous reports where sign- and goal-trackers were investigated [50].

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” [50,53] (for review see: [45]). In the current experiment, reward animals were trained to run through the runway maze to access their daily food ration in a cage attached to the end of the goal arm. We acknowledged that in the maze there was no localizable sign-stimulus specifically paired with the UCS at which attention and behavior could be directed in order to properly analyze sign-tracking [50,53]. However, we took advantage of a behavior that emerged naturally during the runway training, which shared some important features with sign-tracking behavior reported elsewhere [45]: As training progressed, we observed that some animals readily ran down the maze, jumped into the cage and started eating, whereas others reached the cage (often faster), but before and between eating bouts they repeatedly returned to explore the maze (Figure 4). This behavior gradually increased over testing days even though it was unreinforced and opposed to approaching and consuming the food reward, which was only available in the attached cage (see 2. General Material and Methods).

4.2. Methods and results

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. 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 (DxG: $F_{3,81}=7.22$, $p=.0001$;

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 (χ^2_1=7.50, p=.006). 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=.009) (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 (DxG: p>.05). Regarding USVs (Fig. 4D), none of the subgroups differed from controls while FD (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 (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 (Figure 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 no significant difference were found while FD (Figure 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 (Figure 5).

4.3. 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. As we previously showed, 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 [39]. In the runway maze, certain individuals developed a sort of somehow counterintuitive, unreinforced behavior towards the contextual 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 physiological demands were fulfilled– 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 contextual 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 and results

The same 24 rats used in experiment 2 served as experimental subjects, with sweetened condensed milk used as reward. As shown in figure 6A, before reinstatement animals underwent a 7-days training period on FD, and a 3-days 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. We found that 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 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 (DxG: $F_{2,44}$=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 figure 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=.03). 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 figure 6E, reward cues elicited more 50-kHz calls in HR rats than in LR and control conspecifics (G: $F_{2,21}$=9.07, p=.001), which did not differ from each other.

5.3. 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 trails, 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 did not prevent cue-induced calling to occur on reinstatement. In a similar study, 50-kHz calls elicited by cues predicting access to intravenous cocaine were higher after rats being deprived from cues and cocaine during two testing days [38]. One might assume that the one week period of not being exposed to sweetened condensed milk and its related cues reduced reward predictability, which in some cases translates into heightened motivation [60,61]. In Pavlovian conditioning, uncertainty of motivationally relevant stimuli is coded by the mesolimbic DA activity [62]. This DA activity increases as long as the uncertainty of reward does [62], namely, when expectations about reward are augmented. This is consistent with the fact that 50-kHz calls seem to signal sudden changes in reward expectancy as suggested by the phasic pattern of USVs observed after shifting feeding conditions and reward availability [39] or after reinstituting access to cocaine [38], which agrees with the evidence that 50-kHz calls are highly dependent of mesolimbic DA activity [6,9,13,15,63]. On the other hand, individual differences in anticipatory activity –developed during the acquisition phase of conditioning– persisted the time-out period and again, animals that had not attributed incentive salience to cues earlier while FD did not show reward-induced appetitive 50-kHz calls when re-exposed, which is consistent with findings when comparing low and high ranked rats of experiments 1 and 4.

6. Experiment 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 [55]. Behavioral cross-tolerance has widely been demonstrated among drugs with similar mechanism of action (i.e., cocaine vs. amphetamine, [64]), and among drugs and behavioral treatments that recruit similar neurochemical systems (i.e., voluntary exercise attenuating further conditioning for cocaine, morphine, or heroin, [54–57,65]). In the current experiment, animals were challenged with the euphorigenic drug amphetamine. This drug strongly induces unconditioned appetitive 50-kHz calls in rats, and these are thought to be indicative of a DA-dependent positive affective state [9,11,13,15,16]. We anticipated that previous reward experience lead to lessened responses to the psychostimulatory and affective effects of amphetamine. Such a behavioral cross-tolerance between both rewarding stimuli was expected to be more pronounced in rats with higher levels of anticipatory activity displayed during the acquisition phase.

6.2. Methods and results experiment 6

The same 20 rats as in experiment 3 were used here. Before drug administration, 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 7 (see 2. General Materials and Methods). On the following two days, animals were handled and habituated to an injection needle while they continued to being tested.

On the tenth day, all animals were injected with vehicle, which served as a baseline measure. On the next day, d-amphetamine (Sigma St. Louis, MO, USA) was administered ip at a dose of 2.5 mg/kg 10 min before testing, which was conducted exactly as in previous training days. The dose and administration pathway were similar as in our previous reports [11,16]. 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, TxG*:* $F_{1,18}=13.65$, $p=.002$; rearing, TxG: $F_{1,18}=13.93$, $p=.002$) (figure 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 [66]. As depicted in figure 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 (TxG: $F_{1,18}=9.10$, $p=.007$): 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 calls 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 [14,15]. 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 (CxG: $F_{2,36}$=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).

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=.02) and locomotion (G: $F_{2,17}$=5.18, p=.02), 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 (Fig. 8C). Again, both reward subgroups differed significantly from controls (G: $F_{2,17}$=5.47, p=.02). Since amphetamine mainly affected the trill subtype when including all reward subjects, we analyzed trills between LR and HR rats (Fig. 8D). We found that the percentage of trills calls was significantly lower in HR rats compared to CO rats (G: $F_{2,17}$=4.58, p=.03). LR rats, in contrast, did not differ from controls or HR rats (Fig. 8D).

6.3. Methods and results experiment 7

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. The same 24 rats used in experiment 2 served as experimental subjects. After the reinstatement on day 17 (see 5.2. Experiment 6 for details), animals continued to being tested during 2 consecutive days while they were handled and habituated to the injection needle. On the third day, all animals were injected with vehicle (0.9% NaCl) and this measure was used as a baseline. In the subsequent days, animals randomly received either flupenthixol (Sigma St. Louis, MO, USA) or vehicle following a Latin square design in which drug-vehicle days were separated by one drug-free testing day. Flupenthixol was dissolved in vehicle and injected ip at a dose of 0.8mg/kg 30 min before testing. The dose and schedule of administration were chosen based on previous reports [6,67]. As expected, 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, TxG: $F_{1,44}$=4.20, p=.046). 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, TxG: p>.05). When treated with saline, 50-kHz calls, (G: $F_{1,22}$=5.84, p=.02), rearing (G: $F_{1,22}$=10.16, p=.004), but not locomotion (G: p>.05) were 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=.04; Fig. 9E). On the other hand, the analysis of 50-kHz calls 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=.03) 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%) (CxG: $F_{1,22}$=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).

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 2) was used. We found no significant group differences for locomotion, rearing, and USVs (G: all p-values >.05) (Fig. 10A–C). As shown in figure 10C, both reward subgroups showed descriptively less inhibition in call rate as compared to controls, irrespective of the differences in rearing. For the percentage of trills calls (Fig. 10D), however, HR rats showed significantly less inhibition than both LR and control counterparts (G: $F_{1,21}$=4.02, p=.03), which did not differ from each other.

6.4. Discussion

The findings from the amphetamine experiment suggested that repeated activation of DAergic reward system by Pavlovian incentive learning may have decreased the rewarding impact of amphetamine, which comprised both the affective and the psycho-stimulatory drug effects. 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 dopamine turnover in the nucleus accumbens as well [68]. 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 [54,56,57,69] (for review see: [70]), in agreement with our findings. 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 [6,13,14]. 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 [46,67,71]. Spontaneous and reward-induced calling were reduced by blocking D1/D2 receptors as previously described [6,14,63]. 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 [72,73]. In agreement with this idea, it has been found in rat and human studies that food and other rewarding stimuli, which raise DA activity, down-regulate DA receptors [72,74,75] (for review see: [42]). Regarding individual differences, HR rats showed a reduced percentage of amphetamine-induced trills 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 USVs subtype signaling catecholamine-induced euphoric states [7,14–16]. 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 [53]. Previous reports with sign-tracker rats have already pointed out that one of these adaptations is the up-regulation of D1 receptors [50], which may underlie acquisition and early consolidation of appetitive Pavlovian conditioning [76]. Also, levels of DA appear augmented in the nucleus accumbens of animals with high Pavlovian conditioned responses [73]. 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. Further studies are warranted to elucidate these assumptions.

7. General Discussion

The analysis of exploratory activity revealed that rearing behavior appeared consistently conditioned in anticipation of food rewards [43,44,46,47]. 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 [58,59]. High rearing animals have been found to be more efficient in obtaining and consuming food pellets in a radial-maze [77], and to show earlier behavioral sensitization to systemic nicotine [59]. At the neurochemical level, high rearing rats exhibit enhanced ventral and dorsal striatal DA activity as compared to low rearing counterparts [78]. These individual differences in rearing behavior appear to be quite stable in unselected male outbred Wistar rats (for review see: [58]).

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 rearing behavior while FD, predicted cue-induced appetitive 50-kHz calls even though food rewards were devalued. Reducing the salience of the UCS by satiation abolished individual differences in conditioned activity but not in cue-induced appetitive 50-kHz calls, supporting our assumption that affective conditioned responses, such as USVs, can outlast appetitive behaviors [39]. Likewise, in the runway maze only those animals that were attracted by the maze itself during the FD (i.e., sign-trackers) increased calling when the food reward was completely devalued. Again, attribution of incentive salience to environmental cues when the food reward was still valued ensured the emission of appetitive 50-kHz calls during the FAL phase. As with the rearing experiments, 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 were similar in ST and GT rats. Altogether, these data suggest that reward cues were effective in augmenting 50-kHz calling as much as animals had previously developed conditioned activity in anticipation of food. It should be noted, that these findings are in agreement with a previous report in which individual differences in incentive salience attribution to a food cue predicted both conditioned place preference for cocaine and 50-kHz calls induced by cocaine related cues [10].

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 [52]. 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 the last 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. Since animals were FD for 24 h before being re-exposed to the cued setting, such an appetite physiological state seemed to retrieve the hedonic valence of the reward acquired when they were experienced under a state of need. Individuals prone to attribute incentive salience to testing cues, inferred by high and prompted levels of conditioned anticipatory activity while FD, continued showing such a response when tested again one week later under an appetite physiological state. These data, together with findings of the concordance between HR and ST rats in experiments 1 and 4, suggest 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. Interestingly, these effects were greater for 50-kHz calls than for exploratory activity suggesting that USVs may be more sensitive to signal DA-dependent affective states in the rat than traditional psychomotor parameters [16]. The latter was somewhat corroborated by the analysis of individual differences, in which trill calls of HR rats were particularly sensitive to both drugs suggesting that incentive learning recruits DAergic neurons, the activity of which may be even higher in prone animals leading to greater tolerance-like effects expressed as dampened responses to the DAergic agonist and the antagonist.

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 physiological needs being 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 dopaminergic 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.

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LEGENDS FOR FIGURES

Figure 1: Behavioral effects in experiment 1, where animals learned to associate incentive Pavlovian cues with access to daily feeding sessions. 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.

Figure 2: Behavioral effects in experiment 2, where animals learned to associate incentive Pavlovian cues with access to 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.

Figure 3: Behavioral effects in experiment 3, where animals learned to associate incentive Pavlovian cues with access to daily feeding sessions. 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.

Figure 4: Behavioral effects in experiment 4, where animals learned 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.

Figure 5: 50-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.

Figure 6: Behavioral effects in Experiment 5, where re-exposition to reward cues elicited 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 h before reinstatement, animals were food deprived. B. Latencies to drink. C. Drinking times. D. Fluid intakes. E. 50-kHz calls between testing phases. 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. Control vs. reward: *$p<.05$. ++$p<.01$.

Figure 7: Reward-induced behavioral reductions of 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 vs. reward.

Figure 8: Individual behavioral differences in the stimulatory effects of amphetamine on psychomotor activity (A–B) and ultrasonic vocalizations (C–D). 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.

Figure 9: Reward-induced behavioral reductions of the inhibitory effects of flupenthixol. 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 USVs 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 vs. reward. **p*<.05: control vs. reward. #*p*<.05: Saline vs. flupenthixol.

Figure 10: Individual behavioral differences in the inhibitory effects of flupenthixol on psychomotor activity (A–B) and USV (C–D). CO: controls. LR: low rearing. HR: high rearing. A. Locomotion. B. Rearing. C. 50-kHz calls. D. Flupenthixol-reduced 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 group.

FIGURE 1

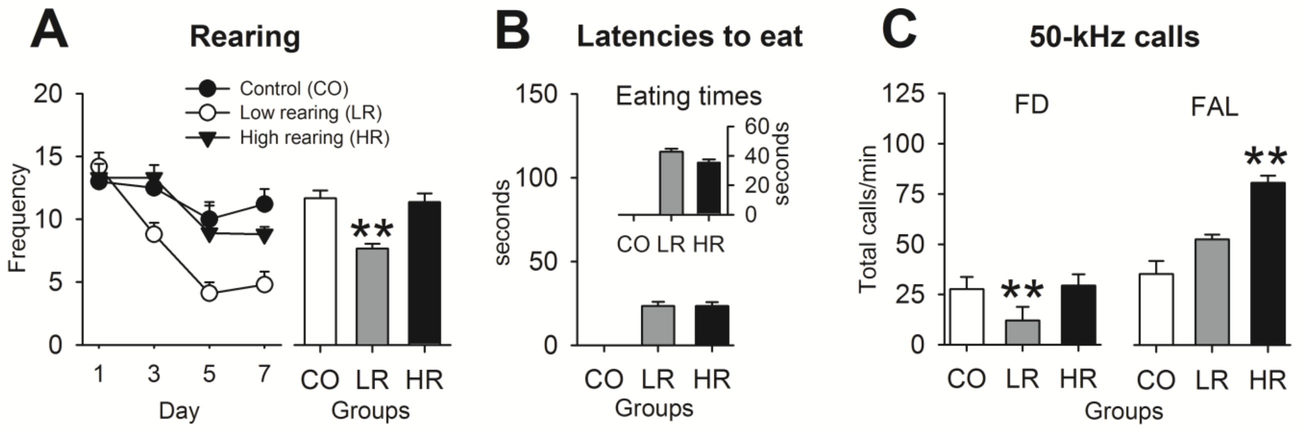


FIGURE 2

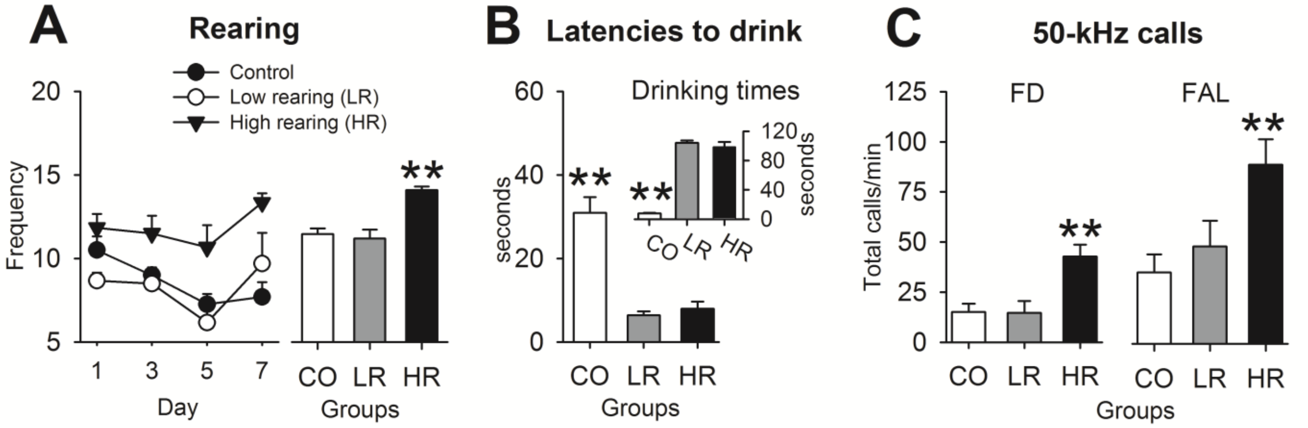


FIGURE 3

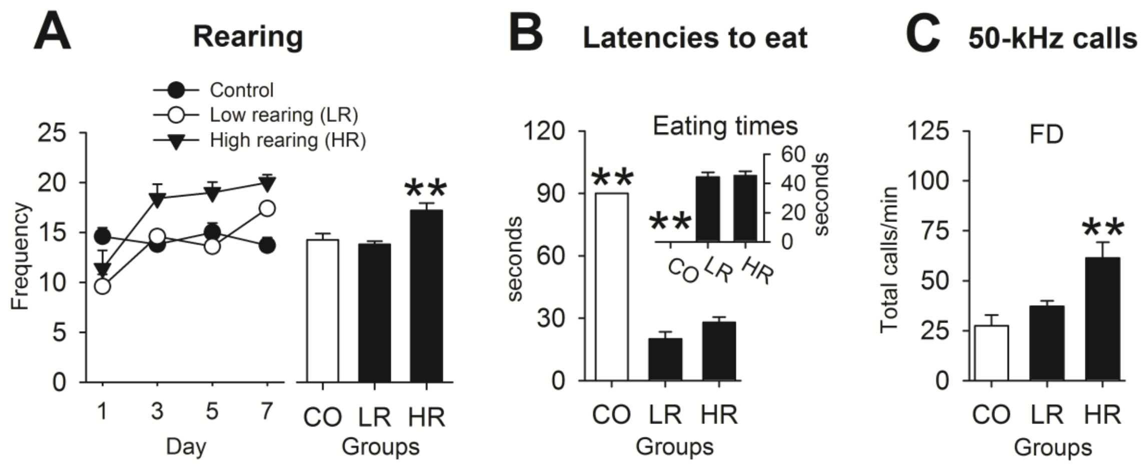

A
Rearing
Control
Low rearing (LR)
High rearing (HR)
Frequency
25
20
15
10
5
0
1
3
5
7
Day
**
CO
LR
HR
Groups
B
Latencies to eat
Eating times
120
90
60
30
0
seconds
**
**
60
40
20
0
seconds
CO
LR
HR
CO
LR
HR
Groups
C
50-kHz calls
FD
125
100
75
50
25
0
Total calls/min
**
CO
LR
HR
Groups

FIGURE 4

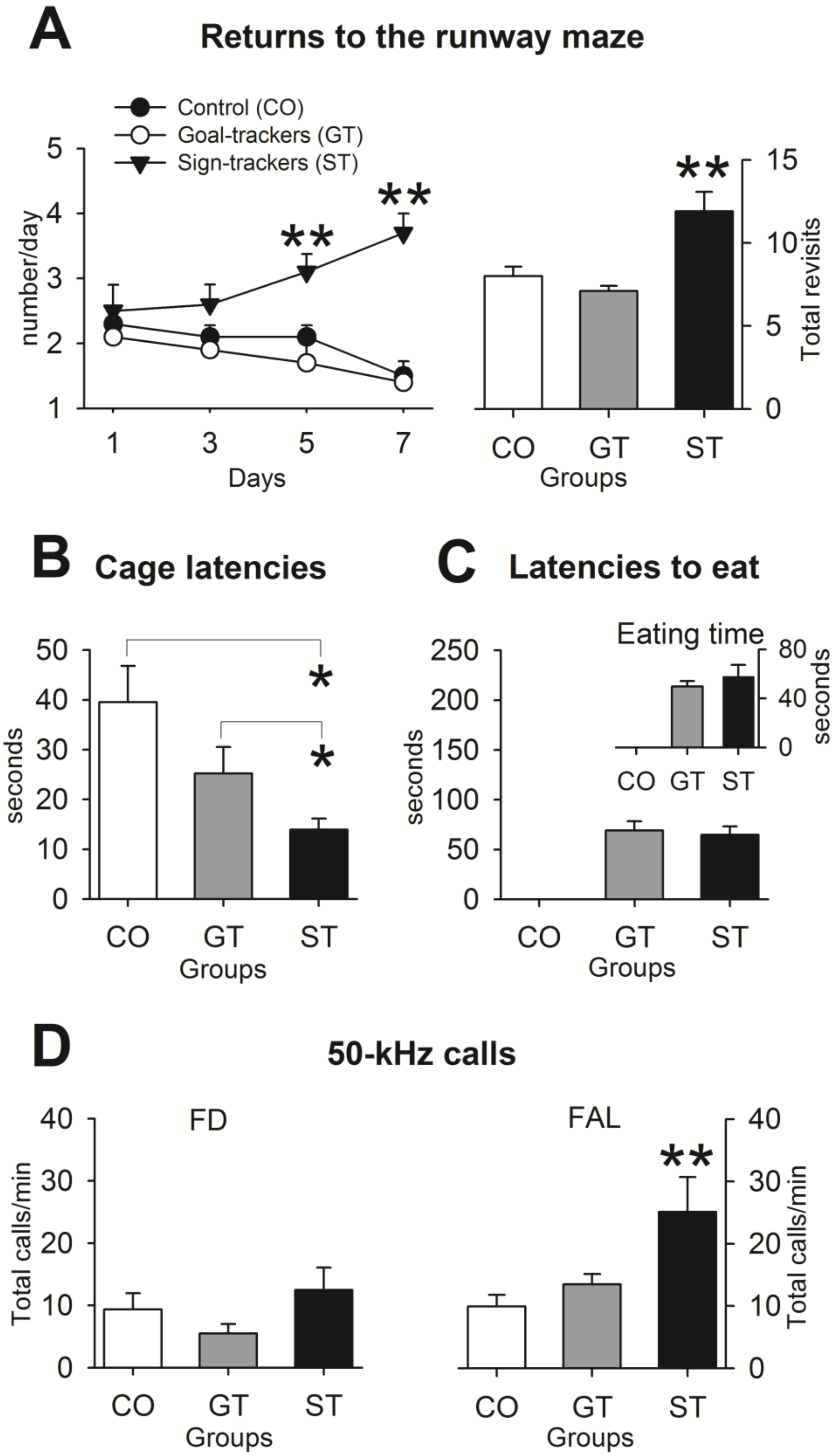

A
Returns to the runway maze
Control (CO)
Goal-trackers (GT)
Sign-trackers (ST)
number/day
5
4
3
2
1
1
3
5
7
Days
**
**
Total revisits
15
10
5
0
CO
GT
ST
Groups
**
B
Cage latencies
seconds
50
40
30
20
10
0
*
*
CO
GT
ST
Groups
C
Latencies to eat
seconds
250
200
150
100
50
0
Eating time
80
40
0
seconds
CO
GT
ST
CO
GT
ST
Groups
D
50-kHz calls
FD
FAL
Total calls/min
40
30
20
10
0
CO
GT
ST
Groups
**
CO
GT
ST
Groups

FIGURE 5

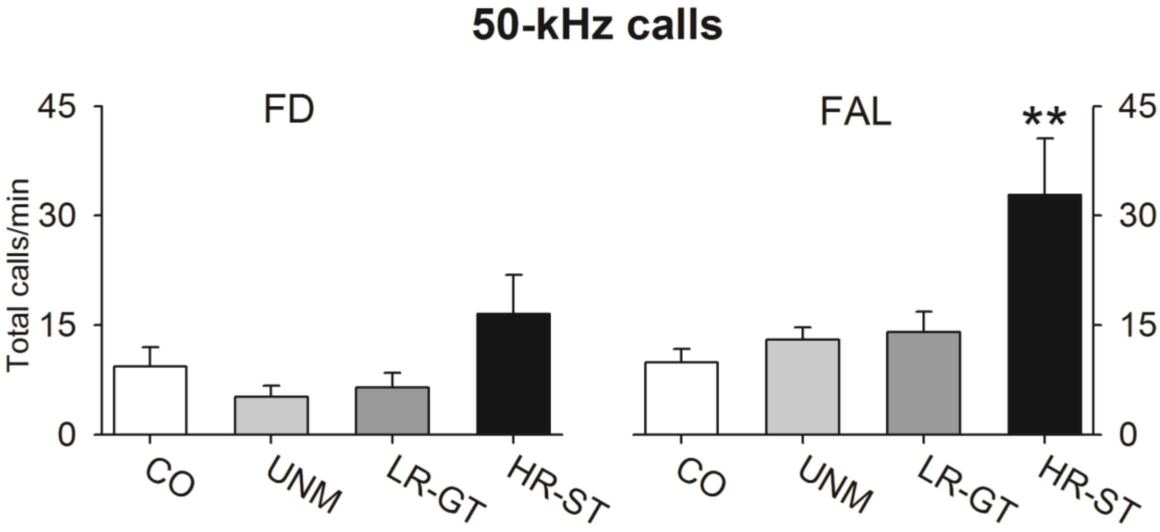

50-kHz calls
FD
FAL
**
Total calls/min
Total calls/min
0
15
30
45
0
15
30
45
CO
UNM
LR-GT
HR-ST
CO
UNM
LR-GT
HR-ST

FIGURE 6

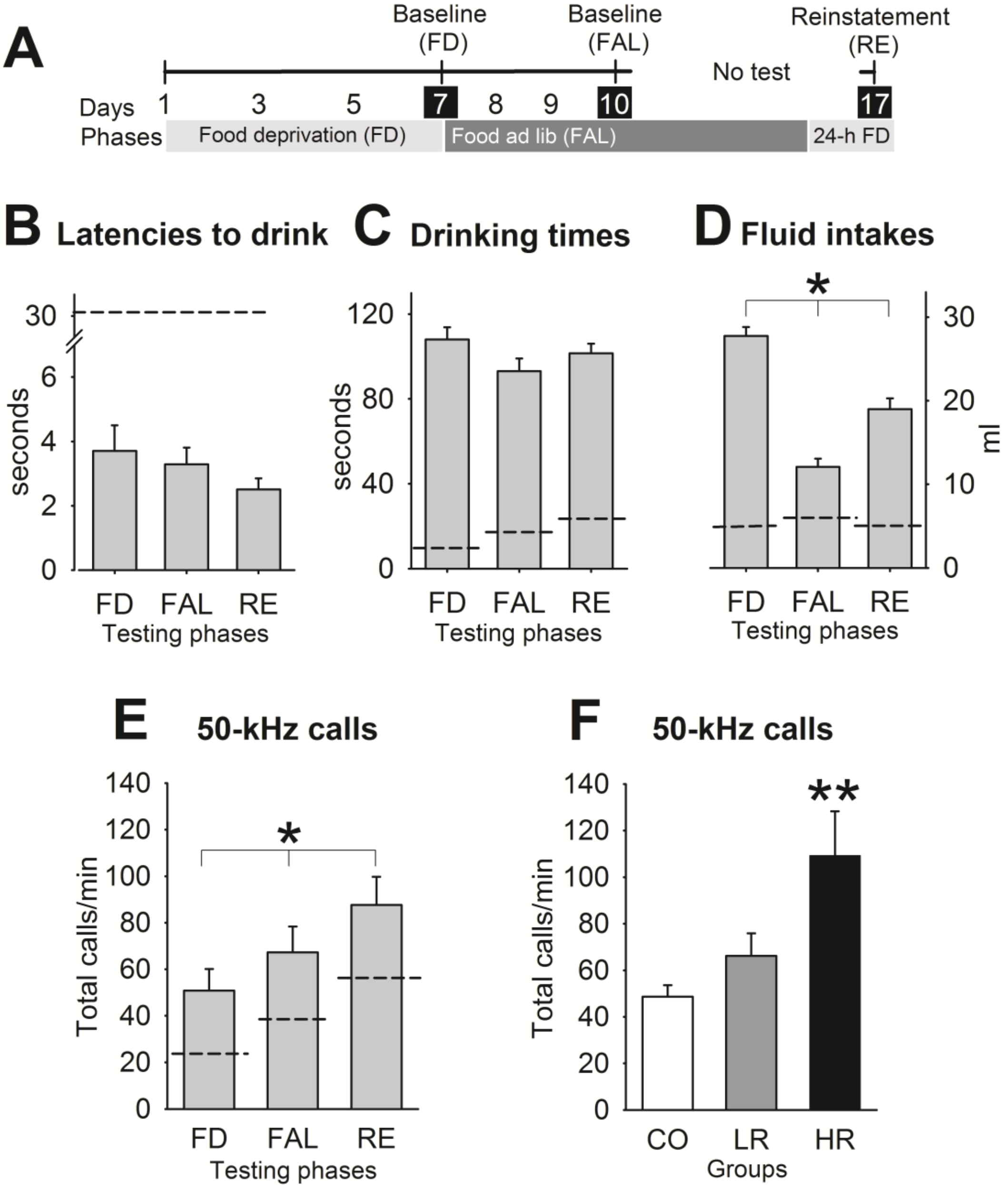

A
Baseline (FD)
Baseline (FAL)
Reinstatement (RE)
No test
Days 1 3 5 7 8 9 10 17
Phases Food deprivation (FD) Food ad lib (FAL) 24-h FD
B Latencies to drink
seconds
30
6
4
2
0
FD FAL RE
Testing phases
C Drinking times
seconds
120
80
40
0
FD FAL RE
Testing phases
D Fluid intakes
*
ml
30
20
10
0
FD FAL RE
Testing phases
E 50-kHz calls
Total calls/min
140
120
100
80
60
40
20
0
*
FD FAL RE
Testing phases
F 50-kHz calls
Total calls/min
140
120
100
80
60
40
20
0
**
CO LR HR
Groups

FIGURE 7

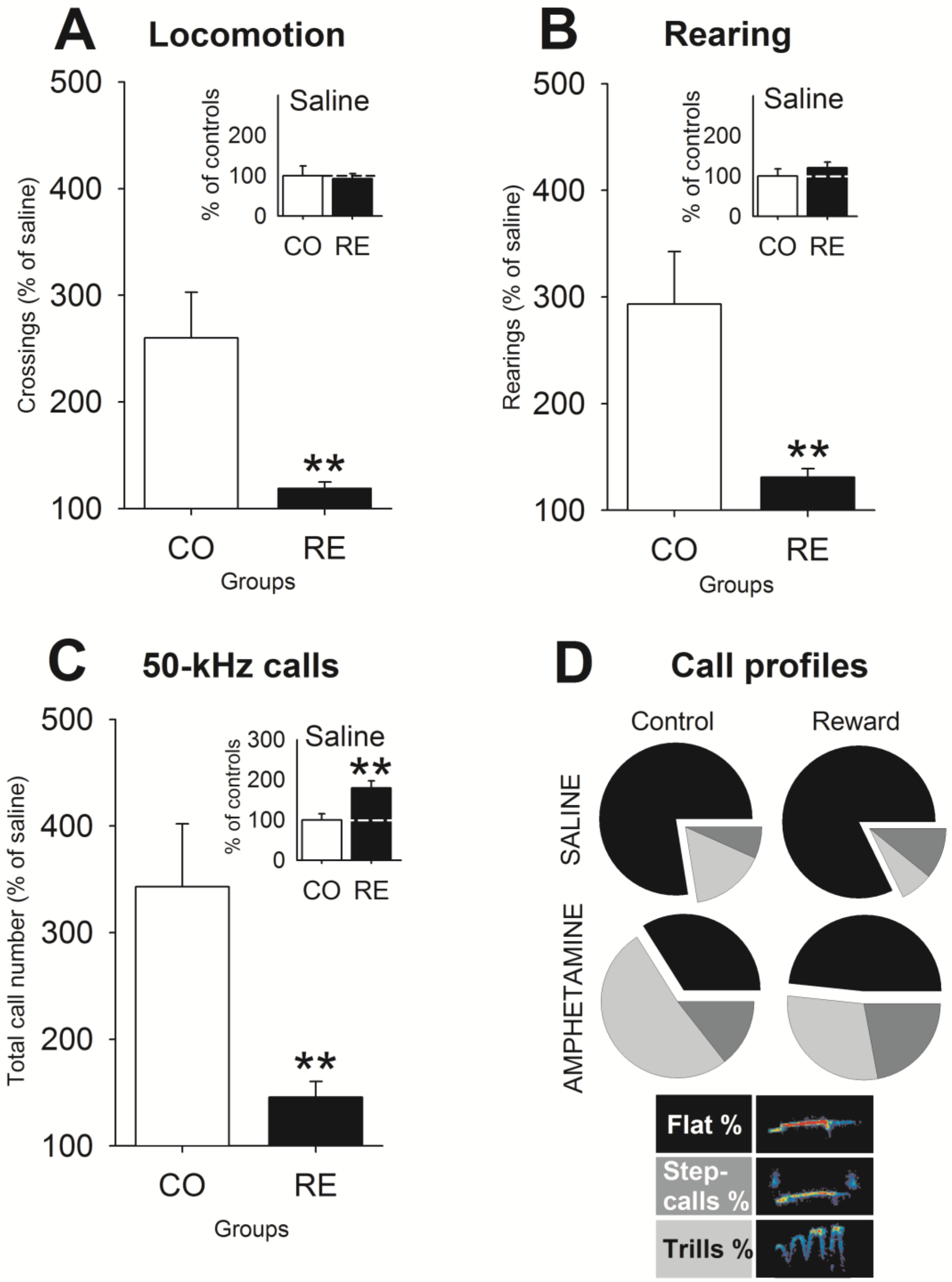
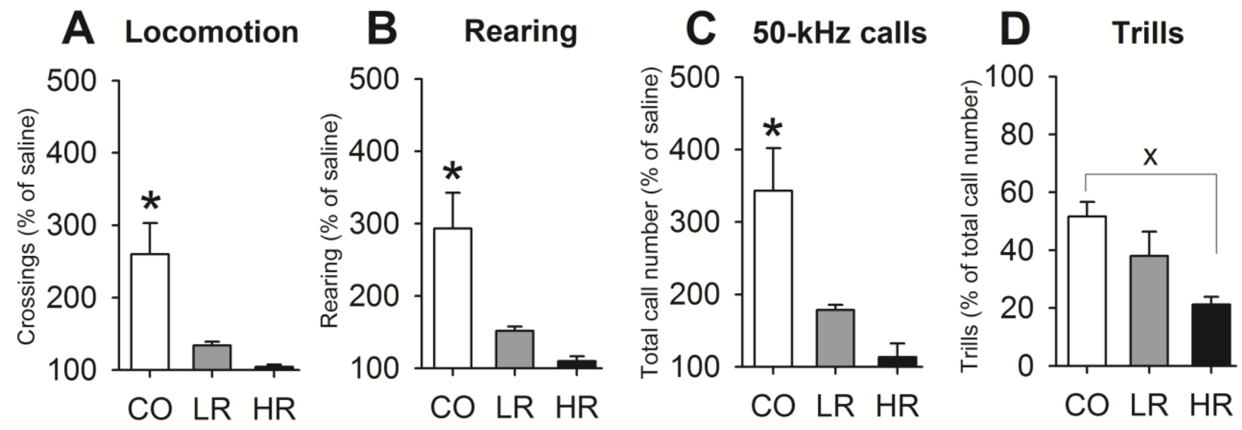

A
Locomotion
Crossings (% of saline)
500
400
300
200
100
CO
RE
Groups
**
Saline
% of controls
200
100
0
CO RE
B
Rearing
Rearings (% of saline)
500
400
300
200
100
CO
RE
Groups
**
Saline
% of controls
200
100
0
CO RE
C
50-kHz calls
Total call number (% of saline)
500
400
300
200
100
CO
RE
Groups
**
Saline
% of controls
300
200
100
0
**
CO RE
D
Call profiles
Control
Reward
SALINE
AMPHETAMINE
Flat %
Step-calls %
Trills %

FIGURE 8



A
Locomotion
Crossings (% of saline)
500
400
300
200
100
*
CO
LR
HR
B
Rearing
Rearing (% of saline)
500
400
300
200
100
*
CO
LR
HR
C
50-kHz calls
Total call number (% of saline)
500
400
300
200
100
*
CO
LR
HR
D
Trills
Trills (% of total call number)
100
80
60
40
20
0
x
CO
LR
HR

FIGURE 9

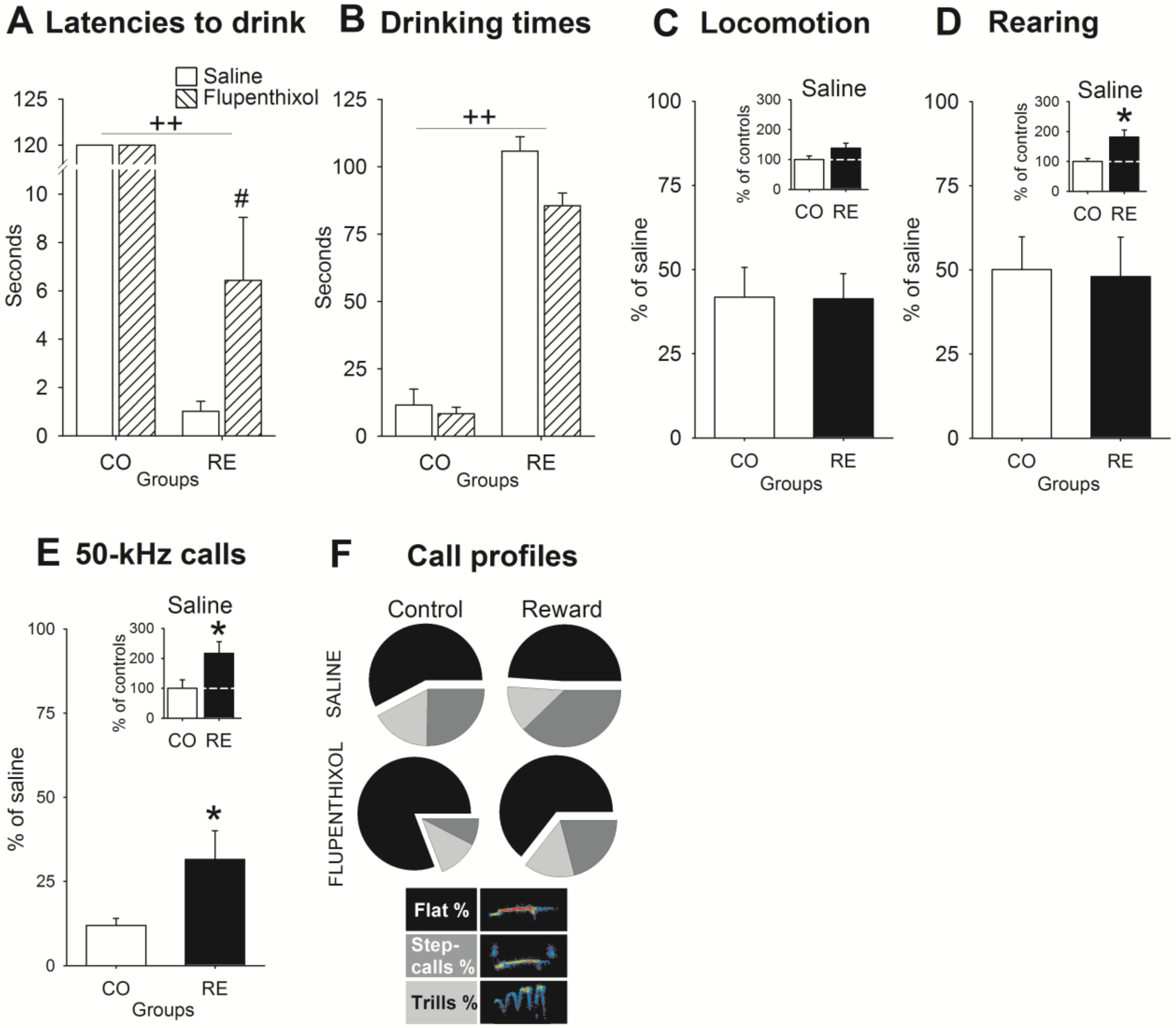
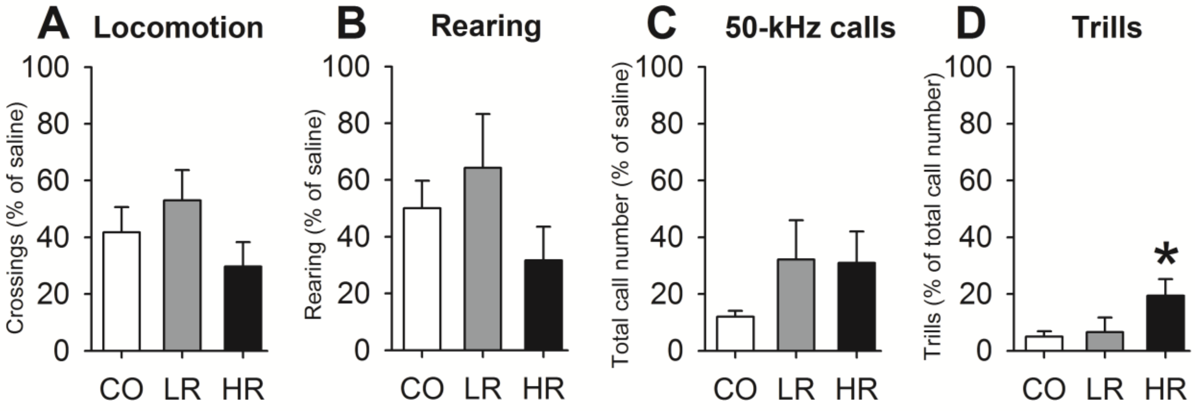

A Latencies to drink
Saline
Flupenthixol
++
#
Seconds
125
120
10
8
6
4
2
0
CO
RE
Groups
B Drinking times
++
Seconds
125
100
75
50
25
0
CO
RE
Groups
C Locomotion
Saline
% of controls
300
200
100
0
CO RE
% of saline
100
75
50
25
0
CO
RE
Groups
D Rearing
Saline
*
% of controls
300
200
100
0
CO RE
% of saline
100
75
50
25
0
CO
RE
Groups
E 50-kHz calls
Saline
*
% of controls
300
200
100
0
CO RE
% of saline
100
75
50
25
0
*
CO
RE
Groups
F Call profiles
Control
Reward
SALINE
FLUPENTHIXOL
Flat %
Step-calls %
Trills %

FIGURE 10


A
Locomotion
Crossings (% of saline)
100
80
60
40
20
0
CO
LR
HR
B
Rearing
Rearing (% of saline)
100
80
60
40
20
0
CO
LR
HR
C
50-kHz calls
Total call number (% of saline)
100
80
60
40
20
0
CO
LR
HR
D
Trills
Trills (% of total call number)
100
80
60
40
20
0
*
CO
LR
HR

Appendix C: Curriculum Vitae

Appendix D: Disclosure of authorship contributions

Study 1

Research idea	Brenes, J.C.; Schwarting R.K.W.
Experimental design	Brenes, J.C.
Execution of the experiments	Brenes, J.C.
Data analysis	Brenes, J.C.
Preparation of the manuscript	Brenes, J.C.
Supervision	Schwarting R.K.W.
Contribution	Brenes, J.C.: 75%; Schwarting R.K.W.: 25%.

Study 2

Research idea	Brenes, J.C.; Schwarting R.K.W.
Experimental design	Brenes, J.C.
Execution of the experiments	Brenes, J.C.
Data analysis	Brenes, J.C.
Preparation of the manuscript	Brenes, J.C.
Supervision	Schwarting R.K.W.
Contribution	Brenes, J.C.: 75%; Schwarting R.K.W.: 25%.

Overall contribution to the studies	Brenes, J.C.: 150%; Schwarting R.K.W.: 50%.

Appendix E: Eidesstattliche Erklärung *[declaration of academic honesty]*

Ich versichere, dass ich meine Dissertation

„*Ultrashallvokalisation und Anreizmotivation bei Ratten*“

selbständig, ohne unerlaubte Hilfe angefertigt und mich dabei keiner anderer als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe.

Die Dissertation wurde in der jetzigen oder einer ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

Marburg, im December 2014

Juan Carlos Brenes Sáenz