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ORIGINAL INVESTIGATION

Failure of rewarding and locomotor stimulant doses of morphine to promote adult rat 50-kHz ultrasonic vocalizations

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Abstract
Rationale Frequency-modulated 50-kHz ultrasonic vocalizations (USVs) are emitted by adult rats in response to psychostimulants and non-pharmacological appetitive stimuli and thus have been proposed to model positive affect.
Objective The main aim was to determine whether rewarding doses of morphine increase 50-kHz call rate or alter the relative prevalence of the trill call subtype.
Methods In experiment 1, USVs were recorded from adult male Long–Evans rats after subchronic morphine (1 mg/kg subcutaneous (SC)) administration, acute challenge with morphine (1 and 3 mg/kg SC) or amphetamine (1 mg/kg IP, positive control), and in conjunction with locomotor activity tests with morphine (1 and 3 mg/kg SC). In experiments 2 and 3, the USV altering, rewarding, and locomotor effects of morphine were examined using a conditioned place preference (CPP) procedure.
Results In experiment 1, morphine (1 mg/kg) initially suppressed calling; rats became tolerant to this effect with repeated exposure. Tested subsequently in singly- and pair-tested rats, morphine markedly decreased USVs but significantly increased locomotor activity. In experiments 2 and 3, morphine produced a significant CPP without increasing either unconditioned or conditioned USV emission. Morphine did not detectably alter the relative prevalence of 50-kHz call subtypes.
Conclusions Although 50-kHz calls, and the trill call subtype in particular, have been proposed as an animal model of positive mood, not *all* euphoriant drugs acutely increase the rate of 50-kHz calling or consistently promote trill calls.

Keywords Ultrasonic vocalizations · Rat · Morphine · Opioid · Amphetamine · Reward · Conditioned place preference · Locomotor activity

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Introduction

Adult laboratory rats emit two broad categories of vocalizations in the ultrasonic range, commonly designated as "50-kHz" and "22-kHz" calls (Brudzynski 2009; Wohr and Schwarting 2010). The 50-kHz call category encompasses a broad frequency range (30–90 kHz) (Kaltwasser 1990; Sales and Pye 1974) and comprises multiple subtypes, including flat (i.e., constant frequency) and at least 12 types of frequency-modulated (FM) calls (Wright et al. 2010). Recently, we have shown that the acoustic profile (i.e., relative prevalence of different call subtypes) can be modulated by drugs or social context (Wright et al. 2010, 2012), adding to the existing evidence that distinct information may be contained within the repertoire of 50-kHz USVs (e.g., Burgdorf et al. 2008; Simola et al. 2009; Wohr et al. 2008).

Whereas the 22-kHz calls appear to express distress or alarm (Covington and Miczek 2003; Litvin et al. 2007), 50-kHz calls are elicited by a number of appetitive stimuli (Burgdorf et al. 2010; Knutson et al. 2002). Consequently, adult rat 50-kHz calls (and FM calls in particular) have been proposed to be a measure of hedonia (Burgdorf and Moskal 2009) and have been described as a reliable indicator (Brudzynski 2007) and validated model (Burgdorf et al. 2010) of positive affective states. Consistent with this notion, the psychomotor stimulants amphetamine (AMPH) and cocaine both increase the 50-kHz

call rate and promote FM calls after systemic injection (e.g., Ahrens et al. 2009; Williams and Undieh 2010; Wright et al. 2010, 2012). Among the FM calls, it is the trill calls in particular that appear to be preferentially increased by these drugs (Wright et al. 2010, 2012). Psychostimulant drugs, however, are not only euphorigenic (Foltin and Fischman 1991) but are also anxiogenic in rodents and humans (Biala and Kruk 2007; File and Hyde 1979; Pellow et al. 1985); in high doses, these drugs can even produce acute psychosis (Angrist et al. 1974; Robinson and Becker 1986). Hence, it is important to note that trill calls are also affected by appetitive non-pharmacological manipulations. In particular, trill calls are also enhanced by testing rats in pairs (Wright et al. 2010), which itself is reported to be rewarding (Calcagnetti and Schechter 1992), whereas they appear less prevalent upon social separation (Wohr et al. 2008). Hence, on present evidence, the trill call subtype appears most closely associated with positive stimuli (Wright et al. 2010).

Opiate agonists form a second major class of euphorigenic drugs (Jasinski and Preston 1986; Zacny et al. 1994), but it is unclear whether they affect 50-kHz USVs in a similar way to psychostimulants. Systemic morphine administration was initially reported to inhibit 50-kHz calling (Haney and Miczek 1994, 1995; Vivian and Miczek 1993); however, these experiments were conducted in an aversive context (social stress) in which the vocalizations were probably acoustically distinct from those proposed to reflect positive affect (Wohr et al. 2008). Acute systemic morphine has also been combined with experimenter-applied tickling-like tactile stimulation, with no apparent drug effect on call rate (Panksepp and Burgdorf 2000). Acute morphine also failed to affect 50-kHz calling rate in a novel testing environment; however, call rates were uniformly very low (i.e., ≤1 call per minute) even in saline-challenged rats (Wohr and Schwarting 2009), suggesting that the environment itself may have generally inhibited USV production. In contrast, morphine reportedly induced 50-kHz USVs in a reward context, i.e., conditioned place preference (CPP) (Burgdorf et al. 2001; Knutson et al. 1999). The latter studies, however, demonstrated only morphine-*conditioned* USVs and, importantly, the unconditioned effects of morphine (i.e., occurring during the CPP acquisition phase) were not reported.

In the absence of other behavioral manipulations, acute morphine administration tended to inhibit 50-kHz call rates according to two recent studies (Hamed et al. 2012; Simola et al. 2012). These apparent (but statistically nonsignificant) suppressant effects occurred at relatively high doses of morphine of 5 or 10 mg/kg which may have nonspecifically inhibited motor output (Fernandes et al. 1977; Fog 1970); lower doses appeared ineffective (Simola et al. 2012). In the latter study, a preliminary analysis of call subtypes revealed no drug effect on trill calls (Simola et al. 2012). These two USV studies suggest a possible dissociation between morphine's rewarding effects and FM 50-kHz calls (and trill calls in particular); this inference is necessarily indirect, however, since rewarding drug effects were not measured.

Since FM 50-kHz calls (and trill calls in particular) have been proposed to reflect positive affect in rats (see above), we hypothesized that after systemic administration morphine would promote trill calls and possibly increase the overall rate of 50-kHz FM calls. This question was addressed in three experiments, using doses of morphine that are reportedly rewarding in CPP tests (Bardo et al. 1995). In experiment 1, we initially tested individual rats with repeated morphine challenge; a low dose (1 mg/kg SC) was chosen to avoid catalepsy, which tends to occur above 5 mg/kg in drug-naïve rats (Fernandes et al. 1977; Fog 1970). We then tested the USV response to different doses of morphine (1 and 3 mg/kg) and also to AMPH (1 mg/kg). Here, rats were tested both individually and with a cage mate. The latter condition served as a test of generalizability, since rats call more frequently and with a distinct acoustic profile in the presence of conspecifics (Brudzynski and Pniak 2002; Wright et al. 2010). Having noted a USV-depressant effect of morphine, we next studied ultrasonic calling in parallel with locomotor activity in order to test for sedation. Next, in experiment 2, the rewarding and locomotor effects of morphine were examined using a CPP procedure; here, USVs were recorded during the acquisition (conditioning) phase and during subsequent drug-free sessions where rats were restricted to either the saline- or morphine-paired floor texture. Finally, in experiment 3, USV recording was performed during the acquisition phase and during the free-choice CPP test session.

Methods

Subjects

Subjects were 64 experimentally-naïve male Long–Evans rats (Charles River Laboratories, St. Constant, Quebec, Canada) weighing 339 ± 14 g (mean ± SD) (i.e., aged approximately 9 weeks) at the start of the experiment. Subjects were housed two per cage (25 × 48 × 20 cm^3) in a temperature- and humidity-controlled colony room (20–22 °C, 50–60 %) at the McGill University Animal Resources Centre. The rats were maintained on a reverse 12:12 light/dark cycle, with lights off at 0700 hours. All behavioral testing took place during the dark phase of the cycle. Food and water were available ad libitum, except during testing. Before the start of the experiment, each rat was handled by the experimenter for approximately 3 min per day for 1–2 days (experiments 1 and 2) or 5 min per day over 6 days (experiment 3). All procedures were approved by the McGill Animal Care

Committee in accordance with the guidelines of the Canadian Council on Animal Care.

Locomotor activity and CPP apparatus

Locomotor activity and CPP were both tested in rectangular, open-topped CPP cages (58-cm long × 29-cm wide × 53-cm high). Two floor textures were used as conditional stimuli: a mesh grid (1 cm^2 stainless steel wire mesh) and a metal panel containing small holes (4.8 mm diameter, set 6.4 mm apart). Rats do not show spontaneous preference for either floor texture (T. Scardochio and P. B. S. Clarke, unpublished observation). Square (29 × 29 cm) tiles made of either flooring were mounted on melamine frames; two tiles completely covered the bottom of each CPP cage and, for experiment 2, one tile fit into each USV recording chamber. A video tracking system (EthoVision v 3.0, Noldus Information Technology, Leesburg, VA, USA) measured locomotor activity (expressed as the total horizontal distance moved) and the time spent on each floor texture (on CPP test day). To minimize visual cues, conditioning and testing in the CPP cages were conducted under darkroom lighting using a Kodak GBX-2 safelight filter (Vistek, Toronto, Ontario, Canada) which provided far-red (wavelength >650 nm) illumination.

Acoustic data acquisition

USV recordings in experiments 1 and 2 took place as previously described in clear Plexiglas experimental chambers (ENV-007CT, Med Associates, St Albans, VT). Please refer to Wright et al. (2012) for details concerning the USV recording apparatus and setup. All lights were off when rats were in the USV test chambers. For experiment 3, two ultrasound microphones were secured inside each CPP chamber at opposite corners, approximately 10 cm from the top (i.e., 40 cm above the floor). Sound-attenuating acoustic foam (Primacoustic, Port Coquitlam, British Columbia) enveloped the outside of the CPP chambers and extended 20 cm above the top of each chamber.

Drugs

Drugs were morphine sulfate pentahydrate (Sandoz, Boucherville, Quebec) and D-amphetamine sulfate (Sigma-Aldrich, Poole, UK). Morphine (1 and 3 mg/kg, dose expressed as free base) was administered by subcutaneous (SC) injection into the flank. AMPH (1 mg/kg, dose as salt) was administered through intraperitoneal (IP) injection. Both drugs were dissolved in sterile 0.9 % saline and administered in a volume of 1 ml/kg.

Experimental protocol

Experiment 1

This experiment comprised three consecutive parts (1.1–1.3). The same 24 rats were used throughout. Rats were left undisturbed in their home cages during the 3 days between parts 1.1 and 1.2, and the 8 days between parts 1.2 and 1.3.

Part 1.1: effects of repeated morphine administration on 50-kHz ultrasonic vocalizations Repeated morphine exposure can produce sensitization to its rewarding effects (Lett 1989), and we anticipated that an analogous effect might occur with ultrasonic calling. Accordingly, the 24 subjects were randomly allocated to receive either morphine (1 mg/kg SC, $n=12$) or saline ($n=12$) daily over 3 days in the home cage prior to USV testing. On each of the following six test days, rats received an injection of morphine (1 mg/kg SC) or saline (presented in alternating sequence) and were placed in a recording test chamber from 30 to 60 min post-injection. The order of testing was counterbalanced such that within each group of 12 rats, six rats on each test day received morphine and six rats received saline.

Part 1.2: effects of morphine and amphetamine on 50-kHz ultrasonic vocalizations in rats tested individually or in pairs Part 1.2 occurred on eight consecutive days according to a fully parametric 2 × 4 within-subjects design, whereby each rat was tested once singly and once paired with a cage mate, under each of four drug conditions: morphine (0, 1 and 3 mg/kg, SC) or AMPH (1 mg/kg, IP). The rats ($n=24$) were placed in the recording chambers 30–60 min post-injection. Cage mates were always tested under the same drug condition. The order of testing was counterbalanced as far as possible using a Williams square design.

Part 1.3: effect of morphine on 50-kHz ultrasonic vocalizations and locomotor activity The same 24 rats were initially habituated to the locomotor test boxes for 10 min. On the following 3 days, each rat was tested once with saline and morphine 1 and 3 mg/kg (SC) (order of testing was counterbalanced). Starting 20 min post-injection, half of the rats received a 20-min locomotor activity test session followed immediately by a 20-min USV recording session. The remaining rats received the same two tests in the reverse order.

Experiment 2: morphine-conditioned place preference and 50-kHz ultrasonic vocalizations

Subjects ($n=24$) were not preexposed to morphine in the home cage since this had had no detectable effect on the USV responses in experiment 1. Behavioral testing consisted

of four main phases, extending over 12 consecutive days in total: habituation (day 1), conditioning (days 2–9), CPP test (day 10), and conditioned USV tests (days 11–12). On the first day (day 1), the 24 rats, which were all drug- and experimentally naïve, were habituated to the CPP chambers for 20 min on a layer of wood-chip bedding. Rats then underwent eight once-daily conditioning trials (days 2–9), whereby morphine (1 or 3 mg/kg, depending on group, $n=12$) and saline were repeatedly paired (on alternating days) with a distinct floor texture which served as a tactile cue. Immediately following injection, half of the rats ($n=12$) were conditioned in the CPP cages for 20 min and then promptly transferred to the USV recording chambers where they received an additional 20 min of contact with the same drug- or saline-paired floor texture; this order of conditioning was reversed for the other rats ($n=12$). On the CPP test day (day 10), rats were placed drug-free in the middle of the CPP cage, straddling the two floor tiles, and for the next 20 min were free to choose between the two floor textures. On the conditioned USV test days (days 11–12), each rat was recorded in the USV test chamber while being exposed for 20 min to the saline- or morphine-paired floor texture. This entire experiment was counterbalanced as far as possible in all respects, i.e., drug/floor-texture pairing, the order of drug vs. saline administration, position of morphine-paired floor texture within the test cage and order of floor texture presentation during the two USV test days. This experiment concluded 1 week later with two additional USV test sessions which served to demonstrate that the rats were capable of emitting high rates of calling in response to a drug. Here, the rats were tested for 20 min immediately after AMPH (1 mg/kg, IP) or saline, given in counterbalanced order.

Experiment 3: 50-kHz ultrasonic vocalizations during acquisition and expression of morphine-conditioned place preference

Experiment 3 differed from experiment 2 in the following respects. (1) All behavioral measures (i.e., USVs, locomotor activity, and place preference) were collected simultaneously in the same (CPP) apparatus. (2) In order to minimize possible experimenter-induced stress or anxiety, rats were handled more (see "Subjects" above) and underwent two (vs. one) habituation sessions to the CPP apparatus before the conditioning phase. (3) All rats ($n=16$) were conditioned with only the lower morphine dose (i.e., 1 mg/kg). (4) Testing for conditioned USVs was conducted during a single CPP test session during which the rats were free to choose between the drug- and saline-paired floor textures.

Analysis and classification of ultrasonic vocalizations

Acoustical analysis was performed as previously described (Wright et al. 2012) using Avisoft SASLab Pro (version 5.1, Avisoft Bioacoustics). Calls were selected manually from spectrograms by an individual who was masked to the treatment condition. Each identified 50-kHz call was classified into 1 of 14 distinct categories: complex, upward ramp, downward ramp, flat, short, split, step up, step down, multi step, trill, flat–trill combination, trill with jumps, inverted U, or composite (see Wright et al. 2010 for criteria for call identification and classification, several examples of each call type, as well as descriptive statistics relating to acoustic parameters). This method of manual call selection has been validated by surgical devocalization, and our method of classification is associated with high inter- and intra-rater reliability (Wright et al. 2010). Twenty-two kilohertz calls were rarely observed in the present study; only a small minority of rats in experiment 1.2 (mostly in the pair-tested condition) emitted any 22-kHz USVs. Therefore these calls were not analyzed further.

Data analysis and statistics

Data were analyzed using commercial software (Systat v11, SPSS, Chicago, IL; GraphPad Prism 4, GraphPad Software, La Jolla, CA). In experiments 1.1 and 1.2, USVs that occurred during min 1, 6, 11, 16, 21, and 26 of the 30-min session were counted and classified. For experiment 1.3, USV analysis was performed for min 1, 11, and 20 of the 20-min session. In experiment 1.1, one rat was a serious outlier (i.e., >3 standard deviations from the mean) rendering the use of ANOVA invalid and was therefore excluded from the statistical analysis. In experiment 1.2, rats were tested both singly and with their cage mate. When rats were tested in pairs, the call rate was divided by two. Therefore, there were $n=12$ pairs for part 2 and the data are expressed as calls per minute per rat. One rat pair was a serious outlier and was therefore excluded from analysis. For experiments 2 and 3, USV analysis was performed for min 3, 8, 13, and 18 of the 20-min session and 3, 8, 13, 18, 23, 28, 33, and 38 of the 40-min session, respectively. However, all USVs that occurred during the entire 20-min CPP test were counted and classified. Due to a technical problem, locomotor data collection failed for four out of the 24 rats during the third conditioning trial in experiment 2. Therefore, these rats were excluded from the locomotor activity analysis for that trial. Repeated measures ANOVA or Friedman's test was performed, where appropriate, and pairwise comparisons were performed using paired t tests or Wilcoxon tests. The choice of test depended on the distribution of the raw data. ANOVA determined the effect of test pair (i.e., morphine minus saline difference scores for USV rate for each of the three morphine/saline tests in experiment 1.1), group (i.e., home cage morphine preexposure), and drug (i.e., morphine dose in experiment 1.3). ANOVA p values were subject to the Huynh–Feldt correction, where appropriate, and multiple

comparisons were subject to Holm–Bonferroni correction. For all analyses, a two-tailed p value <5 % (after any correction) was considered significant.

Results

Experiment 1

Part 1.1 Acute effects of morphine on 50-kHz calling are shown in Fig. 1. Morphine preexposure (i.e., 3 × 1 mg/kg SC in the home cage) failed to alter the acute effects of the drug, and therefore rats from both groups were pooled for all subsequent analyses. Morphine significantly inhibited calling, but only in the first pair of morphine/saline tests (Wilcoxon Z= 3.05, $p < 0.01$; Fig. 1). Tolerance developed by the third test pair as evidenced by a significant difference in the MOR–SAL difference score between the first and third test pairs (test pair, $F_{2,42}$=5.31, p=0.02; t_{22}=2.50, $p < 0.05$).

Part 1.2 Rats tended to emit more calls (on a per rat basis) when tested with their cage mate than when tested alone. This difference was statistically significant in the saline, morphine 1 mg/kg, and AMPH conditions (Wilcoxon Z scores, 2.76–2.93, p<0.02); Fig. 2). Morphine (3 but not 1 mg/kg) significantly decreased the call rate when rats were tested singly or in pairs (Wilcoxon Z=2.31 and 2.80, respectively, $p < 0.05$; Fig. 2). AMPH increased the call rate, as expected, under both conditions (Wilcoxon Z=2.93 and $p < 0.01$; Fig. 2). The time–course of USV emission during the test sessions is shown in Fig. 3.

Part 1.3 USVs were tested either before or after locomotor activity, depending on the group (n=12 rats per group). Data from these two groups were pooled, since neither the locomotor activity nor the USV rate revealed a significant

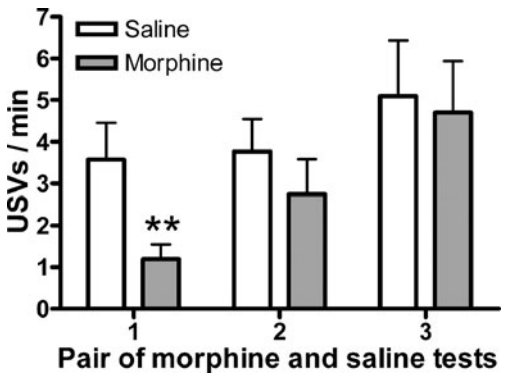

Fig. 1 Experiment 1.1: morphine (1 mg/kg, SC) initially suppressed 50-kHz calling, but rats became tolerant to this effect with repeated testing. The y-axis represents mean + SEM 50-kHz USVs per minute under saline (*open bars*) and 1 mg/kg morphine (*gray bars*). **p < 0.01 (n=24)

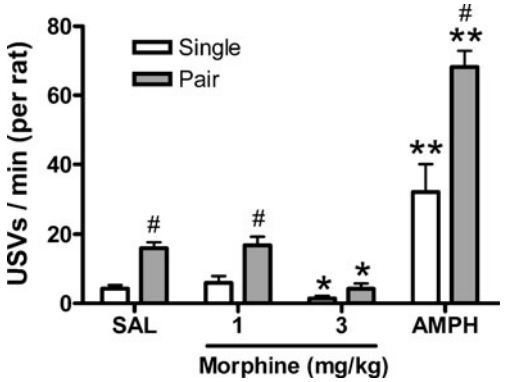

Fig. 2 Experiment 1.2: morphine dose-dependently inhibited calling in both singly- (*open bars*) and pair-tested (*gray bars*) rats. The y-axis represents mean+SEM USVs per minute (on a per rat basis). Each rat was tested under all eight conditions (n=11 rat pairs). *$p < 0.05$, **p < 0.01 vs. respective saline condition; #p<0.02 vs. tested singly under the same drug treatment

between-group difference. Both doses of morphine (1 and 3 mg/kg) increased total locomotor activity per 20-min session (drug: $F_{2,44}$=7.68, $p < 0.01$; 1 mg/kg vs. saline, t_{23}=4.5, p < 0.001; 3 mg/kg vs. saline, t_{23}=3.0, $p < 0.01$; Fig. 4a) but decreased the call rate (Friedman Q_2=25.97, $p < 0.0001$; Wilcoxon tests: saline vs. 1 mg/kg, Z=3.67, $p < 0.001$; saline vs. 3 mg/kg, Z=3.81, $p < 0.001$; Fig. 4b). The higher dose of morphine virtually abolished calling (Fig. 4b).

Experiment 2

During the CPP conditioning phase, the call rate and locomotor activity did not differ significantly between rats tested 0–20 or 20–40 min after injection. Therefore, rats were pooled for the remainder of the analysis. During the conditioning phase, morphine (especially at 3 mg/kg) tended to reduce the USV emission rate, but this apparent effect was nonsignificant (Fig. 5a, c). Morphine also failed to affect locomotor activity, except for a modest, but nonsignificant, stimulant effect that emerged at the 3-mg/kg dose during later conditioning sessions (paired t test between saline and 3 mg/kg morphine for trials 3 and 4, with Holm–Bonferroni corrections: t_9=3.09 and t_{11}=2.73, p=0.052 and 0.059, respectively; Fig. 5b, d). On the CPP test day, both doses of morphine produced a highly significant place preference (one sample t tests for MOR–SAL difference scores: 1 mg/kg, t_{11}=5.94, $p < 0.001$; 3 mg/kg, t_{11}=2.71, $p < 0.05$, n=12 rats per dose; Fig. 6a).

There was no significant difference in USV emission when rats were confined drug-free to the morphine-paired vs. saline-paired floor textures (Fig. 6b). The final tests with AMPH and saline confirmed the well-established unconditioned increase in call rate in response to this drug (Wilcoxon Z=4.11, p < 0.0001, n=24; Fig. 6c).

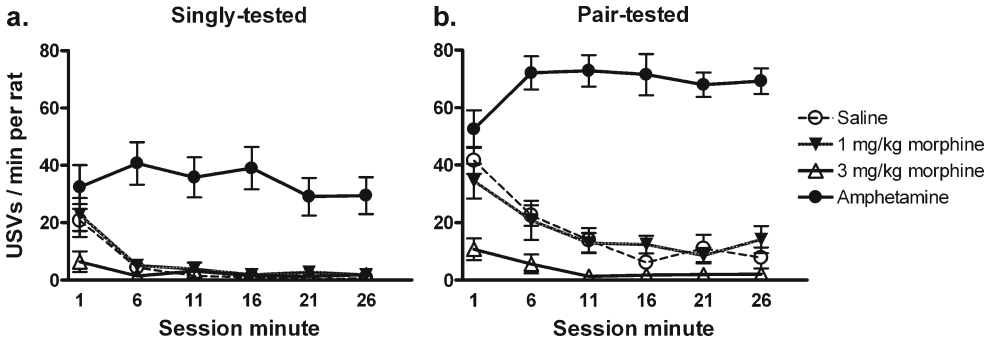

Fig. 3 Experiment 1.2: time course of USV emission following saline (*open circles*), morphine (1 or 3 mg/kg, SC; *downward/solid* and *upward/open triangles*, respectively) or AMPH (1 mg/kg, IP; *solid circle*) when rats were singly tested (**a**) and pair-tested (**b**). The *y*-axes represent mean+SEM USVs per minute (on a per rat basis)

Experiment 3

USV call rate was markedly inhibited by morphine (1 mg/kg) during the first two conditioning trials (saline vs. morphine on trials 1 and 2: Wilcoxon Z=2.71 and 2.63, both $p < 0.05$; Fig. 7a). Locomotor activity, in contrast, was significantly increased from the second morphine conditioning trial onwards (paired t tests between saline and morphine locomotion on trials 2, 3, and 4: t_{15}=2.65, 4.03, 3.12, respectively, $p < 0.05$–0.01; Fig. 7b). On the CPP test day, the subjects greatly preferred the morphine-paired floor texture (one sample t test for MOR–SAL difference score: t_{15}=5.15, p=0.0001, n=16; Fig. 8a), but they did not call at a greater rate (i.e., calls per min) when located on the drug-associated flooring (Fig. 8b). Finally, AMPH produced a significant increase in calling, as expected (Wilcoxon Z=2.73, $p < 0.01$; Fig. 8c)

Analysis of USV subtypes

Subtype analyses were performed in all three experiments. The proportion of trill calls was not significantly enhanced by morphine under any experimental condition, and there was also no discernible trend in this direction (Supplementary Tables S2, S4, S6, S9, S10, and S11). Similarly, there were no consistent changes in either the absolute number or proportion of the other 50-kHz call subtypes (Supplementary Tables S1–S11); however, overall call rates were quite low, producing a high degree of variability in the proportional measures.

Discussion

The present study yielded several novel findings. First, morphine failed to increase the 50-kHz call rate under a variety of experimental conditions, i.e., after morphine pre-exposure, with repeated drug testing, or in different social contexts. Second, low doses of morphine that failed to increase 50-kHz call rates were nevertheless rewarding and non-sedative. Third, detailed call subtype analysis indicated that, contrary to our hypothesis, morphine did not preferentially promote trill calls.

The USV-suppressant effect of morphine, which generalized to pair-tested subjects, did not appear to reflect general behavioral inhibition since it occurred at sub-cataleptic

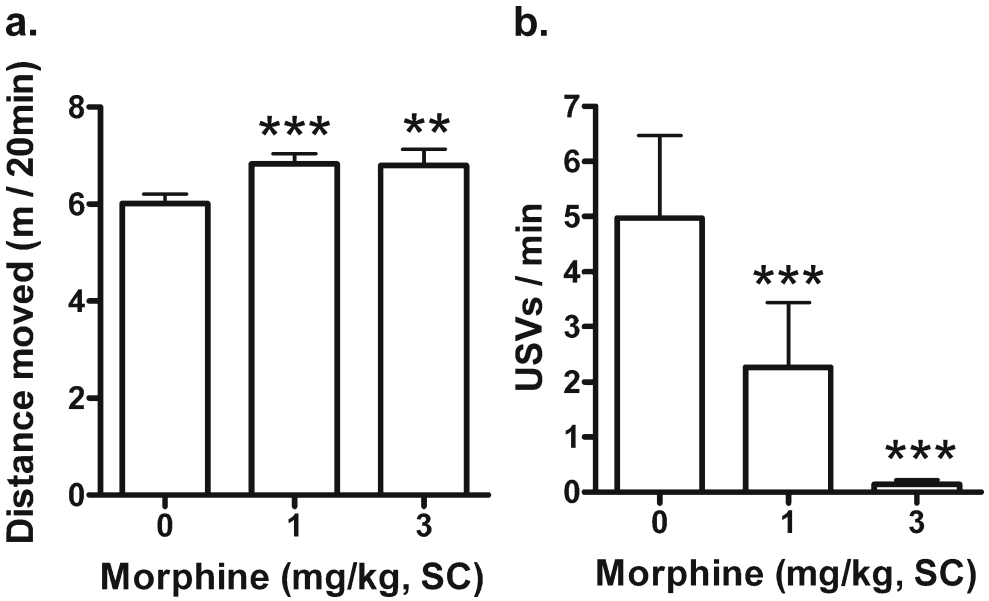

Fig. 4 Experiment 1.3: morphine (1 and 3 mg/kg, SC) significantly increased locomotor activity (**a**), while dose-dependently decreasing emission of 50-kHz calls (**b**). Each rat was tested under all conditions (n=24), **$p < 0.01$, ***$p < 0.001$ vs. saline condition

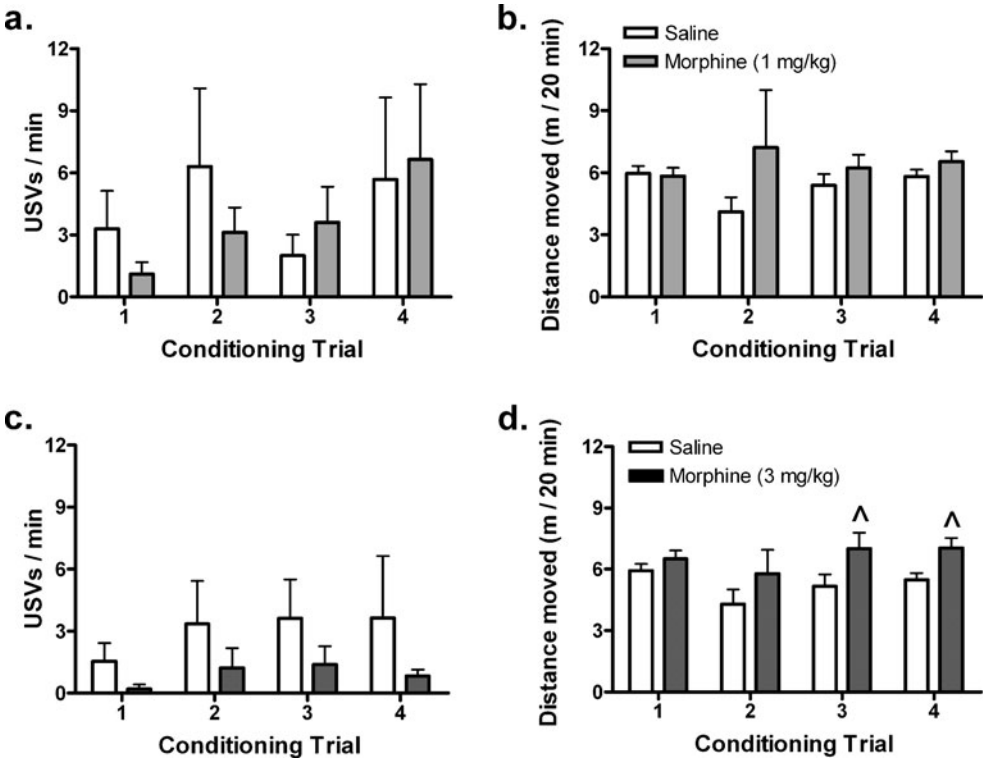

Fig. 5 Experiment 2: call rate was unaffected during morphine conditioning. Each rat received saline (*open bars*) and morphine, given at 1 mg/kg (**a**, **b**) (n=12, *light gray bars*) or 3 mg/kg (**c**, **d**) (n=12, *dark gray bars*). Locomotor activity during conditioning with 1 mg/kg (**b**) and 3 mg/kg (**d**) morphine was unaltered during the four conditioning trials, except for an apparent stimulatory effect at the higher dose (3 mg/kg) on the third and fourth conditioning trial, ^$p < 0.06$

doses (Fernandes et al. 1977; Fog 1970) and was associated with locomotor stimulation rather than sedation (experiments 1.3 and 3). This USV-suppressant effect was unaffected by prior administration of morphine in the home cage (experiment 1.1), whereas it waned rapidly when the drug was later tested repeatedly in the same environment (experiment 1.1 and experiment 3); tolerance to this USV-inhibitory effect was therefore likely context-specific (Siegel and MacRae 1984). The observed tolerance appeared reversible upon drug withdrawal, as evidenced by an inhibition of USVs with morphine after 8 days of abstinence (1.2 vs. 1.3).

Horizontal locomotor activity is inhibited or stimulated by acute systemic administration of morphine, depending on dose and time after injection, with a shift towards locomotor stimulation upon repeated drug testing (Babbini and Davis 1972; Nakamura et al. 1978; Shoaib et al. 1994; Vasko and Domino 1978). Consistent with this literature, a locomotor stimulant effect was immediately apparent in subjects that had already received seven to ten morphine injections (experiment 1.3) whereas such an effect emerged gradually in subjects that were initially drug naïve (experiment 2 and 3).

The conditioned place preference paradigm is widely used to study the rewarding properties of drugs and other stimuli (Tzschentke 1998, 2007). In the present study, the two morphine doses (1 and 3 mg/kg) produced a CPP of similar magnitude, consistent with several published reports (Bardo et al. 1995). Hence, morphine exerted rewarding effects during conditioning but at the same time failed to

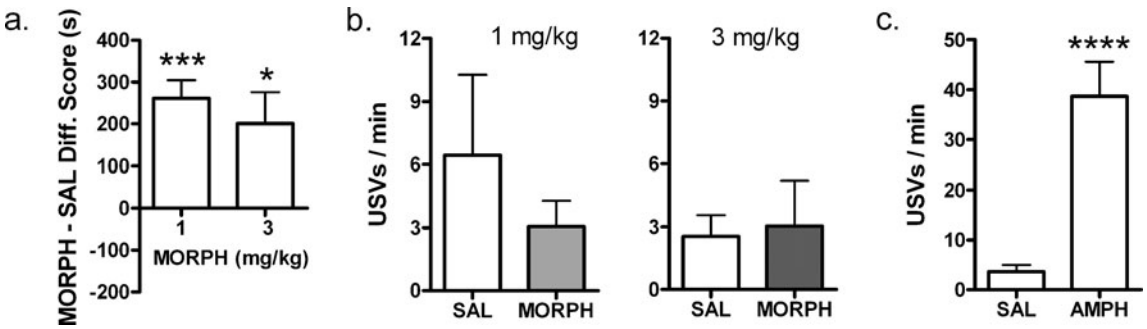

Fig. 6 Experiment 2: post-conditioning and AMPH tests. **a** On the CPP test day (day 10), both doses of morphine produced a significant place preference. **b** On days 11 and 12, there was no significant difference in USV emission when rats were confined and when drug-free to the morphine-paired vs. saline-paired floor textures (*gray and open bars*, respectively). The *y*-axes represent the mean+SEM USVs per minute, n=12 rats per dose. **c** AMPH markedly increased the call rate in a subsequent test, as expected (n=24), *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$

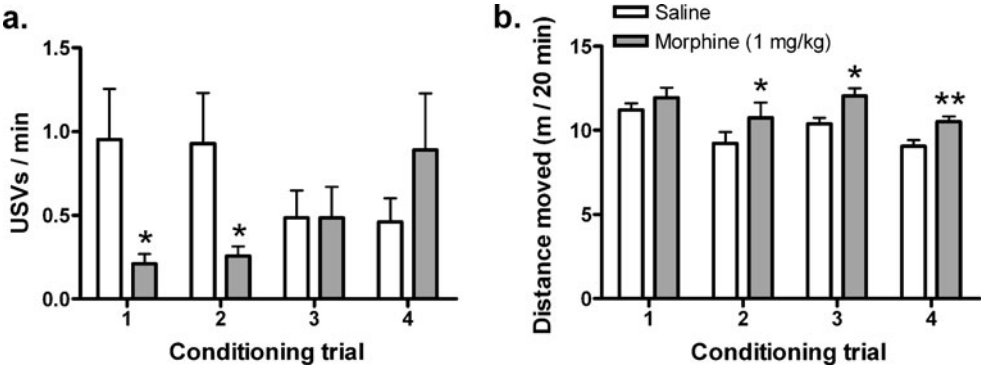

Fig. 7 Experiment 3: during conditioning trials, morphine initially decreased the call rate (**a**). However, morphine significantly increased locomotor activity during the last three trials (**b**). *$p < 0.05$, **$p < 0.01$ ($n=16$)

increase USV production. While 50-kHz USVs are associated with a variety of natural and nonnatural appetitive stimuli, it appears that only certain drugs of abuse, namely AMPH, cocaine, and methylphenidate, lead to an increase in USV emission (Ahrens et al. 2009; Maier et al. 2010; Meyer et al. 2011; Simola et al. 2012; Williams and Undieh 2010; Wright et al. 2010, 2012). Thus, neither morphine (Hamed et al. 2012; Simola et al. 2012; present study) nor MDMA (Sadananda et al. 2012) has been found to stimulate adult rat 50-kHz calling after acute administration.

The observed dissociation between morphine-induced CPP and 50-kHz calling indicates that these two behavioral measures are not generally interchangeable. We cannot at present exclude the possibility that, despite considerable correlative evidence (Knutson et al. 2002), 50-kHz calls are unrelated to reward per se. Alternatively, the concept of reward is multi-faceted (Berridge and Robinson 2003) and ultrasonic vocalizations and CPP possibly reflect different aspects of this phenomenon. A third possibility is that 50-kHz calling and CPP normally track the same type of reward, but that this relationship can be obscured by additional drug effect(s). In this regard, we are not aware of any reports that morphine can directly affect vocal cords or respiratory muscles. However, morphine can produce an aversive effect in rats through activation of peripheral opiate receptors (Bechara et al. 1987; Bechara and van der Kooy 1985); conceivably 50-kHz calling, like conditioned taste aversion, is preferentially sensitive to aversive drug effects. Morphine can also depress the activity of locus coeruleus noradrenergic neurons (Bird and Kuhar 1977; Korf et al. 1974); this action may also be pertinent since 50-kHz USV emission is critically dependent on CNS noradrenergic transmission (Wright et al. 2012).

Although morphine administration produced a robust CPP, it failed to produce *conditioned ultrasonic calling* when the rat was exposed to the drug-paired context, either though passive confinement (experiment 2) or during free choice (experiment 3). In experiment 2, the tests of conditioned USVs occurred after the CPP test session, and so it is possible that the reward–cue associations had already extinguished. However, this is unlikely for two reasons. First, CPP is reported to take several drug-free sessions to extinguish after a similar morphine conditioning regime (Mueller et al. 2002; Parker and Mcdonald 2000; Rutten et al. 2011). Second, extinction of morphine CPP is typically context-dependent (Parker et al. 2006), and the USV tests were performed in a separate room and in a distinct apparatus from the CPP test.

The non-occurrence of morphine-conditioned 50-kHz USVs in experiments 2 and 3 stands in contrast to two positive reports of conditioned calls occurring either during the expression of morphine CPP (Knutson et al. 1999) or in rats passively exposed to drug-paired CPP cues (Burgdorf et al. 2001). Several methodological factors could potentially

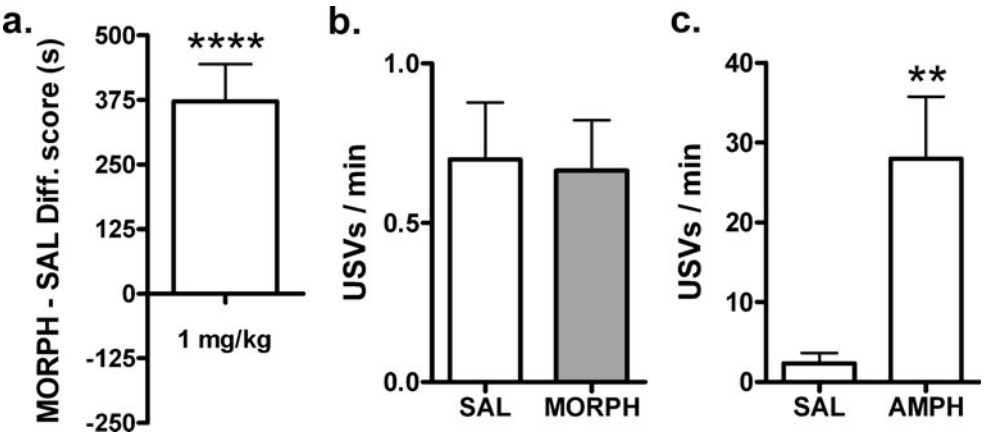

Fig. 8 Experiment 3: **a** On the CPP test day, morphine produced a significant place preference. **b** There was no significant difference in USV emission when rats were on the morphine- vs. saline-paired floor textures during the CPP test (*open and gray bars*, respectively, mean+SEM USVs per minute). **c** Tested subsequently, AMPH markedly increased the call rate, as expected. **$p < 0.01$, ****$p=0.0001$ ($n=16$)

help to explain these disparate findings, for example: the dose of morphine (i.e., 5 mg/kg) (Burgdorf et al. 2001), use of visual as well as tactile cues (Burgdorf et al. 2001; Knutson et al. 1999), biased CPP procedure (Knutson et al. 1999), conditioning session duration and number (Knutson et al. 1999), and rat strain (Burgdorf et al. 2001). Perhaps most significantly, however, call counting in these earlier reports was based on heterodyne and/or frequency-divided acoustic signals, which is less reliable than spectrographic analysis of broadband signals (Hamdani and White 2011; Parsons 2000).

Limitations

Low rates of 50-kHz calling under morphine and saline impeded call subtype analysis. Low call rates, together with high inter-subject variability, likely explains why morphine clearly suppressed calling in experiments 1 and 3, but not in experiment 2. In this context, it would therefore be interesting to examine the effects of morphine on high rates of 50-kHz USVs elicited by positive stimuli, such as sex-relevant odors (e.g., Ciucci et al. 2007) or AMPH (e.g., Wright et al. 2010).

The doses of morphine used in the present study were chosen because they are reliably rewarding and sub-cataleptic. We cannot of course exclude the possibility that doses outside the present range, or more extended dosing (Hamed et al. 2012), would produce unconditioned or conditioned USVs. Finally, whether the morphine-induced suppression of USVs is reversible using an opiate antagonist remains to be investigated.

Conclusion

The findings of the present study clearly show that even if 50-kHz calls, or trill calls in particular, reflect hedonia in some contexts (see "Introduction"), this is not the case after acute morphine administration. Furthermore, while 50-kHz calls are associated with a variety of natural and artificial appetitive stimuli, they also occur in aversive contexts such as aggression (e.g., Haney and Miczek 1994; Vivian and Miczek 1993), morphine withdrawal (Vivian and Miczek 1991), CO_2 exposure (Niel and Weary 2006) and pain (Dinh et al. 1999). The present findings provide an additional reason to exert caution when appraising the significance of 50-kHz calls.

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