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ORIGINAL INVESTIGATION

Effects of acute morphine withdrawal on ultrasonic vocalizations in adult rats: unchanged 50-kHz call rate and altered subtype profile

YiQi C. Lin[1] · Leah L. Zhao[1] · Paul B. S. Clarke[1]

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Abstract

Rationale Adult rat 22- and 50-kHz ultrasonic vocalizations (USVs) are commonly considered as indices of negative and positive affect, respectively. More specifically, we have proposed that positive affective states are revealed by a predominance of trill over flat 50-kHz call subtypes. However, the 50-kHz call subtypes emitted during aversive drug states remain largely uninvestigated.

Objectives To determine whether acute morphine withdrawal affects 50-kHz call rates or alters the relative prevalence of trill and flat calls.

Methods In experiment 1, adult male rats were given saline or morphine (6 mg/kg SC), then acutely challenged 4 h later with saline or naloxone (1 mg/kg SC), and recorded 10–30 min post-injection. In experiments 2 and 3, rats received saline or morphine (6 mg/kg), followed 4 h later by acute saline or naloxone (0.1 mg/kg) challenge; USVs were subsequently recorded during 30-min place conditioning sessions.

Results Naloxone (0.1 mg/kg) produced a strong conditioned place aversion only after acute morphine pretreatment, consistent with antagonist-precipitated morphine withdrawal. The morphine-naloxone combination decreased the relative prevalence of trills and promoted flat calls. Naloxone given alone (0.1 and 1 mg/kg) inhibited trill calls but did not significantly alter the prevalence of flat calls, whereas morphine given alone (4 h pre-session) was largely without effect. Fifty-kHz call rates were inhibited by naloxone given alone, but otherwise unaffected. Twenty-two-kHz calls were sparse.

Conclusions The 50-kHz call subtype shift seen during antagonist-precipitated morphine withdrawal was opposite in direction to that previously associated with rewards, and hence may reveal negative affect.

Keywords Morphine · Withdrawal · Naloxone · Opioid · Ultrasonic vocalization · Aversion

Introduction

Ultrasonic vocalizations (USVs) emitted by adult laboratory rats fall into two broad categories: 22- and 50-kHz calls (Brudzynski 2015; Wohr and Schwarting 2013). Twenty-two-kHz "alarm" calls are often made during aversive situations and are principally thought to reflect negative affect (Brudzynski 2015). In contrast, 50-kHz calls occur in a number of positive social and pharmacological conditions (for reviews, see Burgdorf et al. 2011; Simola 2015), suggesting that positive affective states can be revealed by the rate of 50-kHz calling (Knutson et al. 2002), or more particularly by the rate of frequency-modulated 50-kHz call emission (Burgdorf et al. 2011). On close inspection, 50-kHz vocalizations are highly heterogeneous, with at least 14 identified call subtypes, most of which are frequency-modulated (Wright et al. 2010). The most common call subtypes are the "flat" (constant frequency) call and a narrowly defined "trill" category. Based on findings with euphorigenic drugs, we have hypothesized that positive affect is reflected by an increased relative prevalence of trill calls, either considered alone (Wright et al. 2010) or in relation to a reciprocal decline in flat calls (Best et al. 2017; Wright et al. 2012b).

Although 50-kHz calls have been widely observed in rewarding contexts, little is known about their occurrence in aversive situations. The rate of 50-kHz calling was reported

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✉ Paul B. S. Clarke
paul.clarke@mcgill.ca

[1] Department of Pharmacology and Therapeutics, McGill University, McIntyre Medical Building Rm. 1320, 3655 Promenade Sir William Osler, Montreal, QC H3G 1Y6, Canada

to be reduced by several aversive manipulations, notably painful electric footshock (Schwarting et al. 2007), footshock-predictive cue (Burgdorf et al. 2000), omission of electrical brain stimulation (Burgdorf et al. 2000), and acute naloxone challenge (Burgdorf et al. 2001), although not by acute lithium administration (Pereira et al. 2014). To our knowledge, 50-kHz call *categories* have only been studied in relation to acute lithium administration (Pereira et al. 2014) and electric footshock (Taylor et al. 2017). In the earlier study, lithium did not significantly alter the relative prevalence of flat or trill calls, whereas in the later study, flat calls appeared to be associated with a mildly negative affective state. In these studies, however, 50-kHz calls were subjected to a three- or four-way categorization, as opposed to the 14-subtype scheme used in our own work (Best et al. 2017; Wright et al. 2010, 2012b).

The present study therefore seeks to address a critical knowledge gap: what kinds of 50-kHz calls are emitted in aversive situations? Here, we investigated two opioid-related aversive states. The first occurs after acute administration of the opioid receptor antagonist naloxone, in subjects having little or no experience of morphine (Tzschentke 2007). The second is associated with morphine withdrawal (Harris and Gewirtz 2005). In a previous study (Vivian and Miczek 1991), rats that were undergoing spontaneous morphine withdrawal were found to emit both 22- and 50-kHz calls, but these vocalizations were not categorized further. In the present study, we chose to investigate acute (i.e., single-dose) opioid withdrawal, produced by giving rats a precipitating dose of naloxone a few hours after a pretreatment injection of morphine (Harris and Gewirtz 2005). Human subjects experience dysphoria during acute antagonist-precipitated morphine withdrawal, and also after high-dose naloxone administered alone (Cohen et al. 1981; Harris and Gewirtz 2005; Martin del Campo et al. 1997). In laboratory rats, these two drug states are associated with conditioned place aversion (CPA)(Azar et al. 2003; Bals-Kubik et al. 1989; Harris and Gewirtz 2005; Mucha and Herz 1985; Parker et al. 2002; Tzschentke 2007), and hence, this measure was used to confirm that our drug treatments were aversive.

The present study had three main goals. Specifically, we sought to determine whether aversive drug conditions: (1) reduce the 50-kHz call rate, (2) shift the 50-kHz call subtype profile in the opposite direction to rewarding drugs, and (3) concurrently promote or suppress other 50-kHz calls subtypes.

Methods

Animals

Subjects were 48 male Long-Evans rats (Charles River Laboratories, St. Constant, Quebec, Canada). At the start of testing, they were drug-naïve and weighed 275–301, 261–343, and 292–350 g (in experiments 1–3, respectively). Animals were housed 2–3 per cage in a humidity- and temperature-controlled colony room at the McGill University Animal Resources Centre. Home cage bedding consisted of laboratory grade SaniChips (Harlan Laboratories, Indianapolis, IN). Rats were maintained on a reverse 12:12-h light/dark cycle with lights off at 0700 h, and all testing was performed during the dark phase of the cycle. Food and water were available ad libitum except during testing. Rats were each handled once daily for 2 min, for 3 days prior to the first habituation day. All procedures were approved by the McGill Animal Care Committee in accordance with the guidelines of the Canadian Council on Animal Care.

Testing apparatus

Behavioral testing and recordings were performed in acoustically isolated operant conditioning chambers (experiment 1) or place conditioning boxes (experiments 2 and 3).

The four operant conditioning chambers (ENV-007CT, Med Associates, St Albans, VT) were as described previously (e.g., Wright et al. 2012a). Thus, each chamber was enclosed in a melamine compartment lined with sound-attenuating acoustic foam (Primacoustic, Port Coquitlam, British Columbia). A condenser ultrasound microphone (CM16/CMPA, Avisoft Bioacoustics, Berlin, Germany) was securely inserted through a small (5 cm diameter) hole located centrally in the top panel of each chamber, so that the microphone was 15–30 cm from the rat during testing. The two operant levers were retracted at all times.

Locomotor activity and CPA were both tested in four place conditioning boxes, each comprising a rectangular arena (58 cm long × 29 cm wide) enclosed by melamine walls (53 cm high), as described previously (Wright et al. 2012a), except where noted below. 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 adjacent tiles completely covered the bottom of each CPP cage. In the present study, as an additional refinement, the place conditioning boxes were each equipped with an 8-mm-thick clear Plexiglas™ lid, and the microphone was inserted through a small (9 cm × 6 cm) recess cut halfway along one long side. A video tracking system (EthoVision v 3.0, Noldus Information Technology, Leesburg, VA, USA) measured total horizontal distance moved (conditioning sessions) and the time spent on each floor texture (CPA test day). To minimize visual cues, conditioning and testing were conducted under far-red (wavelength > 650 nm) illumination using a Kodak GBX-2 safelight filter (Vistek, Toronto, Ontario, Canada).

Acquisition and acoustic analysis of ultrasonic vocalizations

Broadband recordings and acoustic analysis were performed as detailed in our recent publications (e.g., Scardochio and Clarke 2013). Briefly, rats were recorded individually in the acoustically isolated testing apparatus, via an ultrasonic microphone connected to an UltraSoundGate 416H data acquisition device (Avisoft Bioacoustics). The sampling rate was 250 kHz with 16-bit resolution. Spectrograms were generated by fast Fourier transform (512 points, 75% overlap, FlatTop window, 100% frame size) using Avisoft SASLab Pro (Version 5.2.07). Calls were manually selected from spectrograms by a single individual (L.Z. in Experiment 1 and YQ.C.L.in Experiments 2 and 3) blinded to the treatment conditions. All calls in a given session were selected; hence, time-sampling was not used. Twenty-two-kHz calls were counted, and 50-kHz calls were categorized according to our 14-subtype scheme (Wright et al. 2010), as indicated in the Fig. 2 legend (below).

Drugs

Test drugs were morphine sulfate pentahydrate (6 mg/kg, Sandoz, Boucherville, Quebec) and μ-opioid antagonist naloxone hydrochloride dihydrate (0.1 or 1 mg/kg; Sigma-Aldrich, Oakville, Ontario). The doses of morphine and naloxone, and the 4-h injection interval, were based on published findings (Azar et al. 2003; Harris and Gewirtz 2005; Schulteis et al. 1997). Both drugs were dissolved in sterile 0.9% saline, given by subcutaneous (SC) injection in a volume of 1 ml/kg. All doses are expressed as the free base. The saline vehicle served as a control for each drug condition.

General testing procedure (all experiments)

On each test day, rats were transported from the colony room, weighed, and then left in their home cages for 15 min to acclimatize to the testing room. Rats were then pre-treated with saline or morphine, 4 h before a challenge injection of saline or naloxone. After the challenge injection, rats were returned to the home cage for 10 min to allow time for withdrawal onset (Harris et al. 2004), before being placed in the test chamber and recorded for 20 min (experiment 1) or 30 min (experiments 2 and 3). In experiments 1 and 3, all rats received all drug conditions. In experiment 2, rats received two out of the four conditions, as imposed by the CPA design. Drug tests were given in counterbalanced order.

Experiment 1: antagonist-precipitated morphine withdrawal: USV emission

Habituation (*days 1–3*) On day 1, rats ($n = 12$) were subjected to an injection-free habituation session in the test apparatus, lasting 20 min. This procedure was repeated on days 2 and 3, except that rats now first received a vehicle injection in the home cage and were placed in the test apparatus 10 min post-injection.

Test phase (*days 4–11*) Each rat received one test session on each of the 8 test days. These test days occurred 48 h apart and were arranged in two consecutive blocks (i.e., day 4–7 and 8–11). Each block comprised a 2 × 2 design, in which each rat received each of four drug conditions, i.e., all four combinations of pretreatment (saline or morphine 6 mg/kg SC) and challenge (saline or naloxone 1 mg/kg SC). Rats were placed in test chambers 10 min after the challenge injection and were recorded for 20 min.

Experiment 2 antagonist-precipitated morphine withdrawal: USV emission and CPA

This experiment used a greater number of drug treatments, in order to produce a more intense form of acute withdrawal. All sessions occurred in the CPA apparatus. A lower (0.1 mg/kg) dose of naloxone was chosen, which is reported to produce withdrawal CPA in morphine-pretreated animals, without producing significant CPA when given alone (see "Discussion"). The following steps occurred on 10 consecutive days.

Habituation (*1 day*) One session was given, with no injections. Rats ($n = 24$) were placed in test boxes for 20 min.

Conditioning phase (*days 2–9*) Rats were randomly allocated to two groups ($n = 12$). Throughout this period of 8 consecutive days, groups 1 and 2 received daily home cage pretreatment injections of saline and morphine (6 mg/kg SC), respectively. Four hours after a home cage injection, each rat was challenged with saline or naloxone (0.1 mg/kg SC), and these two challenges occurred on alternating days, i.e., SNSNSNSN or NSNSNSNS (counterbalanced within group). Ten minutes after the challenge injection, rats were placed in the CPA boxes for 30 min. For a given rat, one of the two floor textures was paired with saline and the other with naloxone, and this pairing was counterbalanced within each group. During these conditioning sessions, USVs were recorded and the rats' movements were tracked by video recording.

Test phase (*day 10*) All rats were subjected to a single drug-free 15-min test session in which their movements were video-tracked. Each test box contained the two floor textures that had been used during conditioning, and these textures were positioned in a counterbalanced fashion. At the start of the session, each rat was placed on the midline, straddling the two floor textures.

Experiment 3 morphine administered alone: USV emission and CPA

The purpose of this experiment was to determine whether morphine alone, given 4 h in advance of the session, would produce a CPA. The same procedure was used as in experiment 2, except

that (1) there was now a single group of rats ($n = 12$), and (2) during the conditioning phase, one floor texture was paired with saline and the other with morphine (counterbalanced across rats).

Data analysis and statistics

Subjects were assigned to groups and conditions in a randomized or counterbalanced manner. Drug solutions were coded so that experimenters were blind to drug conditions. Data were analyzed using Systat v11 software (SPSS, Chicago, IL) and figures were generated using Prism 4 (GraphPad Software, La Jolla, CA). Primary measures were the 50-kHz call rate, the percentages of flat and trill calls, and CPA magnitude. The remaining 50-kHz call subtypes were also of interest (see "Introduction"). Fifty-kHz call profiles were defined by the proportional contributions of all 14 call subtypes (Wright et al. 2010). Locomotor activity provided an additional behavioral measure, measured as the distance moved during conditioning sessions. For data collected in two test sessions performed under the same drug conditions, a mean value was computed, unless otherwise stated.

Parametric tests were used, unless underlying assumptions were violated; this was the case only for 50-kHz call rates, which were positively skewed. Factors were as follows: in experiment 1, morphine and naloxone (both within-subject); in experiment 2, morphine (between-subject) and naloxone (within-subject); and in Experiment 3, morphine (within-subject). Interactions between naloxone and morphine were tested by two-way repeated measures ANOVA, except in the case of 50-kHz call rates. For the latter, a naloxone-saline difference score was computed for each rat and this variable was then compared between the saline and morphine groups using a nonparametric (Mann-Whitney *U*) test. The other main statistical comparisons were between the saline-saline (SS) condition and the other morphine (M) and naloxone (N) conditions, i.e., SS vs. MS (effect of morphine alone), SS vs. SN (effect of naloxone alone), and SS vs. MN (drug combination). For all variables except 50-kHz call rates, these specific comparisons were made using paired or independent *t* tests (or Welch's *t* test in cases of heteroscedasticity). For 50-kHz call rates, comparisons were made with nonparametric tests, i.e., Wilcoxon rank-sum or Mann-Whitney *U* tests (indicated in the text by Z and U values, respectively). No adjustment was made for multiple comparisons, but the alpha level was set at 1% (two-tailed) throughout.

Results

In all three experiments, 50-kHz calls were much more prevalent than 22-kHz calls. Specifically, the total numbers of 22 and 50-kHz calls emitted in experiments 1–3 were, respectively, 0 and 2004, 8 and 5484, and 1 and 8948. The nine 22-kHz calls were emitted by five rats in eight sessions; four of these nine calls occurred after morphine pretreatment followed by saline challenge, and the remaining five calls occurred after morphine pretreatment followed by naloxone challenge. The main findings are summarized in Table 1. *Absolute* emission rates for individual 50-kHz call subtypes were not analyzed, but are presented in Supplementary Tables S1–S3.

Experiment 1 antagonist-precipitated morphine withdrawal: USV emission

50-kHz call rate Call rates, shown in Fig. 1a, were reduced by naloxone (1 mg/kg) when given alone (SS vs. SN: Wilcoxon test: $Z = 2.67$, $p = 0.0076$), but not by morphine alone (SS vs. MS), or by the morphine-naloxone combination (SS vs. MN). Morphine appeared to blunt the inhibitory effect of naloxone, as defined by naloxone-saline difference scores (saline vs. morphine pretreatment, $Z = 2.00$, $p = 0.0454$).

Table 1 Effects of morphine and naloxone on 50-kHz call emission: main findings

Drug conditions	Experiment	Morphine (mg/kg)	Naloxone (mg/kg)	Call rate	% Flats	% Trills
SN vs. SS	1		1	↓	-	↓
SN vs. SS	2		0.1	↓	-	↓? ($p = 0.0130$)
MS vs. SS	1	6		-	↑? ($p = 0.0136$)	-
MS vs. SS	2	6		-	↑	↓
MS vs. SS	3	6		-	-	-
MN vs. SS	1	6	1	-	↑	↓? ($p = 0.0102$)
MN vs. SS	2	6	0.1	-	↑	↓

Pretreatments and treatments are abbreviated as follows: *S* saline, *M* morphine, *N* naloxone. Thus, "MS" refers to morphine given 4 h before saline challenge. Stimulatory and inhibitory effects of drug treatments are shown by ↑ and ↓ symbols, respectively (i.e., $p < 0.01$). An added question mark (e.g., ↓?) denotes a statistically non-significant trend (i.e., $p = 0.01$–0.05). A dash (i.e. "-") indicates no trend. n/a, not applicable, since not tested

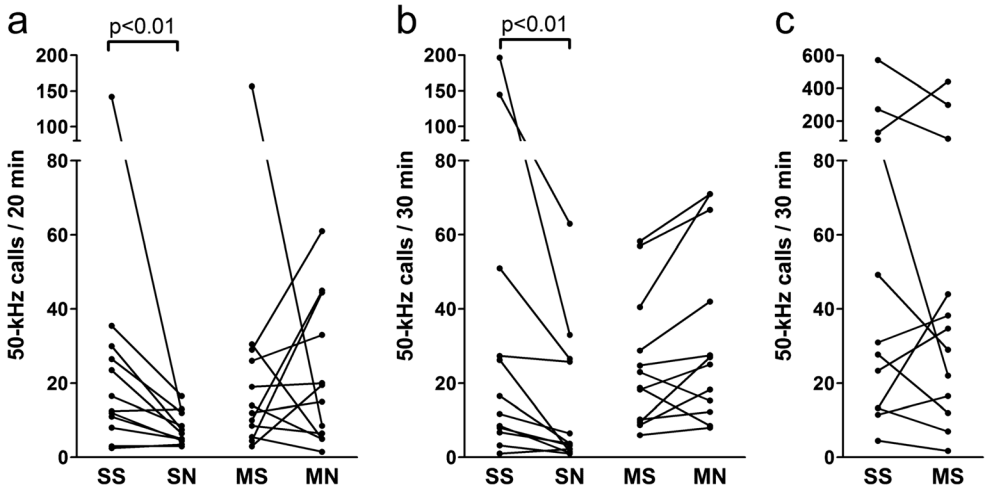

Fig. 1 50-kHz call rate (experiments 1–3, panels **a**–**c**, respectively). Rats received an injection of saline (S) or morphine 6 mg/kg (M), followed by acute saline or naloxone injection (N: 1 mg/kg in experiment 1 and 0.1 mg/kg in experiment 2). Each line represents a single rat. In experiments 1 and 3, each rat was tested under all drug conditions ($n = 12$ rats). In experiment 2, each rat received either saline or morphine pretreatment ($n = 12$ rats per group) and was then challenged with both saline and naloxone

50-kHz call profile Overall, flat and short calls were the most prevalent, with trills accounting for only 11% of all calls even in the saline-saline condition (Fig. 2, left column). The percentage of flat calls was significantly increased by the morphine-naloxone combination ($t_{11} = 3.36$, $p = 0.0064$), with a similar trend for morphine given alone ($t_{11} = 2.93$, $p = 0.0136$), but this measure appeared unaffected by naloxone alone. The percentage of trill calls was suppressed by naloxone alone ($t_{11} = 3.94$, $p = 0.0023$), with a similar trend for the drug combination ($t_{11} = 3.09$, $p = 0.0102$), but this measure appeared unaffected by morphine alone. There was no detectable two-way (morphine × naloxone) ANOVA interaction for either flat or trill calls. No other 50-kHz call subtypes were significantly altered.

Experiment 2 antagonist-precipitated morphine withdrawal: USV emission and CPA

50-kHz call rate As in experiment 1, call rates (Fig. 1b) were not significantly affected by morphine alone or by the morphine-naloxone combination, but were reduced by naloxone when given alone (SS vs. SN: Wilcoxon $Z = 2.98$, $p = 0.0029$). Naloxone differentially affected call rate in the saline and morphine groups (comparison of naloxone-saline difference scores: $U = 14.00$, $p = 0.0008$), such that naloxone appeared to *increase* calling when given after morphine injection (MS vs. MN: $Z = 2.04$, $p = 0.0413$).

50-kHz call profile The most common call subtypes were flat, trill, and short (Fig. 2, middle). The percentage of flat calls was unaffected by naloxone alone (SS vs. SN), but increased by morphine alone (SS vs. MS: $t_{22} = 4.41$, $p = 0.0002$), and by the morphine-naloxone combination (SS vs. MN: $t_{22} = 6.90$, $p = 0.0001$); there was no significant morphine × naloxone ANOVA interaction. The percentage of trill calls was reduced by morphine alone and by the drug combination (respectively: $t_{15} = 3.81$, $p = 0.0016$; $t_{11} = 4.98$, $p = 0.0004$), with a similar trend for naloxone alone ($t_{11} = 2.96$, $p = 0.0130$). There was no detectable morphine × naloxone ANOVA interaction, possibly reflecting a "floor" effect (Fig. 2, middle column). No other 50-kHz call subtypes were significantly altered.

Place conditioning As shown in Fig. 3a, naloxone (0.1 mg/kg) produced a strong CPA in the morphine-pretreated group ($t_{11} = 18.19$, $p = 0.0001$), with a trend in the saline-pretreated group ($t_{11} = 2.00$, $p = 0.0709$). The two groups differed significantly in the percentage of time spent on the naloxone-paired side (Welch's $t_{13} = 4.14$, $p = 0.0011$); respective mean ± SEM values were 16 ± 2 and 39 ± 5%.

Locomotor activity After initial inspection, data were collapsed across test day pairs (Fig. 3b). Distance moved was reduced by the morphine-naloxone combination (SS vs. MN: $t_{22} = 5.82$, $p = 0.0001$), but not by morphine or naloxone given alone. However, there was no detectable morphine × naloxone ANOVA interaction.

Experiment 3 morphine administered alone: USV emission and CPA

50-kHz USVs Morphine did not detectably affect the number of 50-kHz calls emitted (Fig. 1c) or the percentage of flat calls (Fig. 2, right column), but tended to decrease the percentage of

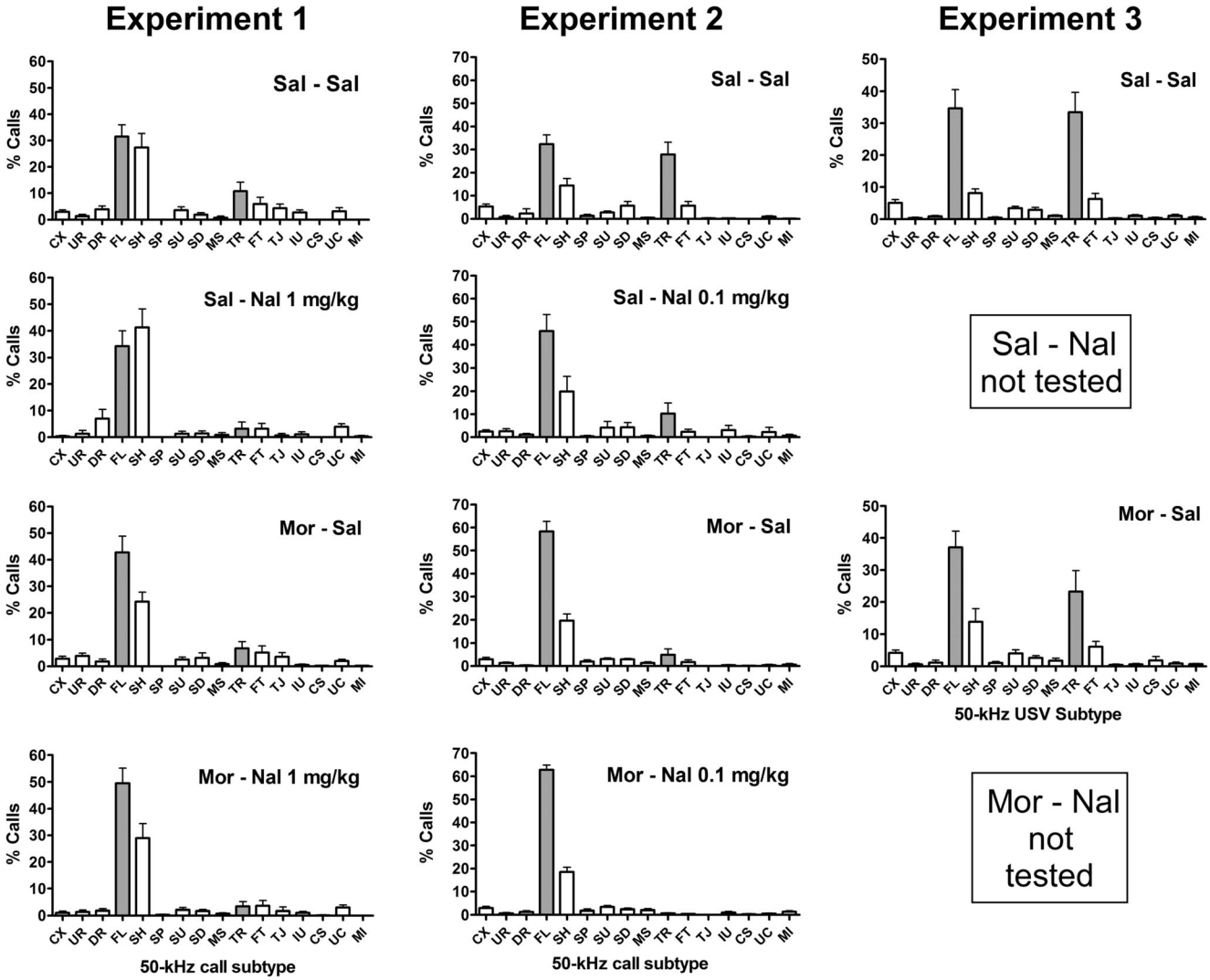

Fig. 2 50-kHz call subtype profiles (experiments 1–3). Rats received an injection of saline (Sal) or morphine 6 mg/kg (Mor), followed by acute saline or naloxone injection (Nal: 1 mg/kg in experiment 1 and 0.1 mg/kg in experiment 2). Y-axes show the relative prevalence of each subtype, expressed as the mean ± SEM percentage of all 50-kHz calls. Each rat was tested under each drug condition, except that in experiment 2, separate groups received saline and morphine pretreatment. Hence, n = 12 rats for each group and drug condition in all experiments. Flat (FL) and trill (TR) calls are shown by gray bars. Call subtype abbreviations: CX complex, UR upward ramp, DR downward ramp, FL flat, SH short, SP split, SU step-up, SD step-down, MS multi-step, TR trill, FT flat-trill, TJ trill with jumps, IU inverted-U, CS composite, UC unclear, MI miscellaneous

trills ($t_{11} = 1.99$, $p = 0.0722$). No other 50-kHz call subtypes appeared affected (Fig. 2, right column).

Place conditioning Morphine pretreatment induced neither a CPA nor a CPP ($t_{11} = 1.51$, $p = 0.1602$), with rats spending 55 ± 4% (mean ± SEM) of the time on the morphine-paired side (Fig. 3c).

Locomotor activity The effect of morphine depended on test day pair (two-way interaction $F_{3,33} = 12.60$, $p = 0.0001$; Fig. 3d), such that morphine significantly inhibited locomotion only when the drug was first tested ($t_{11} = 4.24$, $p = 0.0014$).

Discussion

Main findings

The main findings are summarized in Table 1. In the present study, the morphine-naloxone combination produced a strong CPA, as expected. By this measure, the naloxone-alone condition also appeared somewhat aversive, whereas pretreatment with morphine (4 h pre-session) was not. The 50-kHz call rate was largely unchanged by drug administration, except for an inhibitory effect of naloxone alone. The morphine-naloxone condition produced the most marked effect on 50-

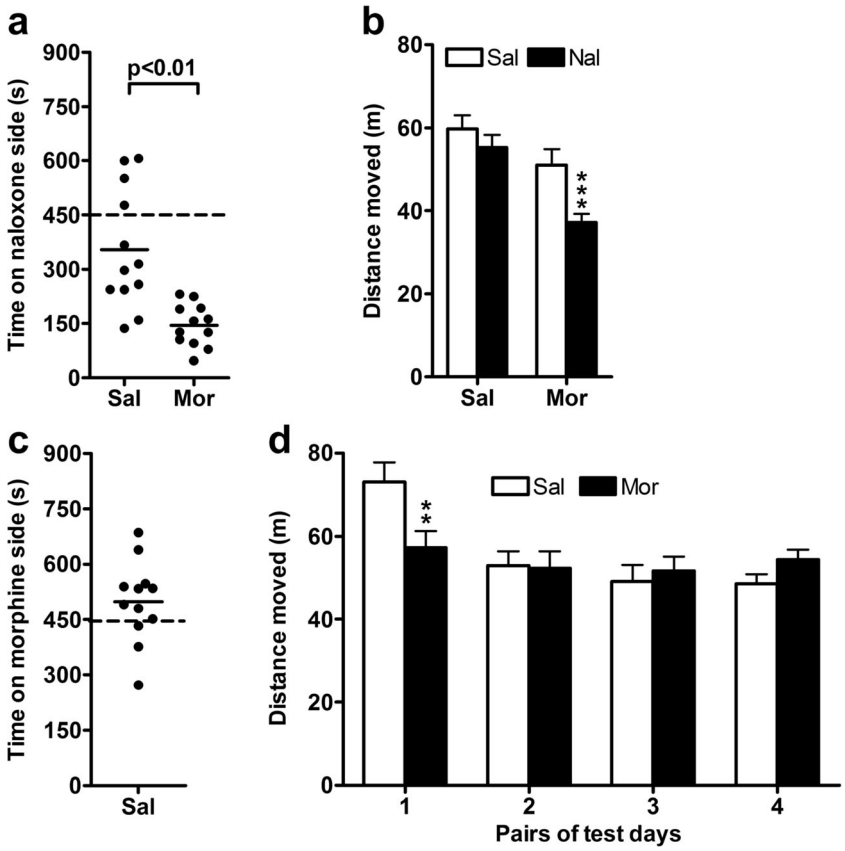

Fig. 3 Place conditioning and locomotor activity in experiments 2 (panels **a** and **b**) and 3 (panels **c** and **d**). Panel **a** shows the time spent on the side previously paired with naloxone (Nal), which was given after either saline (Sal) or morphine (Mor) pretreatment. The solid and dotted horizontal lines represent, respectively, the group mean and the indifference point of the 900-s test session. Each symbol represents an individual rat. Panel **b** refers to the same two pretreatment groups and shows the mean ± SEM distance moved (m) during the 30-min place conditioning sessions. Panel **c** shows the time spent on the side previously paired with morphine (given approx. 4 h pre-session), and panel **d** shows the locomotor activity data from the corresponding conditioning sessions. $n = 12$ rats per group or condition. $^{**}p < 0.01$, $^{***}p < 0.001$

kHz call profile, increasing the relative prevalence of flats at the expense of trill calls. Naloxone alone also decreased the proportion of trills, but did not promote flat calls. The effects of morphine pretreatment alone were inconsistent across experiments. No other 50-kHz call subtypes were consistently associated with any drug condition, and 22-kHz calls were virtually absent in any drug condition.

Naloxone-induced conditioned place aversion

Naloxone was initially tested in operant conditioning chambers (experiment 1). Here, we used a dose of 1 mg/kg, which is reported to produce a CPA in the majority of studies that have used at least three drug pairings (Bals-Kubik et al. 1989; Mucha and Herz 1985; Tzschentke 2007). In experiment 2, we selected a lower dose of naloxone (0.1 mg/kg), which was intended to have little or no aversive effect when given alone (Bals-Kubik et al. 1989; Mucha and Herz 1985; Mucha and Iversen 1984; Shippenberg et al. 1988), while still being capable of precipitating morphine withdrawal (Azar et al. 2003; Schulteis et al. 1997; Young 1986). Correspondingly, in the CPA procedure of experiment 2, naloxone 0.1 mg/kg by itself produced only a non-significant aversive tendency, while it precipitated a strong CPA in rats that had been acutely pretreated with morphine.

Effects of morphine alone

The morphine-alone condition produced mixed results on 50-kHz call emission across experiments (Table 1). Significant effects were observed only in experiment 2. Here, morphine pretreatment by itself decreased the percentage of trills and increased the percentage of flat calls, the same kind of change as seen in the highly aversive morphine-naloxone condition. This finding was unexpected since, at 4 h after injection of morphine, the drug's acute rewarding effects would likely have dissipated and any aversive effects associated with spontaneous withdrawal would also have been weak (Altarifi and Negus 2011; Eisenberg 1982; Harris and Gewirtz 2005; Liu and Schulteis 2004). In this experiment, the effect of morphine alone appears attributable to the particularities of the experimental design, based on the following considerations. Rats in this experiment were allocated to two groups: both groups were conditioned with naloxone, but only one group was acutely pretreated with morphine (i.e., 4 h in advance of each conditioning session). The two groups can be summarized thus: (1) SS vs. SN, and (2) MS vs. MN. Only the latter group had the opportunity to experience the intense aversiveness of the MN condition, and this aversiveness may have partially generalized to the MS condition over the course of multiple conditioning sessions. Accordingly, the final experiment (experiment 3) was designed to test whether the morphine-alone

condition would be aversive in rats that were never exposed to the MN condition. In these rats, no CPA occurred, and the relative prevalence of flat and trill calls was also unaffected by the drug.

22-kHz vocalizations

Rat 22-kHz vocalizations have been reported in a variety of aversive situations and are commonly used as an index of negative affect (Brudzynski 2015; Portfors 2007). However, in the present study, 22-kHz calls were very infrequent, even in the highly aversive morphine-naloxone condition. This observation adds to published findings suggesting that not all negative emotional states are accompanied by 22-kHz calls, at least in singly tested subjects (for review, see Schwarting and Wohr 2012). For example, adult rats tend not to emit 22-kHz calls during the receipt of aversive electric shock, but rather between such stimuli (Schwarting and Wohr 2012; van der Poel and Miczek 1991); such calls potentially reflect anticipation of punishment and may also signal fear (Brudzynski 2015). In other studies, rats failed to make 22-kHz calls after administration of lithium or after a painful intraperitoneal injection of acetic acid (Pereira et al. 2014; Portavella et al. 1993). To our knowledge, only two groups have reported on 22-kHz call emission during morphine withdrawal (Kalinichev and Holtzman 2003; Vivian and Miczek 1991). In the more recent study, morphine withdrawal was found to inhibit 22-kHz calling evoked by a startle stimulus. In contrast, Vivian and Miczek (1991) reported that 22-kHz calling increased during withdrawal, but only when animals were tested in pairs; singly tested animals made few 22-kHz calls, as in the present study.

Study limitations

The use of single acute doses of morphine and naloxone represents an obvious limitation, but served to reproduce aversive drug states that have been extensively characterized in the literature (Harris and Gewirtz 2005). Another limitation is that animals were tested in isolation; to the extent that ultrasonic vocalizations serve to communicate between conspecifics, the present findings may not necessarily generalize to group settings.

50-kHz call subtypes in relation to aversion

Based on the present CPA results and the wider literature, our drug conditions can be considered to range from neutral to highly aversive, as follows: morphine alone (4 h pre-session), naloxone 0.1 mg/kg, naloxone 1 mg/kg, and the morphine-naloxone combination. The following pattern then emerges. Prior administration of morphine alone did not change the relative prevalence of flat or trill calls (experiment 3). The low-dose naloxone-alone challenge, which was minimally aversive, appeared to reduce the percentage of trill calls, but this did not reach our significance level (set at 1%, two-tailed). The high dose naloxone-alone challenge, reported to be reliably aversive, reduced the percentage of trill calls while leaving flat calls unaffected. Finally, the highly aversive morphine-naloxone combination shifted the 50-kHz calls profile in the predicted direction, promoting flat calls at the expense of trill calls. An increased relative prevalence of "constant-frequency" calls has also been reported after the repeated delivery of mild footshock (Taylor et al. 2017), but these vocalizations were defined more broadly (bandwidth < 10 kHz) than our narrowly defined flat call subtype.

Our findings, taken together, suggest that drug aversion may be reflected in an increased prevalence of flat calls and a reduced prevalence of trill calls, with the latter being a potentially more sensitive marker. However, given the limited scope of the present study, it will be necessary to extend this work across multiple drug doses, to other aversive drug states, and to pair- or group-tested animals.

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Compliance with ethical standards

All procedures were approved by the McGill Animal Care Committee in accordance with the guidelines of the Canadian Council on Animal Care.

References

Altarifi AA, Negus SS (2011) Some determinants of morphine effects on intracranial self-stimulation in rats: dose, pretreatment time, repeated treatment, and rate dependence. Behav Pharmacol 22:663–673

Azar MR, Jones BC, Schulteis G (2003) Conditioned place aversion is a highly sensitive index of acute opioid dependence and withdrawal. Psychopharmacology 170:42–50

Bals-Kubik R, Herz A, Shippenberg TS (1989) Evidence that the aversive effects of opioid antagonists and kappa-agonists are centrally mediated. Psychopharmacology 98:203–206

Best LM, Zhao LL, Scardochio T, Clarke PB (2017) Effects of repeated morphine on ultrasonic vocalizations in adult rats: increased 50-kHz call rate and altered subtype profile. Psychopharmacology 234:155–165

Brudzynski SM (2015) Pharmacology of ultrasonic vocalizations in adult rats: significance, call classification and neural substrate. Curr Neuropharmacol 13:180–192

Burgdorf J, Knutson B, Panksepp J (2000) Anticipation of rewarding electrical brain stimulation evokes ultrasonic vocalization in rats. Behav Neurosci 114:320–327

Burgdorf J, Knutson B, Panksepp J, Shippenberg TS (2001) Evaluation of rat ultrasonic vocalizations as predictors of the conditioned aversive effects of drugs. Psychopharmacology 155:35–42

Burgdorf J, Panksepp J, Moskal JR (2011) Frequency-modulated 50kHz ultrasonic vocalizations: a tool for uncovering the molecular substrates of positive affect. Neurosci Biobehav Rev 35:1831–1836

Cohen MR, Cohen RM, Pickar D, Weingartner H, Murphy DL, Bunney WE Jr (1981) Behavioural effects after high dose naloxone administration to normal volunteers. Lancet 2:1110

Eisenberg RM (1982) Further studies on the acute dependence produced by morphine in opiate naive rats. Life Sci 31:1531–1540

Harris AC, Gewirtz JC (2005) Acute opioid dependence: characterizing the early adaptations underlying drug withdrawal. Psychopharmacology 178:353–366

Harris AC, Hanes SL, Gewirtz JC (2004) Potentiated startle and hyperalgesia during withdrawal from acute morphine: effects of multiple opiate exposures. Psychopharmacology 176:266–273

Kalinichev M, Holtzman SG (2003) Changes in urination/defecation, auditory startle response, and startle-induced ultrasonic vocalizations in rats undergoing morphine withdrawal: similarities and differences between acute and chronic dependence. J Pharmacol Exp Ther 304:603–609

Knutson B, Burgdorf J, Panksepp J (2002) Ultrasonic vocalizations as indices of affective states in rats. Psychol Bull 128:961–977

Liu J, Schulteis G (2004) Brain reward deficits accompany naloxone-precipitated withdrawal from acute opioid dependence. Pharmacol Biochem Behav 79:101–108

Martin del Campo AF, Granados-Soto V, Aguirre-Banuelos P, Cardenas M, Castaneda-Hernandez G (1997) The use of the plasma concentration-effect relationship as a tool for the study of the mechanism of action of naloxone effects on mood and endocrine function. J Psychopharmacol 11:361–365

Mucha RF, Herz A (1985) Motivational properties of kappa and mu opioid receptor agonists studied with place and taste preference conditioning. Psychopharmacology 86:274–280

Mucha RF, Iversen SD (1984) Reinforcing properties of morphine and naloxone revealed by conditioned place preferences: a procedural examination. Psychopharmacology 82:241–247

Parker LA, Cyr JA, Santi AN, Burton PD (2002) The aversive properties of acute morphine dependence persist 48 h after a single exposure to morphine: evaluation by taste and place conditioning. Pharmacol Biochem Behav 72:87–92

Pereira M, Andreatini R, Schwarting RK, Brenes JC (2014) Amphetamine-induced appetitive 50-kHz calls in rats: a marker of affect in mania? Psychopharmacology 231:2567–2577

Portavella M, Depaulis A, Vergnes M (1993) 22-28 kHz ultrasonic vocalizations associated with defensive reactions in male rats do not result from fear or aversion. Psychopharmacology 111:190–194

Portfors CV (2007) Types and functions of ultrasonic vocalizations in laboratory rats and mice. J Am Assoc Lab Anim Sci 46:28–34

Scardochio T, Clarke PBS (2013) Inhibition of 50-kHz ultrasonic vocalizations by dopamine receptor subtype-selective agonists and antagonists in rats. Psychopharmacology 226:589–600

Schulteis G, Heyser CJ, Koob GF (1997) Opiate withdrawal signs precipitated by naloxone following a single exposure to morphine: potentiation with a second morphine exposure. Psychopharmacology 129: 56–65

Schwarting RK, Wohr M (2012) On the relationships between ultrasonic calling and anxiety-related behavior in rats. Braz J Med Biol Res 45: 337–348

Schwarting RK, Jegan N, Wohr M (2007) Situational factors, conditions and individual variables which can determine ultrasonic vocalizations in male adult Wistar rats. Behav Brain Res 182:208–222

Shippenberg TS, Millan MJ, Mucha RF, Herz A (1988) Involvement of beta-endorphin and mu-opioid receptors in mediating the aversive effect of lithium in the rat. Eur J Pharmacol 154:135–144

Simola N (2015) Rat ultrasonic vocalizations and behavioral neuropharmacology: from the screening of drugs to the study of disease. Curr Neuropharmacol 13:164–179

Taylor JO, Urbano CM, Cooper BG (2017) Differential patterns of constant frequency 50 and 22 kHz USV production are related to intensity of negative affective state. Behav Neurosci 131:115–126

Tzschentke TM (2007) Measuring reward with the conditioned place preference (CPP) paradigm: update of the last decade. Addict Biol 12:227–462

van der Poel AM, Miczek KA (1991) Long ultrasonic calls in male rats following mating, defeat and aversive stimulation: frequency modulation and bout structure. Behaviour 119:127–142

Vivian JA, Miczek KA (1991) Ultrasounds during morphine withdrawal in rats. Psychopharmacology 104:187–193

Wohr M, Schwarting RK (2013) Affective communication in rodents: ultrasonic vocalizations as a tool for research on emotion and motivation. Cell Tissue Res 354:81–97

Wright JM, Gourdon J, Clarke PBS (2010) Identification of multiple call categories within the rich repertoire of adult rat 50-kHz ultrasonic vocalizations: effects of amphetamine and social context. Psychopharmacology 211:1–13

Wright JM, Deng L, Clarke PB (2012a) Failure of rewarding and locomotor stimulant doses of morphine to promote adult rat 50-kHz ultrasonic vocalizations. Psychopharmacology 224:477–487

Wright JM, Dobosiewicz MR, Clarke PB (2012b) Alpha- and beta-adrenergic receptors differentially modulate the emission of spontaneous and amphetamine induced 50-kHz ultrasonic vocalizations in adult rats. Neuropsychopharmacology 37:808–821

Young AM (1986) Effects of acute morphine pretreatment on the rate-decreasing and antagonist activity of naloxone. Psychopharmacology 88:201–208