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Divergent Acute and Enduring Changes in 50-kHz Ultrasonic Vocalizations in Rats Repeatedly Treated With Amphetamine and Dopaminergic Antagonists: New Insights on the Role of Dopamine in Calling Behavior

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Abstract

Background: Rats emit 50-kHz ultrasonic vocalizations (USVs) in response to nonpharmacological and pharmacological stimuli, with addictive psychostimulants being the most effective drugs that elicit calling behavior in rats. Earlier investigations found that dopamine D_1-like and D_2-like receptors modulate the emission of 50-kHz USVs stimulated in rats by the acute administration of addictive psychostimulants. Conversely, information is lacking on how dopamine D_1-like and D_2-like receptors modulate calling behavior in rats that are repeatedly treated with addictive psychostimulants.

Methods: We evaluated the emission of 50-kHz USVs in rats repeatedly treated (×5 on alternate days) with amphetamine (1 mg/kg, i.p.) either alone or together with (1) SCH 23390 (0.1–1 mg/kg, s.c.), a dopamine D_1 receptor antagonist; (2) raclopride (0.3–1 mg/kg, s.c.), a selective dopamine D_2 receptor antagonist; or (3) a combination of SCH 23390 and raclopride (0.1 + 0.3 mg/kg, s.c.). Calling behavior of rats was recorded following pharmacological treatment, as well as in response to the presentation of amphetamine-paired cues and to amphetamine challenge (both performed 7 days after treatment discontinuation).

Results: Amphetamine-treated rats displayed a sensitized 50-kHz USV emission during repeated treatment, as well as marked calling behavior in response to amphetamine-paired cues and to amphetamine challenge. Antagonism of D_1 or D_2 receptors either significantly suppressed or attenuated the emission of 50-kHz USVs in amphetamine-treated rats, with a maximal effect after synergistic antagonism of both receptors.

Conclusions: These results shed further light on how dopamine transmission modulates the emission of 50-kHz USVs in rats treated with psychoactive drugs.

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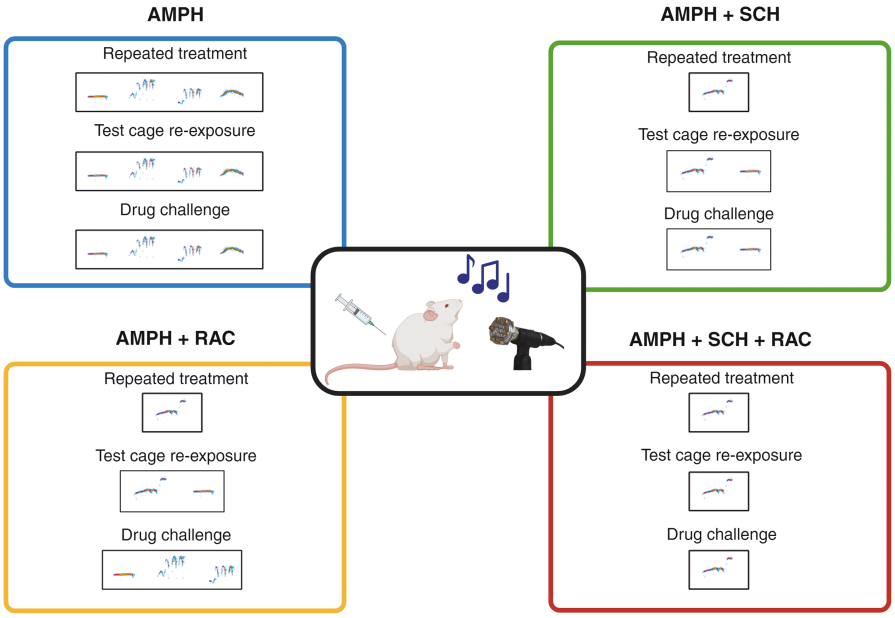
Graphical Abstract



The illustration depicts the divergent effects of dopamine D_1 and D_2 receptor antagonists on the emission of 50-kHz ultrasonic vocalizations in rats following repeated treatment with amphetamine, test cage re-exposure, and drug challenge. Legend: AMPH, amphetamine; SCH, SCH 23390; RAC, raclopride. Image created with Biorender.com.

Keywords: Positive affect, raclopride, reward, SCH 23390, sensitization

Significance Statement

Rat ultrasonic vocalizations (USVs) with an average frequency of 50 kHz (50-kHz USVs) are considered a marker of positive affect and are evaluated in preclinical studies on the neurobiology of emotions and the factors that regulate the affective state, including exposure to psychoactive drugs. We evaluated how dopamine D_1 and D_2 receptors influenced 50-kHz USV emissions in rats repeatedly treated with amphetamine to clarify the mechanisms of calling behavior elicited by psychoactive drugs. Moreover, because dopamine regulates affective processes, this study could further clarify the relationship between 50-kHz USVs and drug-induced changes in the affective state. Antagonism of D_1 or D_2 receptors elicited similar and dissimilar effects on 50-kHz USVs in amphetamine-treated rats. Moreover, D_1 and D_2 receptors synergistically interacted to enable amphetamine's effects on calling behavior. These findings further clarify how dopamine modulates 50-kHz USVs and may enhance our understanding of the significance of calling behavior in rats treated with psychoactive drugs.

INTRODUCTION

Rats emit ultrasonic vocalizations (USVs) in response to and/or in anticipation of stimuli that bear emotional valence (Burgdorf et al., 2011; Brudzynski, 2013; Olszyński et al., 2023). USVs emitted by young and adult rats are classified as 22-kHz USVs and 50-kHz USVs. These calls have dissimilar acoustic features and are emitted in response to aversive or pleasurable stimuli, respectively (Simola and Brudzynski, 2018a). Measuring the emission of USVs is an informative approach in rat studies about the neurobiology of emotional behavior and the factors that modify the affective state, including the exposure to psychoactive drugs (Barker et al., 2015; Simola and Granon, 2019; Wendler et al., 2019; Burgdorf et al., 2020; Premoli et al., 2023). Indeed, several drugs that elevate the affective state increase the emission of 50-kHz USVs in rats (Maier et al., 2012; Simola et al., 2012; Sanchez et al., 2022; Sohn et al., 2022) and so does the presentation of stimuli previously associated with those drugs (Knutson et al., 1999; Ma et al., 2010; Simola et al., 2014; Hamed and Kursa, 2018).

Earlier investigations have demonstrated that the pharmacological manipulation of dopamine D_1-like and/or D_2-like receptors influences the emission of 50-kHz USVs in rats. An increased emission of 50-kHz USVs has been observed after the acute systemic administration of apomorphine, a dopamine D_1/D_2 receptor agonist (Williams and Undieh, 2010; Simola and Morelli, 2015), and after the acute injection of quinpirole, a dopamine D_2/D_3 receptor agonist, into the nucleus accumbens (NAc) (Brudzynski et al., 2012). Besides, the administration of dopamine D_1/D_2 receptor antagonists, either systemic or in the NAc, counteracts the emission of 50-kHz USVs stimulated by the acute administration of the dopaminergic psychostimulants amphetamine or cocaine (Thompson et al., 2006; Williams and Undieh, 2010; Brudzynski et al., 2011; Wright et al., 2013). Dopaminergic psychostimulants are the most effective drugs that elicit the emission of 50-kHz USVs in rats (Simola et al., 2012, 2014). Moreover, it has been suggested that the acute and enduring changes in 50-kHz USV emissions detectable in rats treated with psychostimulants reflect

the occurrence of drug-induced changes in the affective state (Burgdorf et al., 2001; Ahrens et al., 2009; Mu et al., 2009; Browning et al., 2011; Simola and Brudzynski, 2018b). Currently, information is lacking on how dopamine D_1-like and/or D_2-like receptors modulate the emission of 50-kHz USVs in rats repeatedly treated with dopaminergic psychostimulants. Addressing this issue may further elucidate the role of dopamine in the emission of 50-kHz USVs and is relevant to investigate the interplay that may exist between calling behavior and changes in the affective state of rats treated with psychoactive drugs. In fact, dopamine D_1-like and D_2-like receptors regulate the effects of psychoactive drugs on the affective state and the correlated behavioral manifestations in rats (Di Chiara et al., 2004).

The present study evaluated the emission of 50-kHz USVs in rats repeatedly treated with amphetamine, preceded by vehicle or (1) SCH 23390, a dopamine D_1 receptor antagonist; (2) raclopride, a selective dopamine D_2 receptor antagonist; or (3) a combination of SCH 23390 and raclopride. Rats' calling behavior was evaluated immediately after drug administration during repeated pharmacological treatment, as well as after the presentation of amphetamine-paired environmental cues and after amphetamine challenge (both performed after treatment discontinuation). Amphetamine was selected as the psychoactive drug of reference since previous investigations have shown that rats repeatedly treated with amphetamine display (1) sensitization in the emission of 50-kHz USVs, which is considered a marker of persistent heightened affectivity induced by repeated drug experience (Ahrens et al., 2009; Simola and Morelli, 2015); and (2) emission of 50-kHz USVs in response to the presentation of amphetamine-paired environmental cues, which is thought to mark drug-induced environmental conditioning (Knutson et al., 1999; Simola and Morelli, 2015). The total numbers of 50-kHz USVs and the numbers of "flat," "trill," and "frequency modulated" (FM) categorized calls were counted to clarify how D_1-like and/or D_2-like receptors modulated general calling behavior and emission of distinct types of 50-kHz USVs believed to have dissimilar behavioral significance (Burgdorf et al., 2008; Wöhr et al., 2008).

MATERIALS AND METHODS

Animals

A total of 128 male Sprague-Dawley rats (Envigo, Italy) were used. Rats weighed 175–200 g at the beginning of the experiments and were housed 4 per cage under a 12-hour light/dark cycle (lights on at 8:00 AM) with ad libitum access to water and food, except during recordings (performed between 10:00 AM and 4:00 PM). This study was conducted according to the guidelines for animal experimentation of the EU directives (2010/63/EU, L.276; 22/09/2010) and of the Ethical Committee for Animal Experimentation of the University of Cagliari (prot: opec271.20130626160529.28283.02.1.18). All the appropriate procedures were followed to minimize animal discomfort and numbers of animals used.

Drugs

Distinct groups of rats received SCH 23390 (0.1, 0.3, 1 mg/kg), raclopride (0.3, 0.5, 1 mg/kg), the combination of SCH 23390 (0.1 mg/kg) and raclopride (0.3 mg/kg), or vehicle (distilled water). Administration of dopaminergic antagonists was followed by that of either vehicle or amphetamine (1 mg/kg). Drugs were purchased from Sigma Aldrich/Merck Life Science (Milan, Italy). D-Amphetamine (sulphate) was dissolved in distilled water and i.p. administered (injection volume: 3 mL/kg). SCH 23390 (hydrochloride) and raclopride (tartrate) were dissolved in distilled water and s.c. administered (injection volume: 1 mL/kg). Doses of SCH 23390 and raclopride were selected to be in the range of doses that counteract (1) the emission of 50-kHz USVs in rats acutely and systemically treated with psychostimulants (Williams and Undieh, 2010; Wright et al., 2013), and (2) behavioral changes indicative of altered affectivity in rats repeatedly treated with psychostimulants (Caine and Koob, 1994; Shippenberg and Heidbreder, 1995; Bardo et al., 1999). The dose of amphetamine was selected considering that amphetamine persistently modifies the emission of 50-kHz USVs only when administered at low-to-moderate doses (Taracha et al., 2014; Simola and Morelli, 2015). Rats received SCH 23390 and/or raclopride in their home cage 15 minutes before amphetamine/vehicle administration in the test cage.

Experimental Plan

Figure 1 shows the experimental plan, which was designed based on our previous studies that evaluated the enduring changes in 50-kHz USV emissions in rats repeatedly treated with amphetamine (Simola et al., 2014; Costa et al., 2015). Rats were handled daily (5 minutes) for 2 days before experiments, which included 5 phases: (1) test cage habituation (15 minutes) twice a day for 2 days (4 sessions); (2) repeated administration (×5 on alternate days) of amphetamine in the test cage (exposure: 30 minutes) preceded by vehicle, SCH 23390, raclopride, or their combination, to evaluate the occurrence of sensitization in calling behavior; (3) drug withdrawal (7 days) in the home cage; (4) test cage re-exposure (10 minutes) in drug-free conditions to evaluate calling behavior elicited by the presentation of drug-paired environmental cues; and (5) amphetamine challenge in the test cage (exposure: 30 minutes) to evaluate the persistence of drug effects on calling behavior. Locomotor activity was evaluated in

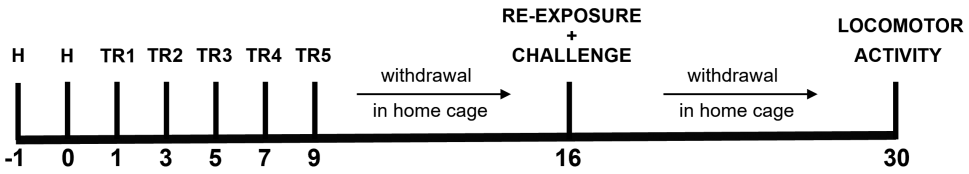


Figure 1. Experimental plan. Rats were first habituated to the test cage for 2 consecutive days. Afterwards, they were repeatedly treated on alternate days with amphetamine (1 mg/kg, i.p.) or vehicle (i.p.) in the test cage (×5 administrations). Amphetamine administration was preceded by vehicle, SCH 23390 (0.1–1 mg/kg, s.c.), raclopride (0.3–1 mg/kg, s.c.), or the combination of SCH 23390 (0.1 mg/kg, s.c.) and raclopride (0.3 mg/kg, s.c.). Dopaminergic antagonists were always administered in the home cage 15 minutes before amphetamine or vehicle. Repeated pharmacological treatment was followed by withdrawal in the home cage (7 days), re-exposure to the test cage to evaluate cue-stimulated calling behavior in treatment-free conditions, and amphetamine challenge. Evaluation of locomotor activity was performed 14 days after drug challenge. The numbers of ultrasonic vocalizations were scored on days 1, 5, 9, and 16 of the experimental protocol. Abbreviations: H, habituation to the test cage; TR, pharmacological treatment.

a subgroup of rats 14 days after the challenge. For USV recordings, rats were randomly assigned to their experimental group. One group of rats was tested with vehicle according to the same schedule and served as the control group. For locomotor activity recordings, experimental groups were organized as follows: (1) the vehicle group included only subjects previously treated with vehicle; and (2) the amphetamine-treated groups included subjects from all groups of rats previously treated with amphetamine, alone or plus SCH 23390 and/or raclopride (1 rat per each group was included). Rats were drug naïve at the beginning of the experiments, and each experimental group included 8 subjects.

USV Recordings

For USV recordings, rats were individually placed in test cages consisting of Plexiglas cylinders (diameter: 30 cm, height: 30 cm). Each cylinder was topped with a lid equipped with an ultrasonic microphone (CM16/CMPA, Avisoft, Berlin, Germany) connected to an ultrasound recording device (Ultrasound Gate 116 Hb, Avisoft). Microphones were placed at an average distance of 27 cm from rats. The recording setup used could identify calls of both the 22-kHz USV family and the 50-kHz USV family. The 50-kHz USVs recorded were further categorized in flat, trill, and FM, according to Wright et al. (2010). Recording procedures, call categorization, and recording times were as previously described (Simola et al., 2012; Costa et al., 2015). Further details are provided in Supplementary Materials.

Locomotor Activity Recordings

For locomotor activity recordings, rats were individually placed in test cages equipped with motor activity counters. Locomotor activity was measured by number of light beams crossed by rats in 16 × 16 light beam apparatuses (Opto-Varimex, Columbus Instruments, Columbus, OH, USA). Recordings were performed according to Simola et al. (2006). Further details are provided in Supplementary Materials.

Statistical Analysis

SASLab Pro 4.52 (Avisoft) was used to convert USV recordings into spectrograms and count the number of calls in each spectrogram, as previously described (Simola et al., 2012). Supplementary Figure 1 demonstrates examples of USVs recorded in this study. The total numbers of 50-kHz USVs, the numbers of categorized 50-kHz USVs, the numbers of 22-kHz USVs, and the acoustic parameters of calls (bandwidth, duration, maximum frequency in a subset of rats only) were scored on the first, third, and fifth administration day of repeated treatment (days 1, 5, and 9 of the experimental protocol), as well as on test cage re-exposure and after challenge (both on day 16 of the experimental protocol). Locomotor activity counts were scored on day 30 of the experimental protocol. Group differences in the total numbers of 50-kHz USVs emitted during repeated treatment were analyzed by 3-way (amphetamine treatment × dopaminergic antagonist(s) treatment × administration day) ANOVA. Group differences in the total numbers of 50-kHz USVs and numbers of categorized 50-kHz USVs emitted on test cage re-exposure or after challenge were analyzed by 2-way ANOVA (previous amphetamine treatment/amphetamine challenge × previous dopaminergic antagonist(s) treatment). Analysis of the categorized 50-kHz USVs emitted during repeated treatment is not presented, given the low magnitude of calling behavior in rats that received SCH 23390 and/or raclopride. Group differences in locomotor activity counts were analyzed by 1-way ANOVA (treatment). ANOVAs were followed by Tukey or Dunnet post hoc test, when appropriate. Spearman test was applied to the subset of rats that emitted 22-kHz USVs during repeated treatment to correlate the numbers of 22-kHz USVs to those of 50-kHz USVs emitted on test cage re-exposure and after challenge. Kolmogorov-Smirnov and Levene tests were used to check data for normality and homoscedasticity. When appropriate, data were log transformed to preserve homoscedasticity, and a constant (+1) was added to the transformed datasets to correct for null values. For the sake of clarity and conciseness, figures demonstrate raw data, main statistical effects and interactions are presented only for significant results, and results of post hoc tests are reported only for significant differences vs vehicle-treated rats. Statistical analysis was performed with Statistica (StatSoft, Tulsa, OK, USA), Prism 8 (GraphPad, La Jolla, CA, USA), and QI Macros (KnowWare International, Denver, CO, USA) for Windows. Significance was set at $P < .05$ for each analysis.

RESULTS

Emission of 50-kHz USVs During Repeated Pharmacological Treatment: General Effects

Repeated amphetamine treatment elicited a robust and sensitized emission of 50-kHz USVs in rats. Conversely, rats emitted few 50-kHz USVs during repeated treatment with amphetamine plus SCH 23390 and/or raclopride (Fig. 2A–C).

Effects of SCH 23390

Three-way ANOVA revealed significant effects of amphetamine ($F_{1,56} = 44.19$, $P < .0001$), SCH 23390 ($F_{3,56} = 48.62$, $P < .0001$), and administration day ($F_{2,112} = 3.67$, $P = .029$) and the following significant interactions: amphetamine × SCH 23390 ($F_{3,56} = 40.69$, $P < .0001$), SCH 23390 × administration day ($F_{3,56} = 4.92$, $P < .0001$), and amphetamine × SCH 23390 × administration day ($F_{6,112} = 3.24$, $P = .005$). Tukey post hoc test showed that amphetamine (1 mg/kg, i.p.) increased the emission of 50-kHz USVs on days 1, 5, and 9 of repeated treatment compared with vehicle ($P < .0002$, all comparisons) and that the magnitude of calling behavior was higher ($P = .0177$) on day 9 compared with day 1. Rats treated with SCH 23390 (0.1–1 mg/kg, s.c.) plus either vehicle or amphetamine (1 mg/kg, i.p.) displayed 50-kHz USV emissions comparable with vehicle-treated rats (Fig. 2A). Supplementary Table 1 reports the complete results of Tukey post hoc test.

Effects of Raclopride

Three-way ANOVA revealed significant effects of amphetamine ($F_{1,56} = 49.85$, $P < .0001$), raclopride ($F_{3,56} = 15.85$, $P < .0001$), and administration day ($F_{2,112} = 4.64$, $P = .01$) and the following significant interactions: amphetamine × raclopride ($F_{3,56} = 14.19$, $P < .0001$), raclopride × administration day ($F_{3,56} = 5.14$, $P < .0001$), and amphetamine × raclopride × administration day ($F_{6,112} = 4.40$, $P = .0005$). Tukey post hoc test showed that amphetamine (1 mg/kg, i.p.) increased the emission of 50-kHz USVs on days 1, 5, and 9 of repeated treatment compared with vehicle ($P < .0002$, all comparisons) and that the magnitude of calling behavior was higher ($P = .0242$) on day 9 compared with day 1. Rats treated with raclopride (0.3–1 mg/kg, s.c.) plus either vehicle or amphetamine (1 mg/kg, i.p.) displayed 50-kHz USV emissions comparable with vehicle-treated rats (Fig. 2B). Supplementary Table 2 reports the complete results of Tukey post hoc test.

Effects of SCH 23390 and Raclopride Combination

Three-way ANOVA revealed significant effects of amphetamine ($F_{1,28} = 121.10$, $P < .0001$), SCH 23390 and raclopride combination ($F_{1,28} = 194.85$, $P < .0001$), and the following significant

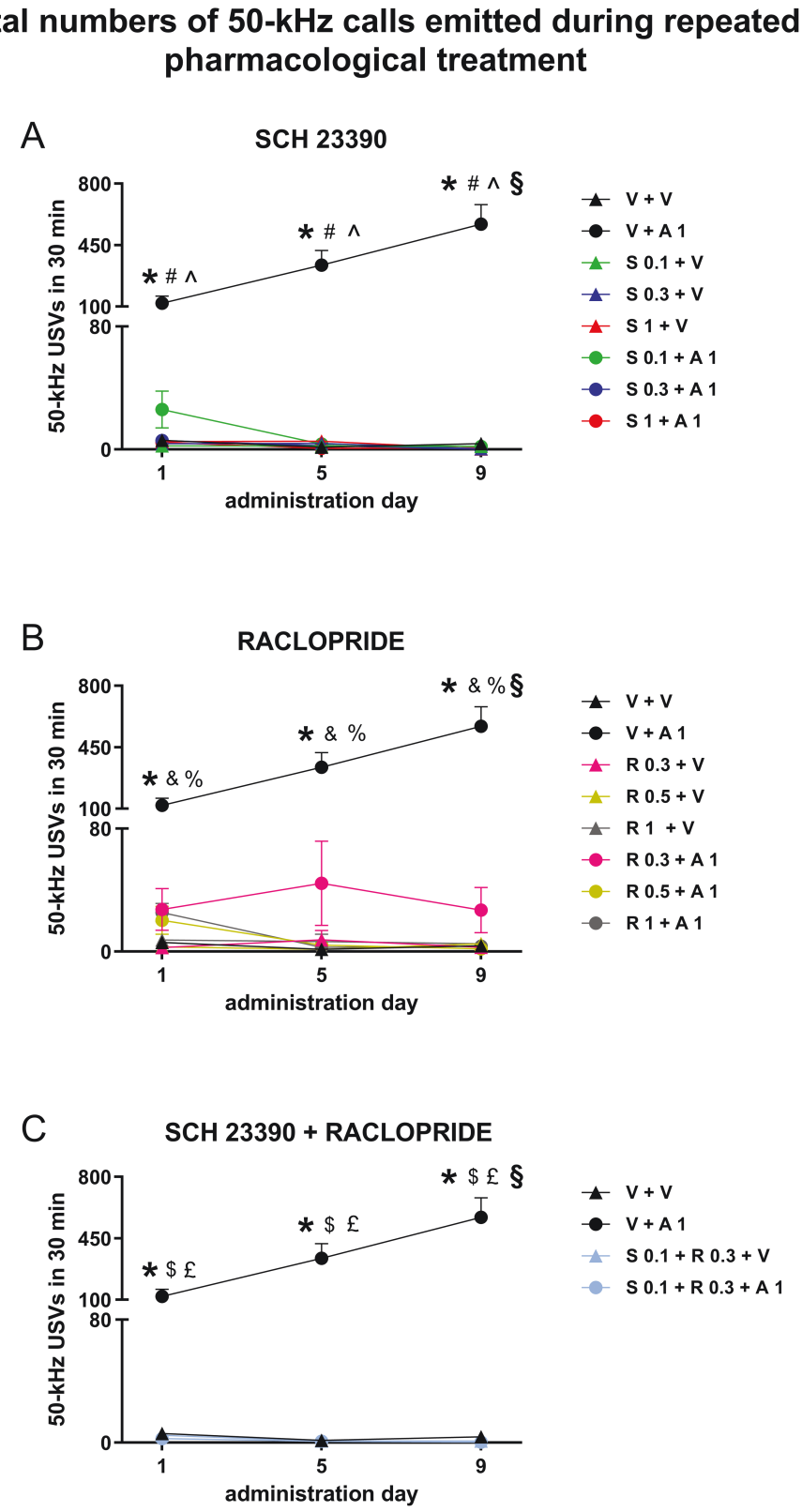


Figure 2. Effects of SCH 23390, raclopride, or the combination of SCH 23390 and raclopride on the emission of 50-kHz USVs stimulated in rats by the repeated administration of amphetamine. Rats received (×5 on alternate days) amphetamine (1 mg/kg, i.p.), preceded by vehicle, SCH 23390 (0.1, 0.3 or 1 mg/kg, s.c.) (A), raclopride (0.3, 0.5 or 1 mg/kg, s.c.) (B), or the combination of SCH 23390 (0.1 mg/kg, s.c.) and raclopride (0.3 mg/kg, s.c.) (C). *Indicates a significant difference vs vehicle-treated rats. #Indicates a significant difference vs rats treated with SCH 23390 (0.1, 0.3, or 1 mg/kg, s.c.) + vehicle. ^Indicates a significant difference vs rats treated with SCH 23390 (0.1, 0.3, or 1 mg/kg, s.c.) + amphetamine (1 mg/kg, i.p.). &Indicates a significant difference vs rats treated with raclopride (0.3, 0.5, or 1 mg/kg, s.c.) + vehicle. %Indicates a significant difference vs rats treated with raclopride (0.3, 0.5, or 1 mg/kg, s.c.) + amphetamine (1 mg/kg, i.p.). $Indicates a significant difference vs rats treated with SCH 23390 (0.1 mg/kg, s.c.) + raclopride (0.3 mg/kg, s.c.) + vehicle. £Indicates a significant difference vs rats treated with SCH 23390 (0.1 mg/kg, s.c.) + raclopride (0.3 mg/kg, s.c.) + amphetamine (1 mg/kg, i.p.). §Indicates a significant difference vs day 1 of repeated treatment with amphetamine (1 mg/kg, i.p.). Emission of 50-kHz ultrasonic vocalizations was recorded for 30 minutes. n = 8 rats for each experimental group. Abbreviations: A, amphetamine; R, raclopride; S, SCH 23390; USVs, ultrasonic vocalizations; V, vehicle. Numbers in the captions indicate drug doses in mg/kg.

interactions: amphetamine × SCH 23390 and raclopride combination ($F_{1,28}$ = 144.39, P < .0001), amphetamine × administration day ($F_{2,56}$ = 4.03, P = .02), and SCH 23390 and raclopride combination × administration day ($F_{2,56}$ = 7.27, P = .002). Tukey post hoc test showed that amphetamine (1 mg/kg, i.p.) increased the emission of 50-kHz USVs on days 1, 5, and 9 of repeated treatment compared with vehicle (P < .0002, all comparisons) and that the magnitude of calling behavior was higher (P = .0085) on day 9 compared with day 1. Rats treated with the combination of SCH 23390 (0.1 mg/kg, s.c.) and raclopride (0.3 mg/kg, s.c.) plus either vehicle or amphetamine (1 mg/kg, i.p.) displayed 50-kHz USV emissions comparable with vehicle-treated rats (Fig. 2C). Supplementary Table 3 reports the complete results of Tukey post hoc test.

Emission of 50-kHz USVs on Test Cage Re-exposure: General Effects

Test cage re-exposure in drug-free conditions after treatment discontinuation (day 16) stimulated a significant emission of 50-kHz USVs in rats previously treated with amphetamine compared with rats previously treated with vehicle. An attenuation in the emission of cue-stimulated 50-kHz USVs occurred in rats previously treated with SCH 23390 and/or raclopride plus amphetamine. The magnitude of this effect depended on the dopaminergic antagonist(s) administered and doses thereof (Fig. 3A–C).

Effects of SCH 23390

Two-way ANOVA revealed a significant effect of previous treatment with amphetamine ($F_{1,56}$ = 13.60, P = .0005) and a significant interaction previous treatment with amphetamine × previous treatment with SCH 23390 ($F_{3,56}$ = 3.102, P = .0338). Tukey post hoc test showed that rats previously treated with amphetamine (1 mg/kg, i.p.) emitted higher numbers of cue-stimulated 50-kHz USVs compared with rats previously treated with vehicle (P = .0045). Rats previously treated with SCH 23390 (0.1–1 mg/kg, s.c.) plus amphetamine (1 mg/kg, i.p.) displayed only nonsignificant trends towards increased emission of cue-stimulated calls compared with rats previously treated with vehicle (Fig. 3A). Supplementary Table 4 reports the complete results of Tukey post hoc test.

Effects of Raclopride

Two-way ANOVA revealed a significant effect of previous treatment with amphetamine ($F_{1,56}$ = 18.36, P < .0001). Tukey post hoc test showed that rats previously treated with amphetamine (1 mg/kg, i.p.) emitted higher numbers of cue-stimulated 50-kHz USVs, compared with rats previously treated with vehicle (P = .0277). Rats previously treated with raclopride (0.3–1 mg/kg, s.c.) plus amphetamine (1 mg/kg, i.p.) displayed only nonsignificant trends towards increased emission of cue-stimulated calls compared with rats previously treated with vehicle (Fig. 3B). Supplementary Table 5 reports the complete results of Tukey post hoc test.

Effects of SCH 23390 and Raclopride Combination

Two-way ANOVA revealed a significant effect of previous treatment with amphetamine ($F_{1,28}$ = 7.347, P = .0113) and of previous treatment with SCH 23390 and raclopride combination ($F_{1,28}$ = 8.624, P = .0066), and a significant interaction previous treatment with amphetamine × previous treatment with SCH 23390 and raclopride combination ($F_{1,28}$ = 9.859, P = .0040). Tukey post hoc test showed that rats previously treated with amphetamine (1 mg/kg, i.p.) emitted higher numbers of cue-stimulated 50-kHz USVs compared with rats previously treated with vehicle (P = .0016). Moreover, rats previously treated with the combination of SCH 23390 (0.1 mg/kg, s.c.) and raclopride (0.3 mg/kg, s.c.) plus amphetamine (1 mg/kg, i.p.) emitted lower numbers of cue-stimulated calls compared with rats previously treated with amphetamine (1 mg/kg, i.p.) (Fig. 3C). Supplementary Table 6 reports the complete results of Tukey post hoc test.

Emission of 50-kHz USVs After Challenge: General Effects

Amphetamine challenge after treatment discontinuation (day 16) significantly increased the emission of 50-kHz USVs in rats previously treated with (1) amphetamine, (2) SCH 23390 (lower dose) plus amphetamine, and (3) raclopride (all doses) plus amphetamine. The emission of 50-kHz USVs after amphetamine challenge was significantly attenuated in rats previously treated with SCH 23390 and raclopride combination plus amphetamine (Fig. 4A–C).

Effects of SCH 23390

Two-way ANOVA revealed a significant effect of amphetamine challenge ($F_{1,56}$ = 41.61, P < .0001) and of previous treatment with SCH 23390 ($F_{3,56}$ = 6.33, P = .0009) and a significant interaction amphetamine challenge × previous treatment with SCH 23390 ($F_{3,56}$ = 3.178, P = .309). Tukey post hoc test showed that rats previously treated with amphetamine (1 mg/kg, i.p.) (P = .0001) and rats previously treated with SCH 23390 (0.1 mg/kg, s.c.) plus amphetamine (1 mg/kg, i.p.) (P = .0002) emitted higher numbers of 50-kHz USVs after amphetamine challenge compared with rats previously treated with vehicle and challenged with vehicle (Fig. 4A). Supplementary Table 7 reports the complete results of Tukey post hoc test.

Effects of Raclopride

Two-way ANOVA revealed a significant effect of amphetamine challenge ($F_{1,56}$ = 119.7, P < .0001). Tukey post hoc test showed that rats previously treated with amphetamine (1 mg/kg, i.p.) (P < .0001) and rats previously treated with raclopride (0.3, 0.5 or 1 mg/kg, s.c.) plus amphetamine (1 mg/kg, i.p.) (P < .0001; P = .0004; P = .0016, respectively) emitted higher numbers of 50-kHz USVs after challenge with amphetamine (1 mg/kg, i.p.) compared with rats previously treated with vehicle and challenged with vehicle (Fig. 4B). Supplementary Table 8 reports the complete results of Tukey post hoc test.

Effects of SCH 23390 and Raclopride Combination

Two-way ANOVA revealed a significant effect of amphetamine challenge ($F_{1,28}$ = 10.92, P = .0026), of previous treatment with SCH 23390 and raclopride combination ($F_{1,28}$ = 8.458, P = .0070), and a significant interaction amphetamine challenge × previous treatment with SCH 23390 and raclopride combination ($F_{1,28}$ = 7.209, P = .0121). Tukey post hoc test showed that rats previously treated with amphetamine (1 mg/kg, i.p.) emitted higher numbers of 50-kHz USVs in response to challenge with amphetamine (1 mg/kg, i.p.) compared with rats previously treated with vehicle and challenged with vehicle (P = .0012) (Fig. 4C). Supplementary Table 9 reports the complete results of Tukey post hoc test.

Emission of Categorized 50-kHz USVs

Rats previously treated with amphetamine displayed a significant emission of categorized 50-kHz USVs (flat, trill, FM) in response to either the presentation of amphetamine-paired cues or amphetamine challenge compared with rats previously treated with vehicle. This effect was suppressed or attenuated by the administration of SCH 23390 and/or raclopride. Rats in all experimental groups emitted numbers of categorized calls

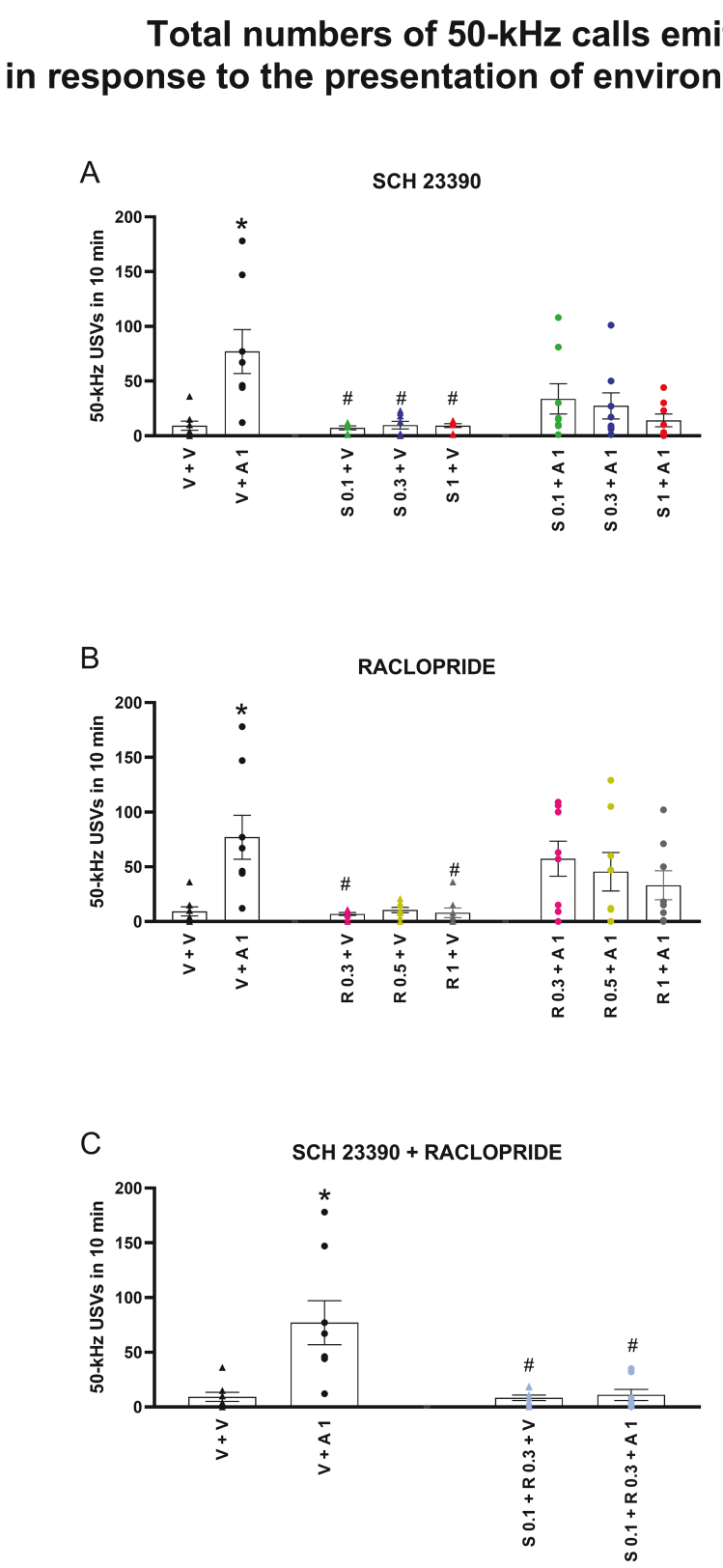


Figure 3. Total numbers of 50-kHz ultrasonic vocalizations emitted on re-exposure to the test cage. Rats were previously treated (×5 on alternate days) with amphetamine (1 mg/kg, i.p.), preceded by vehicle, SCH 23390 (0.1, 0.3, or 1 mg/kg, s.c.) (A), raclopride (0.3, 0.5, or 1 mg/kg, s.c.) (B), or the combination of SCH 23390 (0.1 mg/kg, s.c.) and raclopride (0.3 mg/kg, s.c.) (C). Rats were re-exposed to the test cage in drug-free conditions 7 days after discontinuation of repeated pharmacological treatment. *Indicates a significant difference vs rats previously treated with vehicle. #Indicates a significant difference vs rats previously treated with amphetamine (1 mg/kg, i.p.). Emission of 50-kHz ultrasonic vocalizations was recorded for 10 minutes. n = 8 rats for each experimental group. Abbreviations: A, amphetamine; R, raclopride; S, SCH 23390; USVs, ultrasonic vocalizations; V, vehicle. The sequence of letters reflects the order of drug/vehicle administration during repeated pharmacological treatment. Numbers in the captions indicate drug doses in mg/kg.

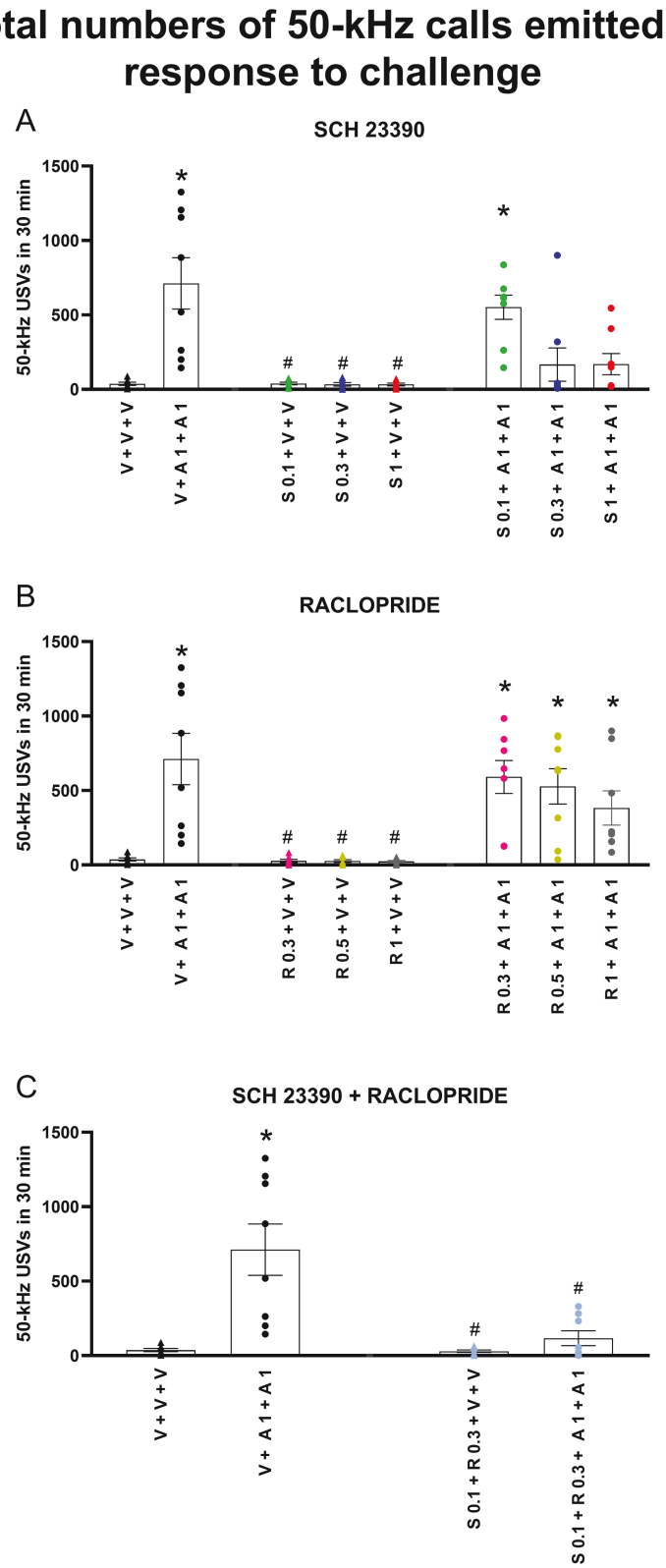


Figure 4. Total numbers of 50-kHz ultrasonic vocalizations emitted after challenge. Rats were previously treated with amphetamine (1 mg/kg, i.p.), preceded by vehicle, SCH 23390 (0.1, 0.3 or 1 mg/kg, s.c.) (A), raclopride (0.3, 0.5 or 1 mg/kg, s.c.) (B), or the combination of SCH 23390 (0.1 mg/kg, s.c.) and raclopride (0.3 mg/kg, s.c.) (C). Rats were challenged with either vehicle or amphetamine (1 mg/kg, i.p.), 7 days after discontinuation of repeated pharmacological treatment, immediately after the evaluation of cue-stimulated calling behavior. *Indicates a significant difference vs rats previously treated with vehicle and challenged with vehicle. #Indicates a significant difference vs rats previously treated with amphetamine (1 mg/kg, i.p.) and challenged with amphetamine (1 mg/kg, i.p.). Emission of 50-kHz ultrasonic vocalizations was recorded for 30 minutes. n = 8 rats for each experimental group. Abbreviations: A, amphetamine; R, raclopride; S, SCH 23390; USVs, ultrasonic vocalizations; V, vehicle. The sequence of letters reflects the order of drug/vehicle administration during repeated pharmacological treatment (first 2 letters) and challenge (third letter). Numbers in the captions indicate drug doses in mg/kg.

that paralleled the total number of 50-kHz USVs emitted, with few exceptions (Supplementary Figs. 2 and 3). The major effects of dopamine receptor antagonists on the emission of categorized 50-kHz USVs in amphetamine-treated rats were as follows: SCH 23390—1 mg/kg significantly reduced the emission of cue-stimulated FM calls; SCH 23390 (0.1 mg/kg, s.c.) + raclopride (0.3 mg/kg, s.c.)—suppressed FM cue-stimulated calls, as well as trill and FM calls after amphetamine challenge. Supplementary Material details the changes in the emission of categorized calls observed.

Emission of 22-kHz USVs During Repeated Treatment and Correlation to Emission of 50-kHz USVs After Treatment Discontinuation

A total of 17 rats treated with amphetamine (1 mg/kg, i.p.) preceded by SCH 23390 (0.1–1 mg/kg, s.c.), raclopride (0.3–1 mg/kg, s.c.), or the combination of SCH 23390 (0.1 mg/kg, s.c.) and raclopride (0.3 mg/kg, s.c.) emitted 22-kHz USVs during repeated treatment. Five rats emitted 22-kHz USVs on all the 3 days evaluated, whereas 12 rats emitted 22-kHz USVs only on 1 or 2 of the days evaluated. Spearman test showed that the total numbers of 22-kHz USVs emitted during repeated treatment significantly correlated neither to the total numbers of 50-kHz USVs emitted on test cage re-exposure ($r=0.155$, $P=.552$, Fig. 5A) nor to the total numbers of 50-kHz USVs emitted after challenge ($r=-0.046$, $P=.861$, Fig. 5B). Moreover, 9 rats treated with vehicle preceded by SCH 23390 (0.1–1 mg/kg, s.c.), raclopride (0.3–1 mg/kg, s.c.), or the combination of SCH 23390 (0.1 mg/kg, s.c.) and raclopride (0.3 mg/kg, s.c.) emitted 22-kHz USVs during repeated treatment (data not shown).

Acoustic Parameters of USVs

Evaluation of bandwidth, duration, and maximum frequency of USVs in a subset of rats confirmed that the acoustic parameters of the recorded calls were in the range of those previously described for 22-kHz calls and 50-kHz calls (Simola and Brudzynski, 2018a). Supplementary Table 10 demonstrates the acoustic parameters of the calls analyzed.

Effects of Dopaminergic Antagonists on Amphetamine-Stimulated Locomotor Activity

Amphetamine significantly stimulated locomotor activity in rats compared with vehicle, and this effect was suppressed by the pretreatment with SCH 23390 and/or raclopride (Supplementary Fig. 4). Supplementary Material details the effects of dopaminergic antagonists on amphetamine-stimulated locomotor activity.

DISCUSSION

The activation of dopamine D_1-like and D_2-like receptors enables the emission of 50-kHz USVs in rats acutely treated with psychostimulants (Thompson et al., 2006; Williams and Undieh, 2010, 2016; Wright et al., 2013). Conversely, little is known about how dopamine D_1-like and D_2-like receptors influence the enduring modifications in calling behavior in rats repeatedly treated with psychostimulants. This study provides new insights in this regard by demonstrating that the enduring increases in the emission of drug- and cue-stimulated 50-kHz USVs occurring in rats repeatedly treated with amphetamine are attenuated by the individual antagonism of D_1/D_2 receptors and suppressed by the combined antagonism of D_1-D_2 receptors.

Rats repeatedly treated with amphetamine emitted 50-kHz USVs and developed sensitized calling behavior during treatment, consistent with earlier findings (Ahrens et al., 2009; Taracha et al., 2014; Simola and Morelli, 2015). Conversely, rats repeatedly treated with SCH 23390 and/or raclopride plus amphetamine emitted few 50-kHz USVs during treatment. This finding agrees

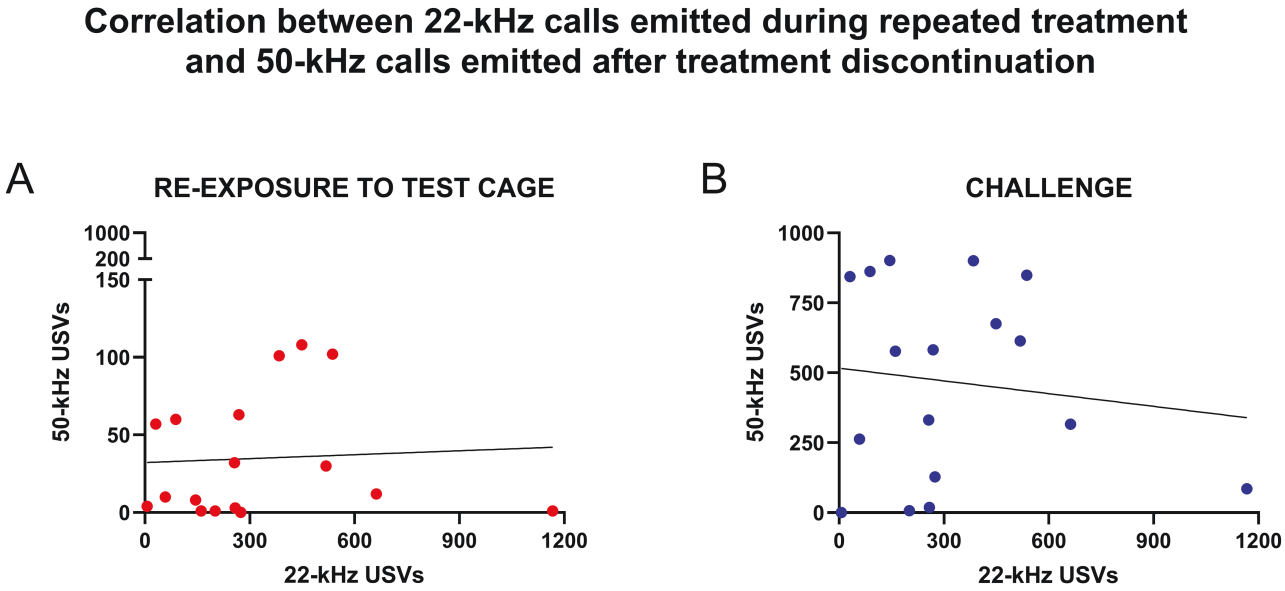


Figure 5. Results of Spearman correlation between 22-kHz and 50-kHz ultrasonic vocalizations. The total numbers of 22-kHz ultrasonic vocalizations emitted during repeated pharmacological treatment were correlated to the total numbers of 50-kHz ultrasonic vocalizations emitted in response to either the presentation of treatment-paired environmental cues (A) or challenge (B), both performed after discontinuation of repeated pharmacological treatment. Correlation analysis was narrowed to the amphetamine-treated rats that emitted 22-kHz USVs on at least one of the days of repeated pharmacological treatment evaluated. n = 17 rats treated with amphetamine (1 mg/kg, i.p.) preceded by SCH 23390 (0.1–1 mg/kg, s.c.), raclopride (0.3–1 mg/kg, s.c.) or the combination of SCH 23390 (0.1 mg/kg, s.c.) and raclopride (0.3 mg/kg, s.c.). Abbreviation: USVs, ultrasonic vocalizations.

with earlier results showing that the antagonism of dopamine D_1-like or D_2-like receptors suppresses the emission of 50-kHz USVs stimulated by acute amphetamine (Thompson et al., 2006; Wright et al., 2013). Besides, the present results extend earlier findings by demonstrating that the simultaneous activation of dopamine D_1 and D_2 receptors is requisite for the emission of 50-kHz USVs stimulated by amphetamine even when the drug is administered repeatedly. These findings further support the evidence that the dopaminergic system prominently contributes to the emission of 50-kHz USVs in rats (Thompson et al., 2006; Burgdorf et al., 2011; Hori et al., 2013; Wright et al., 2013; Buck et al., 2014; Scardochio et al., 2015; Mulvihill and Brudzynski, 2019; Shimoju and Shibata, 2021).

Previous investigations suggested that the 50-kHz USVs emitted after amphetamine administration mark drug-induced elevation in the rats' affective state (Burgdorf et al., 2001; Wintink and Brudzynski, 2001). Besides, D_1-like and D_2-like dopamine receptors modulate the effects of psychoactive drugs on the affective state (Di Chiara et al., 2004; Berridge and Arnstern, 2013; Pardo et al., 2015; Salamone et al., 2016; Zhang et al., 2021). Accordingly, we could speculate that rats emitted few 50-kHz USVs during repeated treatment with SCH 23390 and/or raclopride plus amphetamine because they did not experience drug-induced positive affect. However, this speculation may disagree with the changes in calling behavior observed here after treatment discontinuation if the hypothesis is held that a persistently increased emission of 50-kHz USVs marks enduring elevations in the affective state of amphetamine-treated rats (see Ahrens et al., 2009). Thus, on test cage re-exposure after treatment discontinuation, rats that received SCH 23390/raclopride plus amphetamine displayed a trend toward decreased emission of the total number of 50-kHz USVs, and rats treated with SCH 23390 (1 mg/kg) plus amphetamine showed a significantly decreased emission of cue-stimulated FM calls. Conversely, rats that received each dose of SCH 23390 or raclopride plus amphetamine displayed a robust 50-kHz USV emission after amphetamine challenge, although they also displayed a trend toward decreased calling behavior compared with rats previously treated with amphetamine and challenged with amphetamine. The magnitude of the latter effect varied with the dopaminergic antagonist administered and doses thereof, being more evident in SCH 23390-treated rats. Finally, neither test cage re-exposure nor amphetamine challenge stimulated the emission of 50-kHz USVs in rats that previously received the combination of SCH 23390 and raclopride plus amphetamine. Based on these findings and considering the proposed significance of calling behavior in amphetamine-treated rats, we suggest a new comprehensive hypothesis to explain the results of this study. This hypothesis predicts that the concomitant administration of SCH 23390/raclopride may modify the affective state in rats repeatedly treated with amphetamine, depending on the dose of either dopaminergic antagonist and on the presence of an individual or combined antagonism of dopamine D_1/D_2 receptors.

The first implication of our hypothesis is that the scarce 50-kHz USV emission observed during treatment with SCH 23390/raclopride plus amphetamine was not necessarily related to drug-induced alterations in the rats' affective state. Thus, it is conceivable that rats emitted few 50-kHz USVs during repeated treatment because the simultaneous activation of dopamine D_1-D_2 receptors is requisite for the full manifestation of amphetamine-stimulated calling behavior. Another possibility could be that rats emitted few 50-kHz USVs during repeated treatment because dopamine receptor antagonists inhibited amphetamine's behavioral effects, as it may be suggested by the finding that all doses of SCH 23390 and raclopride suppressed amphetamine-stimulated locomotor activity. However, it is noteworthy that the changes in 50-kHz USV emission and locomotor activity may diverge in rats treated with psychoactive drugs (Burgdorf and Panksepp, 2006; Taracha et al., 2014; Costa et al., 2015; Simola et al., 2021). Moreover, about 30% of the rats that received SCH 23390 and/or raclopride plus amphetamine emitted 22-kHz USVs during repeated treatment. This finding indicates that SCH 23390- and raclopride-treated rats retained the ability to emit USVs. Furthermore, it may imply a dissociation between the impact that dopamine receptor antagonism had on amphetamine-induced locomotor activity and calling behavior. Nevertheless, it is worth recalling that dopamine D_1-like and D_2-like receptors modulate the affective properties of psychoactive drugs. Accordingly, it may be possible that at least in part of the rats the scarce emission of appetitive 50-kHz USVs during repeated treatment originated from a lack of or reduction in amphetamine-induced positive affect. Moreover, since 22-kHz USVs (see above) are considered a behavioral marker of negative affect in rats (Brudzynski, 2001, 2019), it may be speculated that at least some of the rats experienced negative affect during repeated treatment with SCH 23390 and/or raclopride plus amphetamine. However, most of the rats that emitted 22-kHz USVs during repeated treatment emitted high numbers of 50-kHz USVs after amphetamine challenge, and no significant correlation existed between the emission of 22-kHz calls and that of 50-kHz calls. These apparently discrepant findings may be explained by considering that we recorded calling behavior for 30 minutes on each day of repeated treatment, whereas amphetamine's effects on 50-kHz USVs may last longer (Simola et al., 2012; Williams and Undieh, 2016). Moreover, the emission of 22-kHz USVs recorded during repeated treatment faded 20 to 25 minutes after amphetamine administration, suggesting the presence of transient negative affect in rats that received dopamine receptor antagonists. Furthermore, previous studies have suggested that positive and negative states are mutually exclusive, which may be reflected in rats by the emission of 50-kHz USVs or 22-kHz USVs, respectively (Brudzynski et al., 2018). Accordingly, we may suppose that at least some of the rats that received dopaminergic antagonists fluctuated from a positive to a negative emotional state during repeated pharmacological treatment, which could explain why they emitted 22-kHz USVs. Finally, it has been suggested that the emission of 50-kHz USVs in rats repeatedly treated with psychoactive drugs may reflect not only heightened affect but also depend, at least in part, on phenomena unrelated to the affective properties of drugs (Schwarting, 2023), such as drug-induced overactivation of dopamine transmission (Simola and Morelli, 2015). The latter consideration could provide a complementary explanation for the robust emission of 50-kHz USVs observed here after amphetamine challenge.

Another implication of our hypothesis about the significance of calling behavior in rats repeatedly treated with dopaminergic antagonists and amphetamine concerns 50-kHz USV emissions elicited by the presentation of amphetamine-paired cues and by amphetamine challenge, and the similarities and differences in these responses among the different experimental groups. We propose that the combined antagonism of dopamine D_1-D_2 receptors attenuates the enduring effects of amphetamine on the rats' affective state expressed as increased emission of 50-kHz USVs. Moreover, our results indicate that dopamine D_1 and D_2 receptors synergistically interact to enable the manifestation of amphetamine's enduring effects on calling behavior. Indeed, calling behavior elicited by the presentation of amphetamine-paired cues and by amphetamine challenge was suppressed in rats

previously treated with the combination of SCH 23390 and raclopride plus amphetamine, with either dopaminergic antagonist administered at a dose (0.1 or 0.3 mg/kg, respectively) otherwise ineffective on those vocal responses. Nevertheless, rats previously treated with SCH 23390 or raclopride plus amphetamine also displayed trends toward reduced 50-kHz USV emissions elicited by the presentation of amphetamine-paired cues and by amphetamine challenge, an effect more evident in SCH 23390-treated rats. Accordingly, we may propose that dopamine D_1 receptors predominate over dopamine D_2 receptors in enabling the manifestation of the enduring changes in calling behavior that occur in rats repeatedly treated with amphetamine. Additional investigations are needed to verify this hypothesis, and we cannot exclude that enduring changes in calling behavior similar to those of rats treated with SCH 23390 plus amphetamine might occur in rats treated with amphetamine and raclopride at doses different from those tested here, or treated with amphetamine and D_2 receptor antagonists other than raclopride. Indeed, raclopride possesses appreciable antagonistic activity at dopamine D_3 receptors, which could have influenced the results of this study. Moreover, we cannot exclude that the effects of SCH 23390 (1 mg/kg) were influenced by serotonergic mechanisms, because SCH 23390 can interact with $5HT_2$ receptors in vivo, although these effects have been reported for doses of 1.5 mg/kg, i.p., or higher (Bischoff et al., 1986). To fully ascertain the role of dopamine D_1 and D_2 receptors in the emission of 50-kHz USVs in amphetamine-treated rats, it will then be relevant to clarify if and how the activation of either receptor influences the nondopaminergic mechanisms that modulate amphetamine's effects on calling behavior (see Wright et al., 2012; Wöhr et al., 2015; Hamed et al., 2016; Simola et al., 2016).

The results of this study also indicate that dopamine D_1 and D_2 receptors may influence the enduring amphetamine's effects on the emission of categorized 50-kHz USVs. Thus, compared with amphetamine-treated rats, rats that received SCH 23390 (1 mg/kg) plus amphetamine displayed a significantly decreased emission of cue-stimulated FM calls, whereas rats treated with raclopride (0.3 mg/kg) plus amphetamine displayed a trend toward a further increase in the emission of flat calls after amphetamine challenge compared with rats that received amphetamine alone. Amphetamine has been reported to stimulate the emission of trill and other FM 50-kHz USVs in rats and to increase the ratio between trill/FM and flat calls emitted (Simola et al., 2010; Wright et al., 2010; Mulvihill and Brudzynski, 2018). Moreover, the emission of trill and FM 50-kHz USVs has been proposed as *the* vocal response indicative of heightened affectivity in rats treated with psychoactive drugs (Ahrens et al., 2009; Taracha et al., 2014; Barker et al., 2015). However, no definitive evidence exists to conclude that specific categories of 50-kHz USVs communicate distinct effects of psychoactive drugs on the rats' affective state. For example, some studies reported that amphetamine exclusively stimulated the emission of FM 50-kHz USVs in rats (Taracha et al., 2014), while others found that amphetamine-treated rats emitted both FM and flat calls (Simola et al., 2012; Pereira et al., 2014; Wöhr et al., 2015; Simola and Costa, 2018). Furthermore, rats display a marked interindividual variability in 50-kHz USV emissions (Schwarting et al., 2007; Wöhr et al., 2008; Simola and Costa, 2018), which involves both total and categorized calls and may further complicate our understanding of the biological significance of the categorized calls emitted by rats treated with psychoactive drugs. Nevertheless, the effects of SCH 23390 and raclopride on the emission of categorized 50-kHz USVs in amphetamine-treated rats deserve further consideration because they may stimulate future studies about the molecular mechanisms that regulate the emission of different categories of 50-kHz USVs.

CONCLUSIONS

The present study demonstrates that the antagonism of dopamine D_1 or D_2 receptors elicits a divergent influence on the acute and enduring modifications in 50-kHz USV emissions in rats repeatedly treated with amphetamine. Antagonism of either receptor suppressed calling behavior during repeated amphetamine treatment. Conversely, D_1 receptor antagonism more markedly influenced the enduring changes in calling behavior observed after repeated amphetamine discontinuation compared with D_2 receptor antagonism. Finally, D_1 and D_2 receptors synergistically interacted to enable amphetamine's effects on calling behavior. These findings could further elucidate the molecular mechanisms and neurotransmitter systems that regulate the emission of 50-kHz USVs, as well as the significance of calling behavior in rats treated with psychoactive drugs.

Supplementary Materials

Supplementary data are available at *International Journal of Neuropsychopharmacology (IJNPPY)* online.

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Interest Statement

The authors declare no conflict of interest.

Data Availability

The data underlying this article will be shared on reasonable request to the corresponding author.

Author Contributions

Marcello Serra (Formal analysis [Equal], Investigation [Equal], Writing—original draft [Equal]), Giulia Costa (Data curation [Equal], Formal analysis [Equal], Writing—original draft [Equal]), Emmanuel Onaivi (Writing—review and editing [Equal]), and Nicola Simola (Conceptualization [Lead], Funding acquisition

[Lead], Investigation [Lead], Supervision [Lead], Writing—review and editing [Lead])

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