

## REGULAR RESEARCH ARTICLE

# Modulation of Rat 50-kHz Ultrasonic Vocalizations by Glucocorticoid Signaling: Possible Relevance to Reward and Motivation

Nicola Simola, Elena Paci, Marcello Serra, Giulia Costa, Micaela Morelli

Department of Biomedical Sciences, Section of Neuropsychopharmacology, University of Cagliari, Cagliari, Italy (Dr Simola, Dr Serra, Dr Costa, and Dr Morelli); National Institute of Neuroscience, University of Cagliari, Cagliari, Italy (Drs Simola and Morelli); Department of Physiology, Pharmacology and Neuroscience, University of Bristol, Bristol, United Kingdom (Dr Paci); CNR, National Research Council of Italy, Neuroscience Institute, Cagliari, Italy (Dr Morelli).

Correspondence: Nicola Simola, PhD, Department of Biomedical Sciences, Section of Neuropsychopharmacology, University of Cagliari, Via Ospedale, 72, 09124, Cagliari, Italy ([nicola.simola@gmail.com](mailto:nicola.simola@gmail.com)).

## Abstract

**Background:** Rats emit 50-kHz ultrasonic vocalizations (USVs) to communicate positive emotional states, and these USVs are increasingly being investigated in preclinical studies on reward and motivation. Although it is the activation of dopamine receptors that initiates the emission of 50-kHz USVs, non-dopaminergic mechanisms may modulate calling in the 50 kHz frequency band. To further elucidate these mechanisms, the present study investigated whether the pharmacological manipulation of glucocorticoid signaling influenced calling.

**Methods:** Rats were administered corticosterone (1–5 mg/kg, s.c.), the glucocorticoid receptor antagonist mifepristone (40 or 100 mg/kg, s.c.), or the corticosterone synthesis inhibitor metyrapone (50 or 100 mg/kg, i.p.). The effects of these drugs on calling initiation and on calling recorded during nonaggressive social contacts or after the administration of amphetamine (0.25 or 1 mg/kg, i.p.) were then evaluated.

**Results:** Corticosterone failed to initiate the emission of 50-kHz USVs and did not influence pro-social and amphetamine-stimulated calling. Similarly, mifepristone and metyrapone did not initiate calling. However, metyrapone suppressed pro-social calling and calling stimulated by a moderate dose (1 mg/kg, i.p.) of amphetamine. Conversely, mifepristone attenuated calling stimulated by a low (0.25 mg/kg, i.p.), but not moderate (1 mg/kg, i.p.), dose of amphetamine and had no influence on pro-social calling.

**Conclusions:** The present results demonstrate that glucocorticoid signaling modulates calling in the 50 kHz frequency band only in certain conditions and suggest that mechanisms different from the inhibition of corticosterone synthesis may participate in the suppression of calling by metyrapone.

**Keywords:** amphetamine, corticosterone, metyrapone, mifepristone, rat communication

## Introduction

Rats emit the so-called 50-kHz ultrasonic vocalizations (USVs), which are acoustic signals contained within the 35- to 80-kHz

frequency range, to communicate positive emotional states to conspecifics ([Panksepp, 2005](#); [Schwartz et al., 2007](#); [Brudzynski,](#)

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## Significance Statement

Ultrasonic vocalizations (USVs) of 50 kHz are a behavioral marker of reward in rats and are increasingly being investigated in preclinical studies on affect and emotion. On this basis, it appears of interest to elucidate the mechanisms that participate in the emission of 50-kHz USVs. Several preclinical and clinical studies indicate that glucocorticoid receptors may play a major role in the regulation of the emotional state. Therefore, we evaluated whether the emission of 50-kHz USVs could be influenced by drugs that modulate glucocorticoid-mediated signaling. The results of this study may be relevant not only to further elucidate the neuropharmacology of 50-kHz USVs, but also may provide further insight into the interplay between glucocorticoids and positive affect.

2013, 2015). On this basis, preclinical studies on reward and motivation are increasingly investigating the situations that are associated with calling in the 50 kHz frequency band and the mechanisms that underlie this behavior (Barker et al., 2015; Rippberger et al., 2015; Simola, 2015).

The emission of 50-kHz USVs is initiated by the activation of dopamine (DA) receptors in the shell of the nucleus accumbens (NAc) (Burgdorf et al., 2001; Thompson et al., 2006). Nevertheless, either the activation or the antagonism of nondopaminergic receptors may modulate calling in the 50 kHz frequency band (Fu and Brudzynski, 1994; Wright et al., 2012; Costa et al., 2015; Hamed et al., 2015; Wöhr et al., 2015; Simola et al., 2010, 2014, 2016). In this regard, it is noteworthy that rewarding stimuli of a different nature may elevate the plasma levels of corticosterone in rats (Szechtman et al., 1974; Knych and Eisenberg, 1979; Moldow and Fischman, 1987; Buwalda et al., 2012; Egan and Ulrich-Lai, 2015). Endogenous corticosterone, in turn, modulates the release of DA in the NAc shell that is elicited by rewarding stimuli (Barrot et al., 2000). Moreover, additional studies indicate that the administration of exogenous corticosterone may stimulate DA release in the NAc shell and may shape the effects that rewarding stimuli elicit on the emotional state of rats (Piazza et al., 1991; Piazza and LeMoal, 1996; Pecoraro et al., 2005; Der-Avakian et al., 2006). Taken together, these findings may suggest that glucocorticoid receptors could modulate calling in rats that are exposed to rewarding stimuli.

To date, the only studies that provide some evidence of a possible relationship between glucocorticoid signaling and calling in the 50 kHz frequency band were performed in rats previously and repeatedly exposed to stress, which elevates the plasma levels of corticosterone (Smith and Gala, 1977; Giralt et al., 1987). Thus, stress-exposed rats emitted a lower number of 50-kHz USVs in situations that may be rewarding, such as heterospecific playful contacts ("tickling") or amphetamine administration, compared with stress-naïve rats (Popik et al., 2012, 2014; Kövi et al., 2016). Moreover, administration of the corticosterone synthesis inhibitor metyrapone during stress imposition restored calling in response to tickling (Popik et al., 2014). Nevertheless, it has to be remarked that some of the behavioral effects of stress do not depend on the elevation in plasma corticosterone (Ulrich-Lai and Herman, 2009; Mei and Li, 2013). Besides, a previous study has found high levels of calling in stress-naïve rats that were placed in an enriched environment, a situation that elevates the plasma levels of corticosterone (Perez-Sepulveda et al., 2013). However, that study did not clarify whether it was the activation of glucocorticoid receptors by corticosterone that enhanced calling. Therefore, investigating how glucocorticoid signaling modulates the emission of 50-kHz USVs in stress-naïve rats appears to be relevant. Elucidating this issue may further clarify the mechanisms that participate in calling, as well as the interplay between corticosterone and positive affect.

To this end, we administered corticosterone, the glucocorticoid receptor antagonist mifepristone, or the corticosterone

synthesis inhibitor metyrapone to rats. Afterwards, we evaluated whether these drugs initiated the emission of 50-kHz USVs and/or affected calling that was recorded during nonaggressive social contacts or after the administration of amphetamine, two situations that may be rewarding for rats (Burgdorf et al., 2001, 2009). Moreover, we evaluated whether the administration of spironolactone, an antagonist of the mineralocorticoid receptors, affected pro-social and amphetamine-stimulated calling to investigate the mechanisms underlying the effects of metyrapone. In fact, metyrapone may have a greater inhibitory effect on the synthesis of the mineralocorticoid hormone aldosterone than on the synthesis of corticosterone (Rigel et al., 2010; Daniel and Newell-Price, 2015).

## Methods

### Animals

A total of 110 male Sprague–Dawley rats (Harlan, Italy) weighing 150 to 200 g at the beginning of the experiments were used. Rats were housed in groups of 4 to 6 in standard polycarbonate cages with sawdust bedding and maintained on a 12-hour-light/dark cycle (lights on at 8:00 AM). Food and water were freely available, except during USV recordings, which were performed between 12:00 and 4:00 PM. All experiments were conducted in accordance with the guidelines for animal experimentation of the EU directives (2010/63/EU; L.276; 22/09/2010) and with the guidelines approved by the Ethical Committee of the University of Cagliari. Experiments were designed to minimize animal discomfort to the least extent possible and to reduce the number of animals used.

### Doses and Administration Times of Drugs

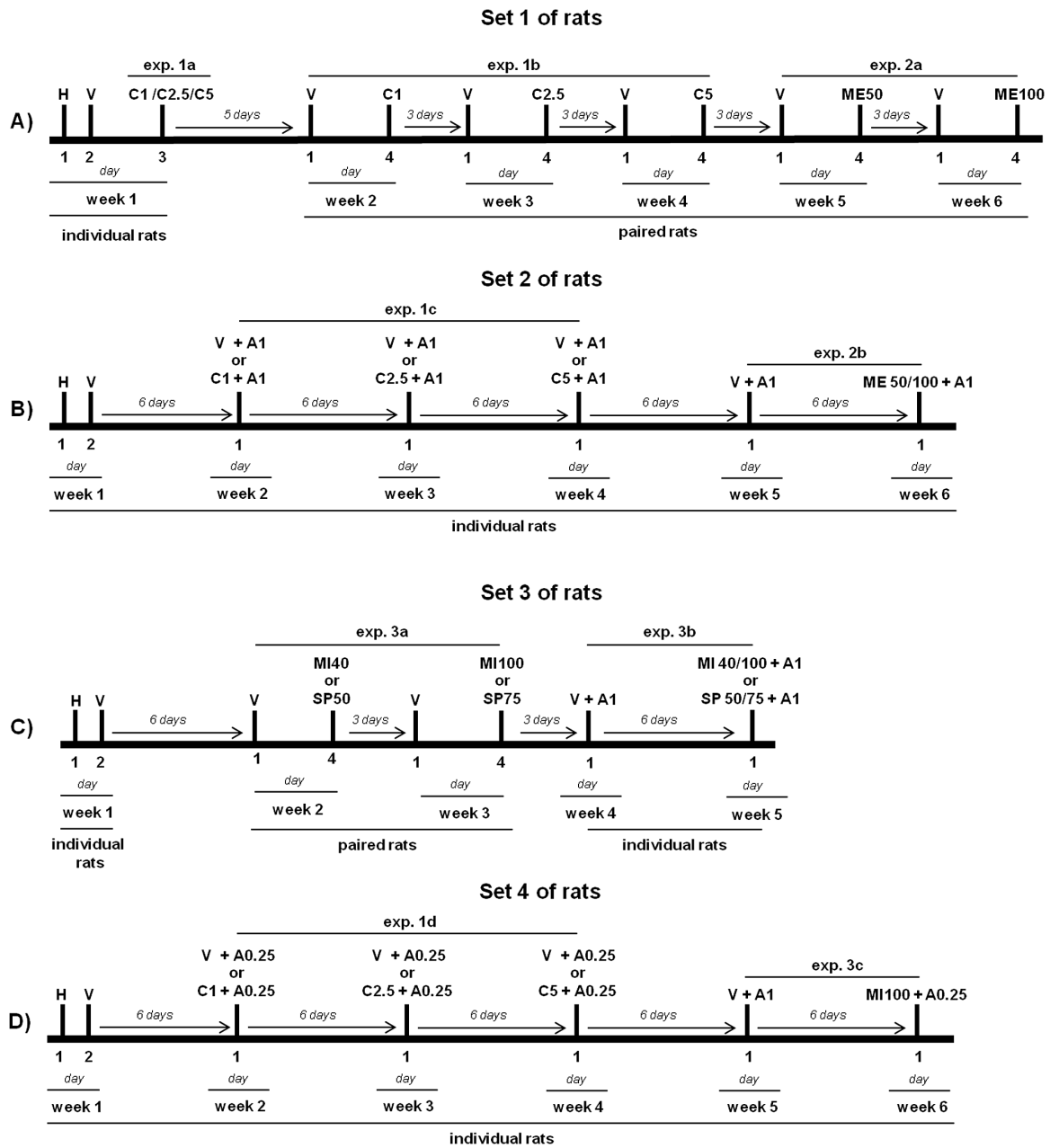
Corticosterone, mifepristone, and spironolactone were purchased from Sequoia Research Products Ltd. D-amphetamine (sulfate) and metyrapone were purchased from Sigma-Aldrich. Corticosterone, mifepristone, and spironolactone were dissolved in 10:90% dimethyl sulfoxide (DMSO)/polyethylene glycol (PEG). Corticosterone was administered at doses of 1, 2.5, or 5 mg/kg (Deroche et al., 1992, 1997; Dietz et al., 2007; Mantsch et al., 1998; Mei and Li, 2013). Mifepristone was administered at doses of 40 or 100 mg/kg (Heikinheimo et al., 1994; De Vries et al., 1996). Spironolactone was administered at doses of 50 or 75 mg/kg (Achterberg et al., 2014). Metyrapone was dissolved in 40:60% PEG/distilled water and administered at doses of 50 or 100 mg/kg (Calvo et al., 1998; Wright et al., 2006). Amphetamine was dissolved in distilled water and administered at doses of 0.25 or 1 mg/kg, both calculated as salt (Simola and Morelli, 2015; Wright et al., 2010). Corticosterone, mifepristone, and spironolactone were administered s.c. in a volume of 1 mL/kg. Amphetamine and metyrapone were administered i.p. in a volume of 3 mL/kg.

Corticosterone (or its vehicle) was administered immediately before recordings in the experiments that evaluated calling initiation, or 10 minutes before recordings in the experiments that evaluated pro-social and amphetamine-stimulated calling (Der-Avakian et al., 2006; Scherer et al., 2011). Metyrapone (or its vehicle) was always administered 90 minutes before recordings (Calvo et al., 1998; Wright et al., 2006). Mifepristone and spironolactone (or their vehicle) were always administered 45 minutes before recordings (De Vries et al., 1996; Fiancette et al., 2010; Mei and Li, 2013; Achterberg et al., 2014; Hofford et al., 2015).

Amphetamine was always administered immediately before recordings (Simola et al., 2012). The supplementary Methods describe how the doses and administration times of drugs were selected.

### Experimental Plan

A series of experiments was performed to evaluate whether the pharmacological manipulation of glucocorticoid signaling influenced the emission of 50-kHz USVs in different conditions. Rats



**Figure 1.** Timeline of the experiments. Four sets of rats were used in this study, and the figure indicates the experiments performed in each set of rats. In the first week, rats in each set were individually housed during recordings. They were habituated (30 minutes) to the test cage on day 1, to evaluate basal calling, and received vehicle (10:90% dimethyl sulfoxide [DMSO]/polyethylene glycol [PEG]) on day 2. Afterwards, rats received pharmacological treatments and were housed either individually or in pairs during recording, as outlined in the figure. Ultrasonic vocalization (USV) recordings were performed on each testing day and lasted for 2 hours in rats individually housed and 15 minutes in rats housed in pairs. A, amphetamine; C, corticosterone; H, habituation to the test-cage; ME, metyrapone; MI, mifepristone; SP, spironolactone; V, vehicle. The numbers indicate the doses of each drug (in mg/kg). The effects of metyrapone, mifepristone, and spironolactone on calling initiation were evaluated in experiment 2b and 3b, before the administration of amphetamine.

received the repeated administration of one or two of the following drugs: corticosterone, metyrapone, mifepristone, and spironolactone. Each drug was administered alone or in combination with amphetamine. Repeated drug administrations in the same rats were performed to reduce the number of animals used in the study. Moreover, drugs were always given at ascending doses. This was done to expose rats to the same experimental conditions across recordings in the attempt to reduce the variability of the results. Finally, 4 sets of rats were used to reduce the possible carryover effects that could arise from repeated drug administration. Rats in each set were drug-naïve before the beginning of the experiments. The individual experiments are listed below and are grouped according to the drugs administered. The timeline of experiments performed in each set of rats is described in [Figure 1](#).

#### Experiments That Evaluated the Effects of Corticosterone on Calling in the 50 kHz Band

**Experiment 1a: calling initiation.** Rats were individually housed during recordings and received the acute administration of corticosterone (1, 2.5, or 5 mg/kg, s.c.) ([Figure 1A](#)).  $n=10$  for each dose of corticosterone.

**Experiment 1b: pro-social calling.** Rats were housed in pairs during recordings to engage in social contacts and evaluated for 3 consecutive weeks. In each week, rats received vehicle (10:90%, DMSO:PEG) on the first day and corticosterone (1, 2.5, or 5 mg/kg, s.c.) on the fourth day ([Figure 1A](#)).  $n=15$  pairs for each evaluation.

**Experiments 1c and d: amphetamine-stimulated calling.** Rats were individually housed during recordings and evaluated for 3 consecutive weeks. In each week, rats received corticosterone (1, 2.5, or 5 mg/kg, s.c.) followed by amphetamine 10 minutes later. Amphetamine was administered at either a moderate dose (1 mg/kg, i.p.) ([Figure 1B](#)) or low dose (0.25 mg/kg, i.p.) ([Figure 1D](#)).  $n=10$  for each dose of corticosterone.

#### Experiments That Evaluated the Effects of Metyrapone on Calling in the 50 kHz Band

**Experiment 2a: pro-social calling.** Rats were housed in pairs during recordings to engage in social contacts and evaluated for 2 consecutive weeks. In each week, rats received vehicle (40:60%, PEG: distilled water) on the first day and metyrapone (50 or 100 mg/kg, s.c.) on the fourth day ([Figure 1A](#)).  $n=15$  pairs for each evaluation.

**Experiment 2b: calling initiation and amphetamine-stimulated calling.** Rats were individually housed during recordings and evaluated for 2 consecutive weeks. In the first week, all rats were administered vehicle (40:60% PEG: distilled water), placed in the test cage for 90 minutes, and thereafter received amphetamine (1 mg/kg, i.p.) to obtain a baseline level of calling. In the second week, rats were administered metyrapone (50 or 100 mg/kg, i.p.,  $n=10$  for each dose) and placed in the test cage for 90 minutes to evaluate calling initiation. Thereafter, metyrapone-treated rats received a moderate dose of amphetamine (1 mg/kg, i.p.) ([Figure 1B](#)).

#### Experiments That Evaluated the Effects of Mifepristone and Spironolactone on Calling in the 50 kHz Band

**Experiment 3a: pro-social calling.** Rats were housed in pairs during recordings to engage in social contacts and evaluated for 3 consecutive weeks. In each week, rats received vehicle (10:90%, DMSO:PEG) and either mifepristone (40 or 100 mg/kg, s.c.) or spironolactone (50 or 75 mg/kg, s.c.) on the fourth day ([Figure 1C](#)).  $n=10$  pairs for each evaluation.

**Experiment 3b: calling initiation and amphetamine-stimulated calling.** Rats were individually housed during recordings and

evaluated for 2 consecutive weeks. In the first week, all rats were administered vehicle (10:90%, DMSO:PEG), placed in the test cage for 45 minutes, and thereafter received amphetamine (1 mg/kg, i.p.) to obtain a baseline level of calling. In the second week, rats were administered either mifepristone (40 or 100 mg/kg, s.c.,  $n=10$  for each dose) or spironolactone (50 or 75 mg/kg, s.c.,  $n=10$  for each dose) and placed in the test cage for 45 minutes to evaluate calling initiation. Thereafter, mifepristone- and spironolactone-treated rats received a moderate dose of amphetamine (1 mg/kg, i.p., [Figure 1C](#)).

**Experiment 3c: calling stimulated by a low dose of amphetamine.** Rats were individually housed during recordings and evaluated for 2 consecutive weeks. In the first week, rats were treated as described for experiment 3b. In the second week, rats were treated with mifepristone (100 mg/kg, s.c.,  $n=20$ ), placed in the test cage for 45 minutes, and thereafter received a low dose of amphetamine (0.25 mg/kg, i.p., [Figure 1D](#)).

In all the experiments that evaluated pro-social calling, rats were always paired with a different rat and both rats were taken from different cages.

#### Recording of USVs

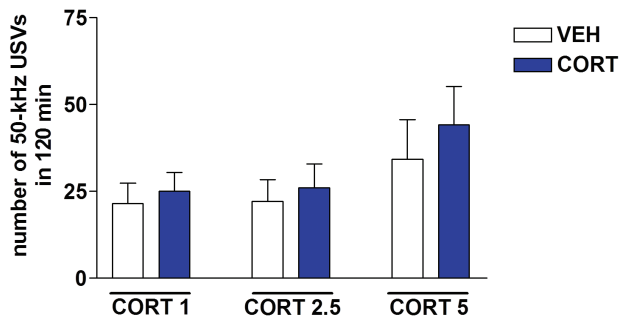
Rats were placed in Plexiglas cylinders (diameter, 25 cm; height, 30 cm). Thereafter, USVs were recorded by means of ultrasonic devices (CM16/CMPA microphones, UltraSoundGate 116 Hb; Avisoft), as described elsewhere ([Simola et al., 2012](#)). Recordings lasted for 2 hours in rats individually housed and 15 minutes in rats housed in pairs. All the recording times were subdivided into 5-minute intervals. The supplementary Methods report further details on the recording procedures. Supplementary Figure 1 shows some examples of 50-kHz USVs recorded in this study.

#### Statistical Analysis

USV recordings were converted into spectrograms by means of the software SASLab Pro 4.52 (Avisoft) with the following settings: 512 Fast Fourier Transform-length, Hamming window, and 75% overlap frame set-up. Spectrograms were visually inspected by an experienced experimenter, and SASLab Pro 4.52 was used to calculate the number of vocalizations after manual cleaning of all the signals that could not be univocally classified as 50-kHz USVs ([Simola et al., 2012](#)).

Means  $\pm$  SEM of the numbers of 50-kHz USVs were calculated for all the experiments. USV data collected in each experiment were tested for normality and homoscedasticity with Levene's test. Logarithmic transformation was applied to preserve homoscedasticity, when necessary. A constant of +1 was added to all data subjected to logarithmic transformation. Figures report raw data for clarity. The analysis of spontaneous calling recorded during habituation in each set of experiments revealed no outliers (data not shown). Moreover, neither aggressive interactions between rats nor emission of aversive 22-kHz USVs ([Brudzynski, 2007](#)) were observed in the present study. Therefore, all the rats used were included in the statistical analysis. The total number of calls emitted and the time course of calling was evaluated for each experiment with 2-way (treatment  $\times$  time) ANOVA to reveal significant differences between pharmacological treatments and vehicle administration. Moreover, data collected in each experiment were subdivided into low vocalization (LV) and high vocalization (HV) groups, based on the median value of the number of 50-kHz USVs emitted. Such a subdivision was done to clarify whether ceiling and/or floor effects could affect the results observed in all rats. Separate ANOVAs were run for all rats and for the LV and the HV groups of rats. Significance was





**Figure 2.** Effects of corticosterone on calling initiation. Acute corticosterone (1–5 mg/kg, s.c.) did not initiate calling. The number of 50-kHz ultrasonic vocalizations (USVs) recorded after corticosterone administration was comparable with that recorded after vehicle administration. CORT, corticosterone; VEH, vehicle.  $n=10$ .

always set at  $P < .05$  and ANOVAs were followed by Tukey's post-hoc tests, when appropriate. For the sake of conciseness, the main effects of ANOVA are reported in supplementary Table 1. Statistical results for the time course of calling recorded in each administration day are described in the legends of the supplementary figures, when appropriate. Statistical analysis was performed with Statistica (Statsoft) and QI Macros (KnowWare International) for Windows.

## Results

This section describes the effects of pharmacological treatment on the total number of 50-kHz USVs emitted in each testing day. Supplementary Figures 2 to 8 demonstrate the effects of pharmacological treatment on the time course of calling in each testing day.

### Effects of Corticosterone on Calling Initiation in Rats Individually Housed during Recording

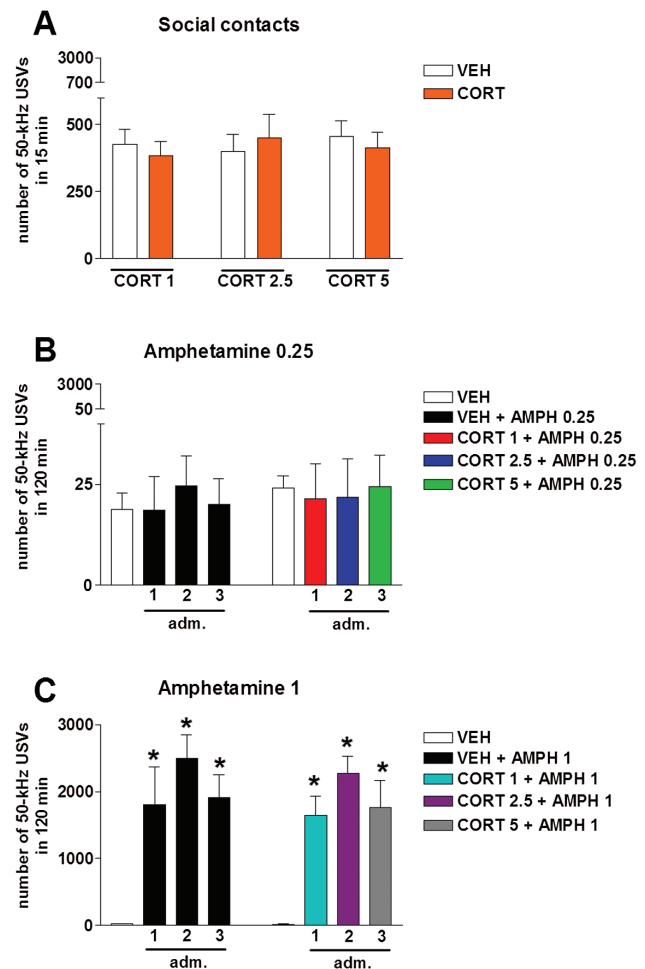
Acute administration of corticosterone (1, 2.5, or 5 mg/kg, s.c.) did not modify the number of 50-kHz USVs emitted by rats, compared with vehicle administration. Two-way ANOVA showed no significant effect of treatment ( $F_{5,53}=1.41$ ,  $P=.23$ ) (Figure 2).

### Effects of Corticosterone on Prosocial Calling in Rats Housed in Pairs during Recording

Administration of escalating doses of corticosterone (1, 2.5, or 5 mg/kg, s.c., once a week) did not modify the number of 50-kHz USVs emitted by rats that were allowed to engage in social contacts, compared with vehicle administration. Two-way ANOVA showed no significant effect of treatment ( $F_{5,84}=0.31$ ,  $P=.91$ ) (Figure 3A).

### Effects of Corticosterone on Calling Stimulated by Amphetamine in Rats Individually Housed during Recording

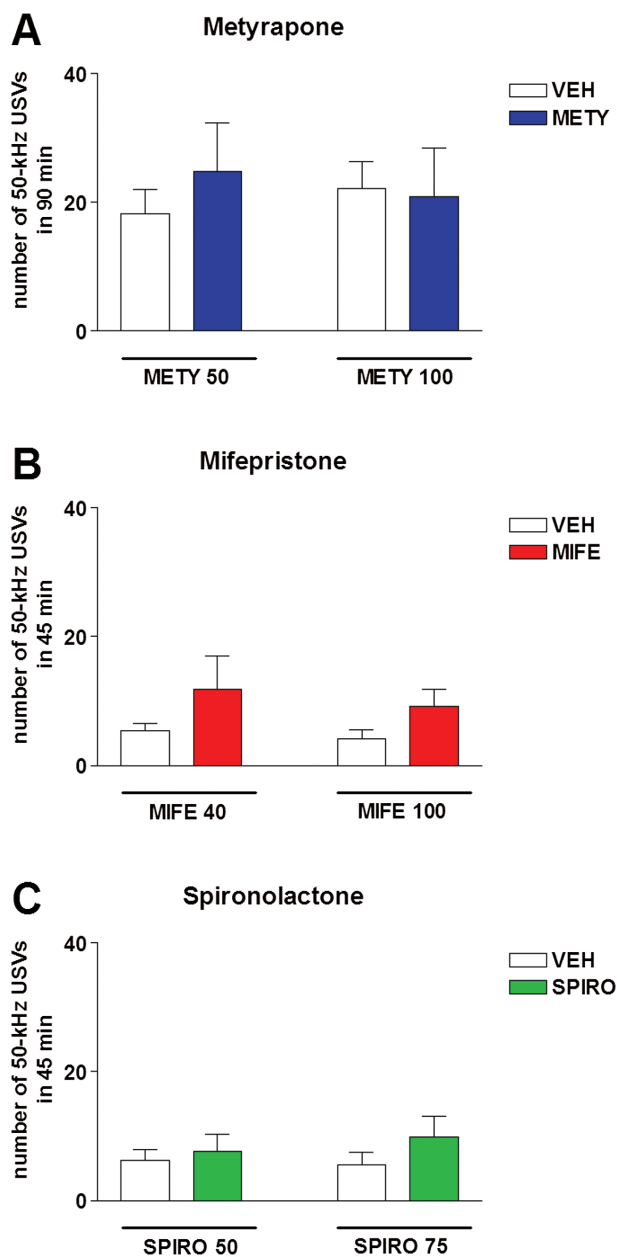
Repeated administration of a low dose of amphetamine (0.25 mg/kg, i.p., once a week for 3 weeks) did not modify the number of 50-kHz USVs emitted by rats compared with vehicle administration. A similar lack of effect was observed when escalating doses of corticosterone (1–5 mg/kg, s.c.) were co-administered



**Figure 3.** Effects of corticosterone on pro-social and amphetamine-stimulated calling. Escalating doses of corticosterone (1–5 mg/kg, s.c.) did not modify the number of 50-kHz USVs emitted by rats that were housed in pairs during recording, compared with vehicle administration (A). Repeated administration of a low dose of amphetamine (0.25 mg/kg, i.p., once a week for 3 weeks) did not modify the number of 50-kHz ultrasonic vocalizations (USVs) emitted by rats that were individually housed during recording, compared with vehicle administration (B). Conversely, repeated administration of a moderate dose of amphetamine (1 mg/kg, i.p., once a week for 3 weeks) significantly elevated the number of 50-kHz USVs emitted by rats that were individually housed during recording, compared with vehicle administration (C). Co-administration of escalating doses of corticosterone (1–5 mg/kg, s.c.) did not influence the effects of amphetamine on calling (B–C). adm., administration; AMPH, amphetamine; CORT, corticosterone; VEH, vehicle. \*  $P < .05$  vs VEH.  $n=15$  for rats housed in pairs.  $n=10$  for rats individually housed.

with amphetamine (0.25 mg/kg, i.p.). Two-way ANOVA showed no significant effect of treatment ( $F_{7,72}=0.56$ ,  $P=.79$ ) (Figure 3B).

Conversely, repeated administration of a moderate dose of amphetamine (1 mg/kg, i.p., once a week for 3 weeks) significantly modified the number of 50-kHz USVs emitted by rats, compared with vehicle administration. Similar effects were observed when escalating doses of corticosterone (1–5 mg/kg, s.c.) were co-administered with amphetamine (1 mg/kg, i.p.). Two-way ANOVA showed a significant effect of treatment: ( $F_{7,72}=29.82$ ,  $P < .01$ ). Tukey's test showed that both amphetamine and corticosterone + amphetamine elevated the number of calls. However, corticosterone + amphetamine increased the number of 50-kHz USVs emitted in a fashion similar to amphetamine alone (Figure 3C).



**Figure 4.** Effects of metyrapone, mifepristone, and spironolactone on calling initiation. Acute metyrapone (50 or 100 mg/kg, i.p.) (A), mifepristone (40 or 100 mg/kg, s.c.) (B), or spironolactone (50 or 75 mg/kg, s.c.) (C) did not initiate calling. The number of 50-kHz ultrasonic vocalizations (USVs) recorded after the administration of each drug was comparable with that recorded after vehicle administration. METY, metyrapone; MIFE, mifepristone; SPIRO, spironolactone; VEH, vehicle.  $n=10$ .

#### Effects of Metyrapone on Calling Initiation in Rats Individually Housed during Recording

Acute administration of metyrapone (50 or 100 mg/kg, i.p.) did not modify the number of 50-kHz USVs emitted by rats, compared with vehicle administration. Two-way ANOVA showed no significant effect of treatment ( $F_{3,36}=0.26$ ,  $P=.86$ ) (Figure 4A).

#### Effects of Metyrapone on Prosocial Calling in Rats Housed in Pairs during Recording

Administration of escalating doses of metyrapone (50 or 100 mg/kg, i.p., once a week) modified the number of 50-kHz USVs emitted by rats that were allowed to engage in social contacts, compared with vehicle administration. Two-way ANOVA showed a significant effect of treatment ( $F_{3,56}=72.96$ ,  $P<.01$ ). Moreover, Tukey's test showed that metyrapone (100 mg/kg, i.p.) significantly reduced the number of calls (Figure 5A).

#### Effects of Metyrapone on Calling Stimulated by Amphetamine in Rats Individually Housed during Recording

Co-administration of metyrapone (50 or 100 mg/kg, i.p.) modified the number of 50-kHz USVs emitted by rats that were treated with amphetamine (1 mg/kg, i.p.), compared with vehicle co-administration. Two-way ANOVA showed a significant effect of treatment ( $F_{5,54}=35.43$ ,  $P<.01$ ). Moreover, Tukey's test showed that metyrapone (100 mg/kg, i.p.) significantly reduced the number of calls (Figure 5D).

#### Effects of Mifepristone and Spironolactone on Calling Initiation in Rats Individually Housed during Recording

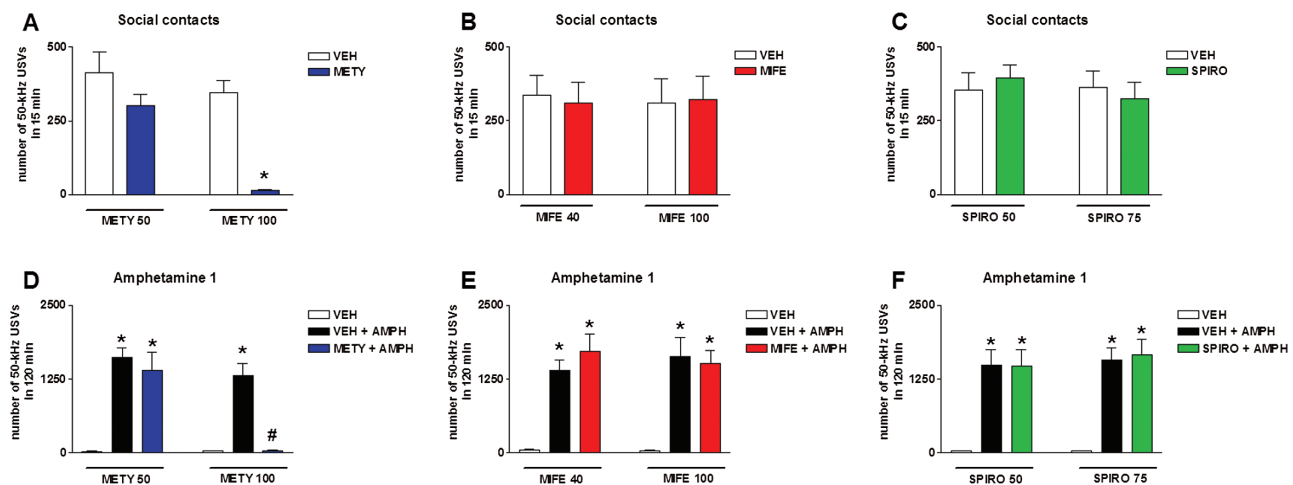
Acute administration of mifepristone (40 or 100 mg/kg, s.c.) or spironolactone (50 or 75 mg/kg, s.c.) did not modify the number of 50-kHz USVs emitted by rats, compared with vehicle administration. Two-way ANOVA showed no significant effects of treatment for mifepristone ( $F_{3,36}=1.77$ ,  $P=.17$ ) and spironolactone ( $F_{3,36}=0.46$ ,  $P=.71$ ) (Figure 4B–C).

#### Effects of Mifepristone and Spironolactone on Prosocial Calling in Rats Housed in Pairs during Recording

Administration of escalating doses of mifepristone (40 or 100 mg/kg, s.c., once a week) or spironolactone (50 or 75 mg/kg, s.c., once a week) did not modify the number of 50-kHz USVs emitted by rats that were allowed to engage in social contacts, compared with vehicle administration. Two-way ANOVA showed no significant effect of treatment for mifepristone ( $F_{3,36}=0.38$ ,  $P=.77$ ) and spironolactone ( $F_{3,36}=0.50$ ,  $P=.68$ ) (Figure 5B–C).

#### Effects of Mifepristone and Spironolactone on Calling Stimulated by Amphetamine in Rats Individually Housed during Recording

Co-administration of mifepristone (40 or 100 mg/kg, s.c.) did not modify the number of 50-kHz USVs emitted by rats that were treated with amphetamine (1 mg/kg, i.p.), compared with vehicle co-administration. Thus, two-way ANOVA showed a significant effect of treatment ( $F_{5,54}=33.95$ ,  $P<.01$ ). However, Tukey's test showed that mifepristone + amphetamine increased the number of 50-kHz USVs emitted in a fashion similar to amphetamine alone (Fig 5E). Nevertheless, co-administration of mifepristone (100 mg/kg, s.c.) influenced calling recorded after the administration of a low dose of amphetamine (0.25 mg/kg, i.p.) (see below).



**Figure 5.** Effects of metyrapone, mifepristone, and spironolactone on pro-social and amphetamine-stimulated calling. Escalating doses of metyrapone (50 or 100 mg/kg, i.p., once a week) almost completely suppressed the number of 50-kHz ultrasonic vocalizations (USVs) emitted by rats that were housed in pairs during recording, compared with vehicle administration (A). Conversely, escalating doses of mifepristone (40 or 100 mg/kg, s.c., once a week) or spironolactone (50 or 75 mg/kg, s.c., once a week) did not affect pro-social calling (B–C). In rats that were individually housed during recordings, co-administration of metyrapone (100 mg/kg, i.p.) almost completely suppressed the number of 50-kHz USVs recorded after the administration of amphetamine (1 mg/kg, i.p.), compared with vehicle co-administration (D). Conversely, co-administration of mifepristone (40 or 100 mg/kg, s.c.) or spironolactone (50 or 75 mg/kg, s.c.) had no influence on amphetamine-stimulated calling (E–F). AMPH, amphetamine; METY, metyrapone; MIFE, mifepristone; SPIRO, spironolactone; VEH, vehicle. \* $P < .05$  vs VEH; # $P < .05$  vs VEH + AMPH.  $n = 10$ .

Co-administration of spironolactone (50 or 75 mg/kg, s.c.) did not modify the number of 50-kHz USVs emitted by rats that were treated with amphetamine (1 mg/kg, i.p.), compared with vehicle co-administration. Thus, two-way ANOVA showed a significant effect of treatment ( $F_{5,54} = 42.04, P < .01$ ). However, Tukey's test showed that spironolactone + amphetamine increased the number of 50-kHz USVs in a fashion similar to amphetamine alone (Figure 5F).

### Emission of 50-kHz USVs in the LV and HV Groups of Rats

Co-administration of mifepristone (100 mg/kg, s.c.) modified the number of 50-kHz USVs emitted in the HV group of rats treated with a low dose of amphetamine (0.25 mg/kg, i.p.). Two-way ANOVA showed a significant effect of treatment ( $F_{2,27} = 5.03, P = .01$ ) (Figure 6). Moreover, Tukey's test revealed that the number of calls emitted after mifepristone + amphetamine was lower than the number of calls emitted after amphetamine alone. The other results obtained in the LV and HV groups of rats were in line with the results obtained in the totality of rats (data not shown).

## Discussion

The present study evaluated whether the pharmacological manipulation of glucocorticoid signaling initiated the emission of 50-kHz USVs and/or influenced calling in situations that may be rewarding for rats. The results obtained showed that metyrapone and mifepristone affected calling, although mifepristone did so only in certain conditions. Conversely, corticosterone had no influence on calling.

Previous studies in rats have reported that corticosterone administration does not induce conditioned place preference (CPP) (Dietz et al., 2007; Mei and Li, 2013). These results would suggest that exogenous corticosterone does not elicit rewarding effects in the rat. In agreement with this view, the present study found that corticosterone administration did not initiate the emission of 50-kHz USVs, which are a behavioral marker

of reward in rats (Panksepp, 2005; Schwarting et al., 2007; Brudzynski, 2013, 2015). Similarly, corticosterone administration did not affect calling during nonaggressive social contacts, a situation that may induce a positive emotional state in rats (Burgdorf et al., 2009). Besides, another previous investigation found that rats after co-administration of corticosterone and morphine, displayed a more marked CPP, compared with those that received morphine alone (Der-Avakian et al., 2006). This finding would suggest that corticosterone may amplify the rewarding effects of morphine. However, corticosterone strengthened morphine-induced CPP in rats that were previously exposed to stress, but not in stress-naïve rats (Der-Avakian et al., 2006). Interestingly, we found that corticosterone administration did not affect calling in stress-naïve rats treated with amphetamine. Therefore, and taken together, the results of the present study may suggest that in stress-naïve rats corticosterone administration neither induces a positive emotional state nor amplifies the effects that rewarding stimuli have on the emotional state.

Similar to corticosterone, mifepristone administration did not initiate calling in the 50 kHz frequency band. This finding would suggest that the antagonism of glucocorticoid receptors does not induce a positive emotional state in rats. Nevertheless, mifepristone affected calling, as it reduced the number of 50-kHz USVs emitted after amphetamine administration. In this regard, it is noteworthy that the suppression of endogenous corticosterone by adrenalectomy attenuates DA release in the NAc shell of rats treated with drugs of abuse (Barrot et al., 2000). Moreover, the activation of DA receptors in the NAc shell is the key mechanism that initiates calling (Burgdorf et al., 2001; Thompson et al., 2006). Therefore, it is conceivable that mifepristone reduced calling in amphetamine-treated rats, because it attenuated DA signaling in the NAc shell. However, mifepristone affected calling only in the HV group of rats treated with a low dose of amphetamine (0.25 mg/kg). Hence, these results indicate that the antagonism of glucocorticoid receptors modulates calling only in certain conditions.

Homospecific social contacts and amphetamine administration increase the plasma levels of corticosterone in rats (Knych and Eisenberg, 1979; File, 1980). Nevertheless, the rise in

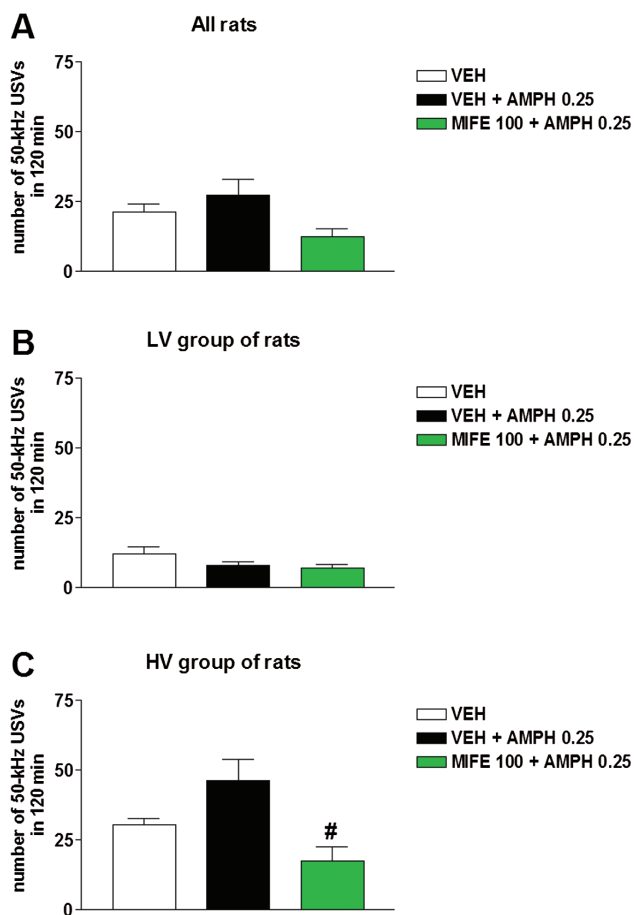


Figure 6. Effects of mifepristone on calling in rats treated with a low dose of amphetamine and individually housed during recordings. Co-administration of mifepristone (100 mg/kg, s.c.) had no influence on calling recorded after the administration of amphetamine (0.25 mg/kg, i.p.) in all rats and in the LV group (A–B). Conversely, mifepristone co-administration reduced the number of 50-kHz ultrasonic vocalizations (USVs) recorded after the administration of amphetamine in the HV group of rats (C). AMPH, amphetamine; HV, high vocalization; LV, low vocalization; MIFE, mifepristone; VEH, vehicle. <sup>#</sup> $P < .05$  vs VEH + AMPH.  $n = 20$  for all rats.  $n = 10$  for the LV and HV groups of rats.

plasma corticosterone might not necessarily predict the affective valence of stimuli. Conversely, it could reflect the metabolic demands of neuronal circuits subsequent to the behavioral activation that is induced by salient stimuli (Buwalda et al., 2012; Egan and Ulrich-Lai, 2015). In agreement with the latter view, it may be speculated that either the stimulation or the antagonism of glucocorticoid receptors would not modify the affective properties of rewarding stimuli in stress-naïve rats. This hypothesis may explain why corticosterone and mifepristone did not affect calling during social contacts or after the administration of a moderate dose of amphetamine (1 mg/kg). Alternatively, it may be speculated that glucocorticoid receptor activation by endogenous corticosterone is an ancillary mechanism that sustains calling in situations featuring the emission of a low number of 50-kHz USVs (i.e., the administration of a low amphetamine dose). Besides, it cannot be ruled out that glucocorticoid receptors influence calling emitted by stress-naïve rats in situations different from those evaluated here. In fact, corticosterone may elicit dissimilar effects on the emotional state of rats depending on the situations considered (Piazza et al., 1991, 1996; Deroche et al., 1997; Mantsch et al.,

1998; Pecoraro et al., 2005; Buwalda et al., 2012; Olausson et al., 2013).

The present study also found that metyrapone did not initiate the emission of 50-kHz USVs when administered at doses that inhibit corticosterone synthesis in rats. However, metyrapone almost completely suppressed calling recorded during nonaggressive social contacts or after the administration of a moderate dose of amphetamine (1 mg/kg). Since mifepristone did not affect calling in the same situations, we investigated whether mechanisms other than the inhibition of corticosterone synthesis participated in the effects of metyrapone observed here. In particular, we evaluated the effects of mineralocorticoid receptor antagonism on calling in the 50 kHz frequency band. In fact, metyrapone inhibits the synthesis of the mineralocorticoid hormone aldosterone (Rigel et al., 2010; Daniel and Newell-Price, 2015). Moreover, studies in cocaine-treated mice have demonstrated that the mineralocorticoid receptor antagonist spironolactone may affect the emotional state (Fiancette et al., 2010). However, spironolactone did not influence pro-social and amphetamine-stimulated calling, and it also failed to initiate the emission of 50-kHz USVs. In this regard, it is noteworthy that metyrapone inhibits the synthesis of both corticosterone and aldosterone (Daniel and Newell-Price, 2015). Therefore, it could be hypothesized that mifepristone and spironolactone did not suppress calling because they partially reproduced the effects of metyrapone. However, we have found in preliminary experiments that the co-administration of mifepristone and spironolactone did not attenuate amphetamine-stimulated calling (see supplementary Table 2). Moreover, previous studies have demonstrated that metyrapone may exert extra-adrenal effects in rats (Shaham et al., 1997; Guerin et al., 2014; Schmoutz et al., 2014). Therefore, we may suggest that mechanisms of actions other than the inhibition of steroidogenesis may participate in the suppression of calling by metyrapone. In this regard, it is also noteworthy that the present study employed a protocol of repeated metyrapone administration, at least in the rats that were housed in pairs during recording. Therefore, such an administration protocol could have favored the sensitization in the inhibitory effects of metyrapone on steroidogenesis and/or the emergence of additional mechanisms of action of metyrapone.

The effects of metyrapone observed here may appear to be in contrast to earlier findings in rats exposed to immobilization stress (Popik et al., 2014). Thus, rats that were previously and repeatedly immobilized emitted a lower number of 50-kHz USVs when later subjected to tickling, compared with stress-naïve rats. Metyrapone administration concomitantly with immobilization did not further suppress tickling-stimulated calling, but rather restored it. Conversely, metyrapone did not affect tickling-stimulated calling in stress-naïve rats. In this regard, it is noteworthy that the study by Popik and coworkers employed a protocol of repeated metyrapone administration throughout 7 consecutive days. Conversely, in the present study metyrapone was given in a single or double, but spaced, administration. Notably, spaced and continuous administration of metyrapone may elicit dissimilar behavioral effects in rats (Shaham et al., 1997). Moreover, the study by Popik and coworkers evaluated the effects of metyrapone in a situation (tickling) that is different from those examined in the present study. Nevertheless, the present results are of great relevance as they demonstrate, for the first time, that metyrapone affects the emission of 50-kHz USVs in stress-naïve rats. Moreover, the present results further elucidate the effects that metyrapone



may have on the emotional state of rats, and can contribute to the preclinical characterization of the anti-addiction and anti-depressant properties of metyrapone, which have been previously described (Murphy, 1997; Healy et al., 1999; Goeders and Guerin, 2008; Guerin et al., 2014).

In conclusion, the present study further characterizes the mechanisms that regulate the emission of 50-kHz USVs and the interplay between glucocorticoid signaling and positive affect. The results obtained may help to define the usefulness of 50-kHz USVs in studying the modifications in the emotional state of rats that can be induced by natural and pharmacological rewards.

## Supplementary Material

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

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## Statement of Interest

None.

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