Psychopharmacology (2014) 231:2567–2577
DOI 10.1007/s00213-013-3413-1

ORIGINAL INVESTIGATION

Amphetamine-induced appetitive 50-kHz calls in rats: a marker of affect in mania?

Marcela Pereira · Roberto Andreatini · Rainer K. W. Schwarting · Juan C. Brenes

Received: 29 May 2013 / Accepted: 18 December 2013 / Published online: 11 January 2014

Abstract
Rationale Animal models aimed to mimic mania have in common the lack of genuine affective parameters. Although rodent amphetamine-induced hyperlocomotion is a frequently used behavioral model of mania, locomotor activity is a rather unspecific target for developing new pharmacological therapies, and does not necessarily constitute a cardinal symptom in bipolar disorder (BD). Hence, alternative behavioral markers sensitive to stimulants are required.
Objectives Since D-amphetamine induces appetitive 50-kHz ultrasonic vocalizations (USV) in rats, we asked whether established or potential antimanic drugs would inhibit this effect, thereby possibly complementing traditional analysis of locomotor activity.
Methods Amphetamine-treated rats (2.5 mg/kg) were systemically administered with the antimanic drugs lithium (100 mg/kg) and tamoxifen (1 mg/kg). Since protein kinase C (PKC) activity has been implicated in the pathophysiology of bipolar disorder and the biochemical effects of mood stabilizers, the new PKC inhibitor myricitrin (10, 30 mg/kg) was also evaluated.
Results We demonstrate for the first time that drugs with known or potential antimanic activity were effective in reversing amphetamine-induced appetitive 50-kHz calls. Treatments particularly normalized amphetamine-induced increases of frequency-modulated calls, a subtype presumably indicative of positive affect in the rat.
Conclusions Our findings suggest that amphetamine-induced 50-kHz calls might constitute a marker for communicating affect that provides a useful model of exaggerated euphoric mood and pressured speech. The antimanic-like effects of the PKC inhibitors tamoxifen and myricitrin support the predictive and etiological validity of both drugs in this model and highlight the role of PKC signaling as a promising target to treat mania and psychosis-related disorders.

Keywords Mania · Bipolar disorder · Emotion · Dopamine · Ultrasonic vocalizations · Mood stabilizer · Protein kinase C · Lithium · Tamoxifen · Myricitrin

M. Pereira · R. Andreatini
Department of Pharmacology, Federal University of Paraná (UFPR), Curitiba PR 81540-990, Brazil

R. K. W. Schwarting (✉) · J. C. Brenes
Behavioral Neuroscience, Experimental and Biological Psychology, Philipps-University of Marburg, Gutenbergstr. 18, 35032 Marburg, Germany
e-mail: schwarti@staff.uni-marburg.de

J. C. Brenes
Institute for Psychological Research, University of Costa Rica, Rodrigo Facio Campus, 2060 San Pedro, Costa Rica

J. C. Brenes (✉)
Neuroscience Research Center, University of Costa Rica, Rodrigo Facio Campus, 2060 San Pedro, Costa Rica
e-mail: brenesaenz@gmail.com

Introduction

Bipolar disorder (BD) is a prevalent and severe psychiatric disease affecting approximately 2–7 % of the worldwide population (Belmaker 2004; Friedman et al. 2006). Its defining feature is mania, which sets it apart from other mood disorders (Belmaker 2004; Nierenberg 2010). In a mania episode, euphoric or irritable mood can be accompanied by a cluster of symptoms such as hyperactivity, little need for sleep, increased risk-taking behavior, reward seeking, pressured speech, and flight of thoughts (Belmaker 2004).

Understanding the neurobiological basis of mania and development of new treatments has taken limited progress partly due to a paucity of relevant animal models with translational

potential (Young et al. 2007). In contrast to anxiety or depression, animal models of mania are less developed and refined. The prevalent behavioral models use locomotion as one of the main markers, primarily because hyperactivity is an important disease symptom and because it is easy to measure in rodents (Harrison-Read 2009; Young et al. 2011). In fact, the current most frequently used behavioral model is amphetamine (AMP)-induced hyperlocomotion in rodents (Gould et al. 2007; Young et al. 2011). This model is based on the observation that AMP can produce mania-like symptoms in healthy individuals, and can precipitate manic episodes or aggravate current symptomatology in patients (Smith and Daves 1977; Willson et al. 2005). Besides its face validity, AMP-induced hyperlocomotion exhibits good predictive validity as mood stabilizers like lithium and valproate reverse AMP-induced hyperactivity both, in humans and rodents (Gould et al. 2007; Van Kammen and Murphy 1975; Willson et al. 2005). However, the use of locomotor activity as a core parameter does have some limitations (Harrison-Read 2009; Young et al. 2011). First, locomotor activity is a general behavioral measure used in various disease models and drug screening tests. Particularly, hyperlocomotion induced by AMP or methylphenidate constitutes a central marker in animal models of other psychiatric entities such as psychosis and addiction (Pereira et al. 2011; Young et al. 2011). Secondly, such hyperactivity is not exclusively linked to exacerbated emotional states. For instance, in animal models of depression such as olfactory bulbectomy and unpredictable chronic mild stress increases in locomotor activity are considered a behavioral sign as well (Grønli et al. 2005; Song and Leonard 2005). Thirdly, hyperactivity is not a cardinal symptom of BD, and mania episodes may take place without noticeable hyperactivity (Young et al. 2011). In general, although evaluation of locomotion is mandatory in behavioral pharmacology, it might be quite an unspecific target for developing new pharmacological therapies. Therefore, the inclusion of additional or alternative behavioral markers sensitive to AMP is required.

In this sense, the analysis of 50-kHz ultrasonic vocalizations (USVs) emerges as a good candidate, since AMP induces this type of positive affect-related USVs in rats (Burgdorf et al. 2001; Thompson et al. 2006; Wright et al. 2010). High-frequency calls are normally emitted in social and nonsocial rewarding situations such as mating, rough-and-tumble play, during rewarding brain stimulation, and following administration of cocaine and apomorphine (Burgdorf et al. 2000, 2008; Williams and Undieh 2010). Besides their affective character, USVs have a prominent communicative function (Brudzynski 2005; Wöhr et al. 2008) which has motivated the use of USV to model speech/voice impairment in Parkinson disease (Ciucci et al. 2007, 2009), or social communicational deficits in autism (Scattoni et al. 2009; Wöhr et al. 2011). Since hyperactivity, euphoria, and pressured speech are three of the typical mania-like symptoms induced by AMP in humans (Van Kammen and Murphy,1975; Willson et al. 2005), we assume that AMP-induced 50-kHz calls can serve as an unparalleled behavioral marker to model euphoria and pressured speech, which can complement traditional behavioral analysis of locomotor activity. To assess the predictive validity of USV in the AMP model, the antimanic drugs lithium and tamoxifen were used. Tamoxifen is a protein kinase C (PKC) inhibitor and it was used to further evaluate both the predictive validity of AMP-induced appetitive 50-kHz calls and the etiological validity of PKC activity in this model. PKC, a family of enzymes that play a pivotal role in signal transduction, was found to be augmented in BD (Hahn et al. 2005; Yildiz et al. 2008; Zarate et al. 2007). Mood stabilizers such as lithium and valproate partly exert their clinical effects by reducing PKC activity (Hahn et al. 2005; Manji and Lenox 1999). In human trials and in rodent AMP-induced hyperactivity models, the PKC inhibitor tamoxifen has shown antimanic and antimanic-like effects, respectively (Einat et al. 2007; Sabioni et al. 2008; Yildiz et al. 2008; Zarate et al. 2007). In addition to tamoxifen, the new drug myricitrin was also assessed, a natural plant flavonoid extracted from genus *Eugenia*, which has recently shown preclinical antipsychotic-like effects presumably due to the inhibition of PKC and nitric oxide (NO) activity (Meotti et al. 2006; Pereira et al. 2011). We hypothesized that increases in appetitive 50-kHz calls and locomotor activity in AMP-treated rats can be prevented by the well-established drug lithium, the novel antimanic drug tamoxifen, and that these effects may be shared by myricitrin.

Methods and materials

Subjects and general procedure

Ninety-four male Wistar rats (200–210 g; Harlan-Winkelmann, Germany) were used. Animals were group-housed in polycarbonate cages. Lab chow and water (0.0004 % HCl solution) were available ad libitum. Animals were maintained in an animal room with a 12:12 h light/dark cycle (lights on 7–19 h) and a room temperature of 23–29 °C. One week before testing, animals were habituated to the animal facilities and handled and gentled in a routine lab procedure for three consecutive days (5 min each) in order to accustom them to the experimenter and to being manipulated. Thereafter, the initial screening cage test was performed, followed by the final drug testing procedure.

Screening cage test

Since rats show substantial and rather stable inter-individual variability in 50-kHz calls (e.g., Schwarting et al. 2007), we applied a screening test, where rats are tested for their levels of

spontaneous USVs in a clean cage with fresh bedding (Natusch and Schwarting 2010; Schwarting et al. 2007; Wöhr et al. 2008). The cage test was conducted on two consecutive days (5 min each), and according to the number of 50-kHz calls emitted on both days, rats were equally assigned to one of six groups (n=7–8 each) per experiment. In individual rats, call numbers in this test ranged between 9 and 134 calls (experiment 1) and 4 and 102 calls (experiment 2). All subjects were used in these experiments.

Drugs

D-AMP (Sigma, St. Louis, MO, USA) was dissolved in saline and administered intraperitoneally (ip) at a dose of 2.5 mg/kg. Myricitrin (ABCR GmbH & Co.KG, Germany) was suspended in two drops of Tween 80, diluted in saline and administered subcutaneously (sc) at doses of 10 and 30 mg/kg. Lithium carbonate (Sigma) was dissolved in saline and administered sc at 100 mg/kg. Tamoxifen (Sigma) was suspended in two drops of Tween 80, diluted in saline and administered sc at doses of 1 mg/kg. All drugs and their vehicles were administered at a constant volume of 1 ml/kg. All routes of administration and doses were chosen based on our previous reports (Natusch and Schwarting 2010; Pereira et al. 2011; Sabioni et al. 2008).

Open field test

As previously described (Schwarting et al. 2007), open-field activity was automatically monitored under red light (TruScan, Photo beam Sensor-E63-22, Coulbourn Instruments, PA, USA) in two acrylic boxes (40×40×40 cm) equipped with infrared sensor beams that allowed measuring total distance traveled (cm), and an ultrasonic microphone (UltraSoundGate Condenser Microphone CM16; Avisoft Bioacoustics, Berlin, Germany) affixed centrally at 45 cm above the floor of the box. To promote 50-kHz calls, the floors were covered with 1-cm bedding that was changed between subjects (according to Natusch and Schwarting 2010).

Drug-testing procedure

The procedure consisted of three consecutive days, each with 10-min exposures to the open field. The first day was used to habituate the subjects to the testing environment (without data sampling), and the second day was used to habituate them to being injected (0.9 % saline ip 15 min before being test). During this test, locomotion and USV were recorded to verify that the screening cage test had resulted in homogenous groups. On day 3 of experiment 1, rats were first administered with lithium (100 mg/kg sc), tamoxifen (1 mg/kg sc), or saline (0.9 % sc) 25 min before testing, and 15 min later, all groups received a single dose of AMP (D-AMP 2.5 mg/kg or saline, ip). On day 3 of experiment 2, rats received myricitrin (10 or 30 mg/kg sc) or saline (sc) 25 min before testing, and 15 min later all groups were injected with AMP or saline (ip) exactly as in experiment 1. Groups were named according to the combinations of treatments received on day 3 (see Figs. 1, 2, 3, 4).

Ultrasonic vocalizations recording and analysis

As previously reported (Wöhr et al. 2008; Natusch and Schwarting 2010), USV was monitored with an UltraSoundGate Condenser Microphone (CM16; Avisoft Bioacoustics, Berlin, Germany) and recorded with Avisoft Recorder 2.7 software. Experienced observers manually counted the USVs off-line from the spectrograms. All USVs emitted over 33 kHz were considered as 50-kHz calls. If two call elements were at least 0.048 s apart, two independent calls were counted. Based on peak frequency and shape, 50-kHz calls were classified into four different subtypes according to the following criteria: Flat calls: a call was scored when peak frequency changes within a single call element were equal or lower than 5 kHz. However, the difference between start and end peaks could be higher than 5 kHz, that is, calls with a flat shape in either upward or downward direction were also considered as flat calls. Step-calls: When a fundamental flat call had at least one short flat element overlapped at the start and/or at the end of the call. At least one of these short ‘steps’ had to be about 5 kHz higher than the fundamental call. Trills: a single call element either with one peak frequency change higher than 5 kHz or with two or more peak frequency changes in opposed directions at least 5 kHz apart (i.e., zigzag-shaped call). Mixed calls: Frequency-modulated calls (FM) that did not fall within the previous categories of step calls and trills, for example, trills with one or more flat and/or step component, or two or more trills overlapping. Whenever appropriate, step-calls, trills, and mixed calls were summed up and simply referred to as FM calls. Since 22-kHz calls were rarely observed, they were omitted from the analysis. In addition to counting their rate, calls were manually marked by a section label to be included in the automated parameter measurement of the Avisoft SASLabPro software (Wöhr et al. 2008). Then, mean peak frequency, bandwidth, call duration, and call frequency modulation were determined automatically from the average spectrum of the entire element. Peak frequency was defined as the frequency at the location of the peak amplitude, and frequency modulation was computed as the difference between the lowest and the highest peak frequency within each specific call. Bandwidth refers to call thickness measured at the highest amplitude peak.

Statistical analysis

Data were expressed as means±SEM (7–8 rats per group). The following questions were analyzed: First, we verified that

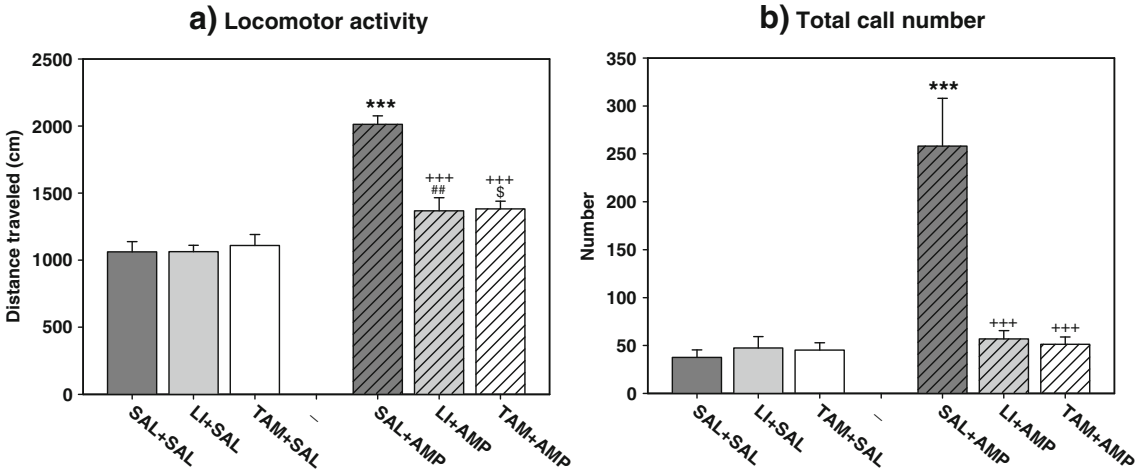

Fig. 1 Experiment 1 - Effects of lithium and tamoxifen on hyperlocomotion and appetitive 50-kHz calls induced by amphetamine. **a** Locomotor activity (distance traveled in cm). **b** Ultrasonic vocalizations (number of 50-kHz calls). Abbreviations: *SAL* 0.9 % saline, *LI* lithium 100 mg/kg, *TAM* tamoxifen 1 mg/kg, *AMP* amphetamine 2.5 mg/kg. *** $p<.001$ vs. SAL+SAL, +++ $p<0.001$ vs. SAL+AMP, ## $p<0.01$ vs. LI+SAL, $ $p<0.05$ vs. TAM+SAL. Data are expressed as mean±SEM (7–8 rats per group)

the screening cage procedure had resulted in homogenous groups. For this purpose, locomotor activity and 50-kHz call numbers measured during the saline test (day 2 of the three-day drug testing procedure) were compared between the later treatment groups by means of one-way ANOVAs. Secondly, we asked whether and how the drug treatments affected locomotion and USV (day 3 of the drug testing procedure), again using one-way ANOVAs. In case of significant differences, LSD-Fisher protected *post hoc* tests (PLSD) were then used to test (A) whether the antimanic drugs alone affected locomotor activity or USV as compared to saline, and (B) whether AMP (group SAL+AMP) led to the expected enhancement in locomotor activity and 50-kHz calls as compared to saline (group SAL+SAL), and whether the antimanic drugs (given drug+AMP group) were able to reduce the effects of AMP (group SAL+AMP) or even normalize them as compared to their saline controls (given drug+SAL group). Call features after drug treatments were analyzed in a similar manner. Statistical significance was defined as $p<0.05$.

Results

Experiment 1

The saline test (second day of drug testing procedure) did not yield indications for differences between the later treatment groups (p values>.05; data not shown) indicating that the

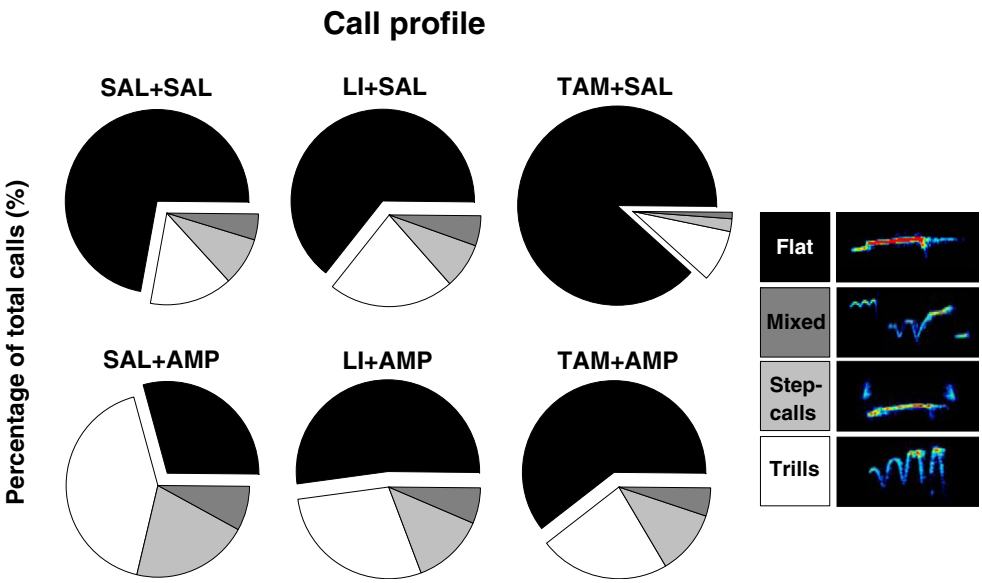

Fig. 2 Experiment 1 - Call profile of 50-kHz calls. *Left*, each area represents the number of calls of a given subtype, expressed as the percentage of all 50-kHz calls. *Right*, exemplary sonograms of the four call subtypes analyzed. Abbreviations: *SAL* 0.9 % saline, *LI* lithium 100 mg/kg, *TAM* tamoxifen 1 mg/kg, *AMP* amphetamine mg/kg. In undrugged animals, flat calls predominated. Amphetamine led to a decrease in flat calls and increases in step-calls and trills, which were not normalized by pretreatment with lithium but with tamoxifen. For statistical details, see text

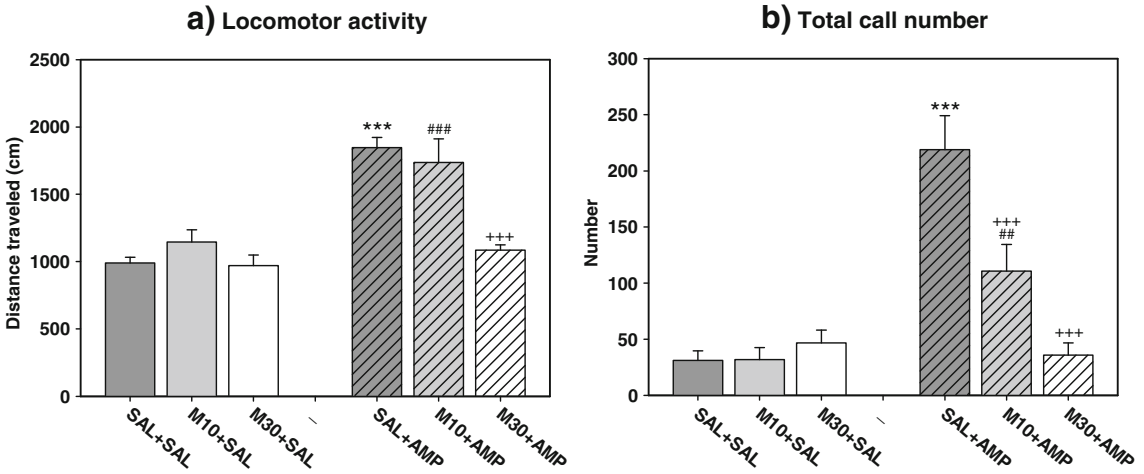

Fig. 3 Experiment 2 - Effects of myricitrin on hyperlocomotion and appetitive 50-kHz calls induced by AMP. **a** Locomotor activity (distance traveled in cm). **b** Ultrasonic vocalizations (number of 50-kHz calls). Abbreviations: *SAL* 0.9 % saline, *M10* myricitrin 10 mg/kg, *M30* myricitirn 30 mg/kg, *AMP* amphetamine 2.5 mg/kg. *** $p<.001$ vs. SAL+SAL, +++ $p<0.001$ vs. SAL+AMP, ### $p<0.001$, ## $p<0.01$ vs. M10+SAL. Data are expressed as mean±SEM (7–8 rats per group)

group assignment based on the initial screening cage test had led to homogenous groups.

Lithium- and tamoxifen-reduced hyperlocomotion induced by AMP Overall, there was a significant difference in locomotor activity between groups ($F_{5,41}=24.93$, $p<.001$; Fig. 1a). The post-hoc tests showed that AMP (SAL+AMP) led to the expected enhancement in locomotion (vs. SAL+SAL: $p<.001$). This effect that was largely reduced but not completely abolished by lithium and tamoxifen, since locomotor activity was lower as compared to SAL+AMP (vs. LI+AMP: $p<.001$; vs. TAM+AMP: $p<.001$), but was still higher in both cases as compared to respective controls (LI+AMP vs. LI+SAL: $p=.005$; TAM+AMP vs. TAM+SAL: $p=.014$). There were no differences between lithium (LI+SAL) or tamoxifen (TAM+SAL) as compared to saline (SAL+SAL: all p values>.05), indicating that these drug treatments alone did not affect spontaneous locomotion.

Lithium and tamoxifen blocked AMP-induced appetitive 50-kHz calls. Similar to locomotion, there was an overall group difference in the numbers of 50-kHz calls ($F_{5,41}=15.08$, $p<.001$; Fig. 1b). The post-hoc tests showed that AMP (SAL+AMP) led to the expected enhancement in 50-kHz call rates (vs. SAL+SAL: $p<.001$), which ranged between 13 and 436 calls between subjects. This effect was completely

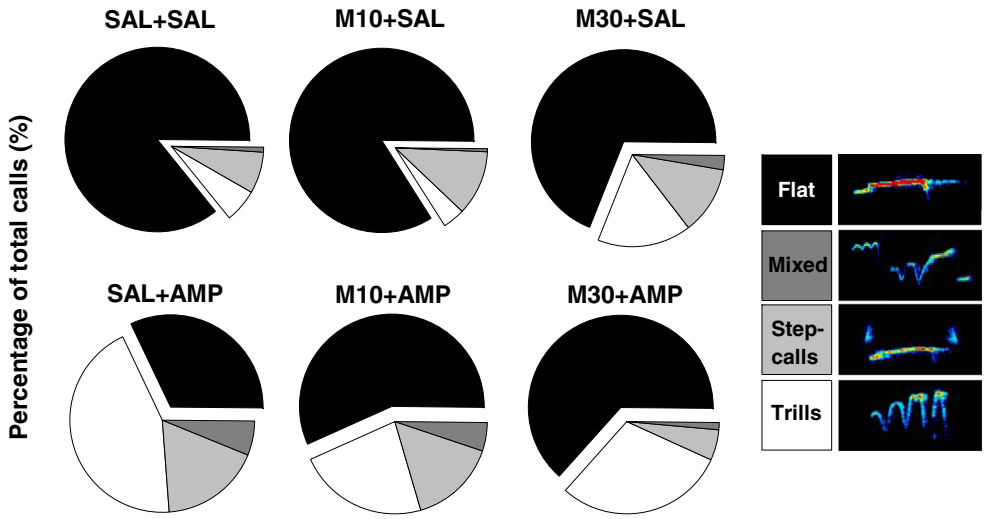

Fig. 4 Experiment 2 - Call profile of 50-kHz calls. *Left*, each area represents the number of calls of a given subtype, expressed as the percentage of all 50-kHz calls. *Right*, exemplary sonograms of the four call subtypes analyzed. Abbreviations: *SAL* 0.9 % saline, *M10* myricitrin 10 mg/kg, *M30* myricitrin 30 mg/kg, *AMP* amphetamine 2.5 mg/kg. Data are expressed as mean±SEM (7–8 rats per group). Amphetamine led to a decrease in flat calls, and increases in and trills, which were partly normalized by pretreatment with myricitrin. For statistical details see text

abolished by lithium and tamoxifen since call rate was lower as compared to SAL+AMP (vs. LI+AMP: $p<.001$; vs. TAM+AMP: $p<.001$), and did not differ from respective controls (LI+AMP vs. LI+SAL; TAM+AMP vs. TAM+SAL, p values>.05). There were no differences between lithium (LI+SAL) or tamoxifen (TAM+SAL) as compared to saline (SAL+SAL: all p values>.05), indicating that these drug treatments did not affect spontaneous call rates.

Lithium and tamoxifen reversed the increase in frequency-modulated calls induced by AMP Besides total call numbers, the drug treatments also affected the emission of call subtypes: AMP (SAL+AMP) increased the number of trills ($F_{5,41}$= 8.121, $p<.001$) and mixed calls ($F_{5,41}$=5.388, p=0.001) compared to saline-treated groups (i.e., SAL+SAL, LI+SAL and TAM+SAL; LSD, $p<0.05$) (Table 1). Both lithium and tamoxifen (LI+AMP, TAM+AMP) significantly reduced the number of trills and mixed calls (LSD, $p<0.05$) in a way that calling in these groups no longer differed from saline controls (i.e., SAL+SAL, LI+SAL, TAM+SAL; LSD, $p>0.05$). When given alone, lithium and tamoxifen did not affect call subtypes.

Besides absolute call numbers (see Table 1), the drug treatments also affected the percentages of 50-kHz call subtypes (Fig. 2), that is, flat calls ($F_{5,41}$=5.30, p=.001), step calls ($F_{5,41}$=4.38, p=.003), and trills ($F_{5,41}$=2.74, p= 0.03), but not mixed calls ($p>.05$). Thus, in saline-treated rats (SAL+SAL), the majority of calls emitted was of the flat subtype (~72 %), followed by trills (~15 %), step (~9 %), and mixed calls (~4 %). These patterns were not affected by lithium (LI+SAL) or tamoxifen (TAM+SAL) as compared to SAL+SAL (all p values>.05). AMP (SAL+AMP), in contrast, decreased the percentage of flat calls (compared to SAL+SAL: p=.001), and increased the percentages of step calls (p=.006) and trills (p=.009), but not mixed calls ($p>.05$). Lithium (LI+AMP) was not effective to normalize these AMP-induced (SAL+AMP) effects since the call percentages did not differ significantly between the two groups (p values>.05). Tamoxifen (TAM+AMP), on the other hand, partly prevented the decrease in the percentage of flat calls (p=.018), and the increase in the percentage of step calls (p=.038), but not trills or mixed call (p values>.05).

To complement the description of these call profiles, quantitative USV parameters were analyzed, which yielded group differences in case of peak frequency modulation ($F_{5,40}$=2.86, p=.029; see Table 2), but not mean peak frequency, call duration, and bandwidth (p values>.05). Post-hoc tests showed that AMP (SAL+AMP) increased peak frequency modulation by about 40 % as compared to saline (SAL+SAL, p=.015), an effect which was prevented by lithium (SAL+AMP vs. LI+AMP: p=.038) and tamoxifen (SAL+AMP vs. TAM+AMP, p=.033). Lithium (LI+SAL, p=.997) and tamoxifen alone had no effect on peak frequency modulation as compared to SAL+SAL (p values>.05).

Experiment 2

Again, the saline test (second day of drug testing procedure) did not yield indications for differences between the later treatment groups (p values>.05; data not shown).

The high dose of myricitrin attenuated AMP-induced hyperlocomotion Overall, there was a significant difference in locomotor activity between groups ($F_{5,40}$=29.67, $p<.001$; Fig. 3a). The post-hoc tests showed that AMP (SAL+AMP) again led to enhanced locomotion (vs. SAL+SAL: $p<.001$). This effect was not affected by the lower dose of myricitrin (M10+AMP), since locomotor activity did not differ from AMP+SAL ($p>.05$) but was higher as compared to M10+SAL ($p<.001$), whereas AMP-induced locomotion was reduced by the higher dose (M30+AMP: $p<.001$; as compared to SAL+AMP), which also did not differ from its respective control ($p>.05$). There were no differences between M10+SAL or M30+SAL compared to saline (SAL+SAL: all p values>.05), indicating that these drug treatments alone did not affect spontaneous locomotion.

Myricitrin dose-dependently reduced appetitive 50-kHz calls induced by AMP Again, there was an overall difference in the numbers of 50-kHz calls between groups ($F_{5,40}$=18.71, $p<.001$; Fig. 3b). The post-hoc tests verified that AMP

Table 1 Call subtypes - experiment 1

	SAL+SAL	LI+SAL	TAM+SAL	SAL+AMP	LI+AMP	TAM+AMP
Flat calls	23.87±3.67	30.00±9.06	38.62±5.97	64.00±14.81	27.25±6.69	30.14±7.95
Step-calls	5.00±2.49	4.75±2.38	1.50±1.00	55.50±14.82	7.75±2.04	5.86±2.51
Trills	6.75±3.00	9.37±4.04	4.25±2.18	117.87±36.40*	17.62±6.15^{+}	12.57±4.36^{+}
Mixed calls	2.00±0.73	2.00±0.57	0.75±0.62	20.62±7.61*	4.37±1.59^{+}	2.57±0.57^{+}

The analysis of call subtypes was restricted to the last testing day when the experimental treatments, including amphetamine were administered. Data are expressed as mean±SEM (n=7–8 rats per group) of call number per min. *SAL* 0.9 % saline, *LI* lithium 100 mg/kg, *TAM* tamoxifen 1 mg/kg, *AMP* amphetamine 2.5 mg/kg. * $p<.05$ vs. SAL+SAL, LI+SAL, Tam+SAL; + $p<0.05$ vs. SAL+AMP

Table 2 Quantitative USV parameters - experiment 1

	SAL+SAL	LI+SAL	TAM+SAL	SAL+AMP	LI+AMP	TAM+AMP
Peak frequency modul.	8.1±1.3	8.1±1.1	6.0±1.6	12.7±1.2*	9.2±1.2^{+}	8.9±1.2^{+}
Mean peak frequency	57.7±2.5	57.3±2.2	58.0±3.0	57.3±2.2	57.3±2.2	56.5±2.3
Call bandwidth	3.2±0.2	2.9±0.3	3.1±0.2	3.2±0.2	3.0±0.2	3.1±0.2
Call duration	25.3±2.7	29.0±4.3	28.6±4.9	32.7±2.7	27.0±1.9	22.0±2.5

The analysis of the different call features was restricted to the last testing day when the experimental treatments, including amphetamine, were administered. USV parameters were computed including all 50-kHz calls subtypes. Peak frequency modulation, mean peak frequency, and mean call bandwidth values are given in kHz, whereas mean call duration is given in milliseconds. Data are expressed as mean±SEM (n=7–8 rats per group). *SAL* 0.9 % saline, *LI* lithium 100 mg/kg, *TAM* tamoxifen 1 mg/kg, *AMP* amphetamine 2.5 mg/kg. * p<.05 vs. SAL+SAL, + p<0.05 vs. SAL+AMP

(SAL+AMP) again led to enhanced 50-kHz call rates (vs. SAL+SAL: p<.001), which ranged between 2 and 339 calls between subjects. This effect was partly reduced by the lower dose of myricitrin (M10+SAL), which led to lower call rates than SAL+AMP (p<.001) but still higher ones than SAL+SAL controls (p=.002). The higher dose of myricitrin (M30+AMP) led to a complete prevention of the AMP effect, since call rates were lower than those of the SAL-AMP group (p<.001), and did not differ from saline controls (p>.05). There were no differences between M10+SAL or M30+SAL as compared to saline (SAL+SAL, all p values>.05), indicating that these drug doses alone did not affect spontaneous call rates.

Besides absolute call numbers (see Table 3), the drug treatments also affected the percentages of 50-kHz call subtypes (Fig. 4), that is, flat calls ($F_{5,40}$=5.96, p=.001), trills ($F_{5,40}$=5.15, p=.001), and mixed calls ($F_{5,40}$=4.68, p=.002), but not step calls (p>.05). AMP (SAL+AMP) again decreased the percentage of flat calls (compared to SAL+SAL: p=.001), and increased the percentages of trills (p=.001) and mixed calls (p=.001). The lower dose of myricitrin (M10+AMP) partly normalized the AMP-induced effect, since the percentage of flat calls was higher than of the SAL+AMP group (p=.038) but still lower than of the SAL+SAL group (p=.013). In case of trills, however, the percentage in the M10+AMP group was lower than that in the SAL+AMP group (p=.038), without differing from the SAL+SAL group (p>.05). In contrast, in the M10+AMP group, the relative number of mixed calls was similar to that of the SAL+AMP group (p>.05) and higher than that in the SAL+SAL group (p=.009). A largely similar pattern was obtained with the higher dose of myricitrin: the percentage of flat calls was higher than of the SAL+AMP group (p=.009) but still lower than that of the SAL+SAL group (p=.044). In case of trills, the percentage in the M30+AMP group did not differ from that of the SAL+AMP group (p>.05) and was higher than that of the SAL+SAL group (p=.012). The percentages of mixed calls, in contrast, were significantly reduced in the M30+AMP group compared with both the M10+AMP group (p=.017) and the SAL+AMP group (p=.003) and did not differ from the SAL+SAL group (p>.05).

The quantitative analysis of USV parameters again yielded group differences in case of peak frequency modulation ($F_{5,40}$=7.21, p<.001; see Table 4), but not mean peak frequency, call duration, and bandwidth (p>.05). Post-hoc tests showed that AMP (SAL+AMP) increased peak frequency modulation compared to saline (SAL+SAL: p<.001), an effect which was reduced by both doses of myricitrin (M10+AMP: p=.025; M30+AMP: p=.001). Neither doses alone affected peak frequency modulation as compared to SAL+SAL (p values>.05).

Discussion

All behavioral models aimed to mimic manic episodes have in common the lack of a genuine affective parameter. The present study was therefore designed to measure the utility of appetitive 50-kHz USV as a genuine affective-communicational target in the well-established AMP-induced hyperlocomotion model of mania. Our results demonstrate for the first time that drugs with known specific or potential antimanic activity were all effectively able in reversing AMP-induced appetitive 50-kHz calls, besides diminishing locomotor activity. The predictive validity of USV in this model was assessed by administrating the antimanic drugs lithium and tamoxifen. As it is known for hyperactivity (Gould et al. 2007; Sabioni et al. 2008), lithium and tamoxifen demonstrated to be effective in preventing AMP-induced USV at doses that on their own did not affect either spontaneous calling or locomotor activity.

Clinical and preclinical studies have implicated PKC in both the pathophysiology of BD and the biochemical effects of mood stabilizers such as lithium and valproate (Hahn et al. 2005; Manji and Lenox 1999; Yildiz et al. 2008). Evidence from our lab (Dr. Andreatini) and others has demonstrated that the inhibition of PKC reverses AMP-induced hyperactivity in mice and rats (Einat et al. 2007; Sabioni et al. 2008). Here, we further evaluated the predictive validity of USV in the AMP

Table 3 Call subtypes - experiment 2

	SAL+SAL	M10+SAL	M30+SAL	SAL+AMP	M10+AMP	M30+AMP
Flat calls	24.87±5.37	25.00±7.20	32.75±8.18	68.29±12.84	57.29±12.00	15.62±5.01
Step calls	3.37±1.57	4.50±2.88	5.62±1.76	38.14±6.25	16.00±3.88	3.25±1.93
Trills	2.50±1.52	1.87±1.26	7.25±2.14	98.43±23.56*	31.29±17.10	16.37±8.09
Mixed calls	0.50±0.50	0.12±0.12	1.37±0.71	14.00±4.33*	6.14±2.00*	0.62±0.32

The analysis of call subtypes was restricted to the last testing day when the experimental treatments, including amphetamine, were administered. Data are expressed as mean±SEM (n=7–8 rats per group) of call number per min. *SAL* 0.9 % saline, *M10* myricitrin 10 mg/kg, *M30* myricitrin 30 mg/kg, *AMP* amphetamine 2.5 mg/kg. * p<.05 vs. SAL+SAL, M10+SAL, M310+SAL

model showing that prior administration of the PKC-inhibitor tamoxifen effectively prevented AMP-induced appetitive 50-kHz calls, besides attenuating hyperlocomotion in agreement with previous reports (Einat et al. 2007; Pereira et al. 2011; Sabioni et al. 2008). Also, tamoxifen did not affect basal USV and locomotor activity. Tamoxifen showed similar effects as lithium in both parameters, indicating that the doses selected here were equipotent in this model. In addition, the efficacy of tamoxifen in comparison with lithium supports the potential of tamoxifen as an antimanic treatment in humans (Yildiz et al. 2008; Zarate et al. 2007) and highlights the role of PKC activity in mania-like symptoms, at least in those mimicked by AMP.

It was previously shown that haloperidol also blocked the AMP-induced increases in 50-kHz USV, which would increase predictive validity of the model. However, other antipsychotics used to treat mania (e.g., risperidone) exerted this effect at a dose that also impaired 50-kHz USV after saline administration (Wright et al. 2013). However, these authors used a different protocol (e.g., previous AMP challenge to subject selection) that could influence the results. Moreover, antipsychotics also have other clinical effects compared with the antimanic one, which may make its use difficult as a unique class of drug for pharmacological validation of animal models of mania. In this line, lithium and tamoxifen appeared as good tools.

Although mood stabilizers and antipsychotic drugs have been successfully used to treat acute mania, many patients do not respond adequately to these drugs or fail to tolerate them (Nierenberg 2010; Gitlin 2006). Moreover, tamoxifen used for breast cancer has important side effects (Amir et al. 2011). Thus, the development of new therapies is needed. In this regard, one drug with putative antimanic properties was tested: myricitrin. This drug reduced AMP-induced 50-kHz calls in a dose-dependent way, whereas only the higher dose was able to reduce AMP-induced hyperlocomotion. None of the doses affected spontaneous calling or basal locomotor activity. The high dose of myricitrin showed equivalent effects compared with lithium and tamoxifen, supporting the predictive validity of myricitrin in this model. Myricitrin has recently shown antipsychotic-like effects (Pereira et al. 2011) which supports the promising profile of this drug in attenuating both mania and psychotic-like symptoms, probably due to its dual inhibitory action upon PKC and NO activity (Meotti et al. 2006; Pereira et al. 2011). In general, data from both experiments revealed that USV were more sensitive than locomotor activity to the effects of both AMP and antimanic drugs, providing a wider dose range for drug detection as well.

USV are complex signals, which can be classified into different subtypes, often clustered into two general categories of flat and FM calls (Burgdorf et al. 2008; Wright et al. 2010). Out of these, flat calls may serve a social communicative function (Schwarting et al. 2007), while FM calls, including step-calls and trills (Wöhr et al. 2008), seem to signal a dopamine (DA)-dependent affective state in the rat (Burgdorf et al. 2008; Wright et al. 2010). As AMP and

Table 4 Quantitative USV parameters - experiment 2

	SAL+SAL	M10+SAL	M30+SAL	SAL+AMP	M10+AMP	M30+AMP
Peak frequency modul.	5.8±0.7	6.1±0.9	7.2±0.8	13.0±0.9***	9.6±1.3^{+}	7.7±1.3^{++}
Mean peak frequency	54.4±2.0	53.4±0.8	55.5±1.9	55.9±0.7	55.1±1.1	57.4±1.8
Call bandwidth	3.1±0.2	3.1±0.2	3.0±0.2	2.9±0.1	3.3±0.3	3.4±0.3
Call duration	23.9±4.6	21.0±2.4	25.9±2.5	30.4±1.6	25.4±3.7	23.0±3.6

The analysis of the different call features was restricted to the last testing day when the experimental treatments, including amphetamine, were administered. USV parameters were computed including all 50-kHz calls subtypes. Peak frequency modulation, mean peak frequency, and mean call bandwidth values are given in kHz, whereas mean call duration is given in milliseconds. Data are expressed as mean±SEM (n=7–8 rats per group). SAL (0.9 % saline), M10 (myricitrin 10 mg/kg), M30 (myricitrin 30 mg/kg), AMP (amphetamine 2.5 mg/kg). *** p<.001 vs. SAL+SAL, + p<0.05 vs. SAL+AMP, ++ p<0.01 vs. SAL+AMP

cocaine increase the relative number of FM calls (Williams and Undieh 2010; Wright et al. 2010, 2012), a shift in the call profile has been taken as indicative of a high positive affective state provoked by these euphorigenic drugs (Fig. 4; Wright et al. 2010, 2012). We found that AMP preferentially increased the percentage of FM calls over those with a flat frequency, whereas lithium, tamoxifen, and myricitrin restored the AMP-induced shift in call profiles reducing the percentage of FM calls, especially the trill subtype. It has been suggested that only FM calls reflect appetitive behavior, reward and positive affect, out of which the trills are the most consistent and recurrent FM call types obtained in those situations (Burgdorf et al. 2008; Wright et al. 2010, 2012). Although all drugs exhibited a very similar effect on AMP-induced call rate, tamoxifen showed a larger reduction of FM calls, suggesting a putative role of PKC signaling in spontaneous and reward-induced appetitive 50-kHz calls. In the case of myricitrin, both doses were almost equally effective in reversing the AMP-induced shift in call profiles. The measurement of quantitative USV parameters confirmed that all treatments did reduce the AMP-induced increase in frequency-modulation of the 50-kHz calls without affecting their mean peak frequency, which is considered the most distinctive communicational call element (Brudzynski 2005). Previously, we found that playback of artificial 50-kHz sine waves calls without any frequency modulation is equally effective as playback of natural 50-kHz calls in inducing social approach behavior (Wöhr and Schwarting 2007). Thus, it is likely that in our experiment, 50-kHz calls may have retained their normal communicative function even under the effect of the antimanic drugs.

Pharmacological and lesion studies have repeatedly shown that the mesolimbic DA system is critically involved in the production of 50-kHz calls, especially the FM subtype observed in appetitive situations (Burgdorf et al. 2001; Ciucci et al. 2009; Williams and Undieh 2010). Acute or repeated administration of AMP, either systemically or directly into the nucleus accumbens, elicits 50-kHz USVs that are blocked by DA and norepinephrine antagonists (Ahrens et al. 2009; Thompson et al. 2006; Wright et al. 2012). On the other hand, PKC signaling mediates endogenous and psychostimulants-induced DA release and hyperactivity, effects that can be reduced by the administration of PKC inhibitors including tamoxifen (Browman et al. 1998; Cowell et al. 2000; Einat et al. 2007; Giambalvo 1992; Kantor and Gnegy 1998; Steketee 1993). Since lithium, tamoxifen, and myricitrin have in common the ability to directly or indirectly inhibit PKC signaling (Manji and Lenox 1999; Meotti et al. 2006; Pereira et al. 2011) the reduction in AMP-induced hyperlocomotion and 50-kHz calls may be the consequence of PKC-inhibition on DA, probably by reducing DA transporter availability (Giambalvo 1992; Kantor and Gnegy 1998; Kantor et al. 2001).

In conclusion, lithium attenuation of psychostimulant-induced hyperlocomotion has been used both to study the therapeutic action of mood stabilizers and to develop novel lithium-mimetic drugs (Gould et al. 2007). In our present model, appetitive USVs seem to provide a more sensitive parameter than traditional psychomotor parameters, which might offer a new avenue for drug testing and dose-response studies including drugs with different sensitivities and profiles. The analysis of the USV subtypes provided a unique kind of information regarding the affective state of the rat which might not be otherwise obtained. We suggest, therefore, that AMP-induced appetitive 50-kHz calls might constitute a genuine affective marker useful to model exaggerated euphoric mood and pressured speech, with both good face and predictive validity. The antimanic-like effects shown by lithium and tamoxifen, two clinically effective antimanic drugs, support the predictive validity of the model. Furthermore, the effects of tamoxifen and myricitrin in this model highlight the role of PKC signaling as a promising pharmacological target to treat mania and psychosis-related disorders. Although the present results provide substantial evidence that analyzing drug effects on AMP-induced 50-kHz calls may provide an interesting and new model for mania, more tests are necessary in the future for further validation. Among others, these should comprise wider dose ranges of the drugs (especially lithium) used here, extended drug testing periods, the analysis of repeated drug treatments (both, in case of AMP and of the antimanic drugs to be tested), other psycho-stimulant challenges (like methylphenidate), and the test of other drugs with anti-manic properties, or drugs which lack such properties.

Acknowledgements This work was supported by the grant Schw 559/10-1 from the Deutsche Forschungsgemeinschaft. M. Pereira was funded by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil). R. Andreatini received a researcher fellowship from CNPq. JC Brenes was funded by Deutscher Akademischer Austauschdienst (DAAD, Germany), and the University of Costa Rica.

Conflict of interest The authors declare no conflicts of interest.

References

Ahrens AM, Ma ST, Maier EY, Duvauchelle CL, Schallert T (2009) Repeated intravenous amphetamine exposure: rapid and persistent sensitization of 50-kHz ultrasonic trill calls in rats. Behav Brain Res 197:205–209

Amir E, Seruga B, Niraula S, Carlsson L, Ocaña A (2011) Toxicity of adjuvant endocrine therapy in postmenopausal breast cancer patients: a systematic review and meta-analysis. J Natl Cancer Inst 103:1299–1309

Belmaker RH (2004) Bipolar disorder. N Engl J Med 351:476–486

Browman KE, Kantor L, Richardson S, Badiani A, Robinson TE, Gnegy ME (1998) Injection of the protein kinase C inhibitor Ro31-8220 into the nucleus accumbens attenuates the acute response to amphetamine: tissue and behavioral studies. Brain Res 814:112–119

Brudzynski SM (2005) Principles of rat communication: quantitative parameters of ultrasonic calls in rats. Behav Genet 35:85–92

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, Ikemoto S (2001) Nucleus accumbens amphetamine microinjections unconditionally elicit 50 kHz ultrasonic vocalizations in rats. Behav Neurosci 115:940–944

Burgdorf J, Kroes RA, Moskal JR, Pfaus JG, Brudzynski SM, Panksepp J (2008) Ultrasonic vocalizations of rats (*Rattus norvegicus*) during mating, play, and aggression: behavioral concomitants, relationship to reward, and self-administration of playback. J Comp Psychol 122: 357–367

Ciucci MR, Ma ST, Fox C, Kane JR, Ramig L, Schallert T (2007) Qualitative changes in ultrasonic vocalization in rats after unilateral dopamine depletion or haloperidol. Behav Brain Res 182:284–289

Ciucci MR, Ahrens AM, Ma ST, Kane J, Cormack L, Schallert T (2009) Reduction of dopamine synaptic activity: degradation of 50-kHz ultrasonic vocalization in rats. Behav Neurosci 123:328–336

Cowell RM, Kantor L, Hewlett GH, Frey KA, Gnegy ME (2000) Dopamine transporter antagonists block phorbol ester-induced dopamine release and dopamine transporter phosphorylation in striatal synaptosomes. Eur J Pharmacol 389:59–65

Einat H, Yuan P, Szabo ST, Dogra S, Manji HK (2007) Protein kinase C inhibition by tamoxifen antagonizes manic-like behavior in rats: Implications for the development of novel therapeutics for bipolar disorder. Neuropsychobiology 55:123–131

Friedman JN, Hurley RA, Taber KH (2006) Bipolar disorder: imaging state vs. trait. J Neuropsychiatry Clin Neurosci 18:296–301

Giambalvo CT (1992) Protein kinase C and dopamine transport – 1. Effects of amphetamine in vivo. Neuropharmacology 31:1201–1210

Gitlin M (2006) Treatment-resistant bipolar disorder. Mol Psychiatry 11: 227–240

Gould TD, O'Donnell KC, Picchini AM, Manji HK (2007) Strain differences in lithium attenuation of d-amphetamine-induced hyperlocomotion: a mouse model for the genetics of clinical response to lithium. Neuropsychopharmacology 32:1321–1333

Grønli JT, Murison R, Fiske E, Bjorvatn B, Sørensen E, Portas CM et al (2005) Effects of chronic mild stress on sexual behavior, locomotor activity and consumption of sucrose and saccharine solutions. Physiol Behav 84:571–577

Hahn CG, Umapathy HY, Wang R, Koneru DF, Friedman LE (2005) Lithium and valproic acid treatments reduce PKC activation and receptor-G protein coupling in platelets of bipolar manic patients. J Psychiatr Res 39:355–363

Harrison-Read PE (2009) Models of mania and antimanic drug actions: progressing the endophenotype approach. J Psychopharmacol (Oxf) 23:334–337

Kantor L, Gnegy ME (1998) Protein kinase C inhibitors block amphetamine-mediated dopamine release in rat striatal slices. J Pharmacol Exp Ther 284:592–598

Kantor L, Hewlett GH, Park YH, Richardson-Burns SM, Mellon MJ, Gnegy ME (2001) Protein kinase C and intracellular calcium are required for amphetamine-mediated dopamine release via the norepinephrine transporter in undifferentiated PC12 cells. J Pharmacol Exp Ther 297:1016–1024

Manji HK, Lenox RH (1999) Protein kinase C signaling in the brain: molecular transduction of mood stabilization in the treatment of manic-depressive illness. Biol Psychiatry 46:1328–1351

Meotti FC, Luiz AP, Pizzollati MG, Kassuya CAL, Calixto JB, Santos ARS (2006) Analysis of the antinociceptive effect of the flavonoid myricitrin: evidence for a role of the L-arginine-nitric oxide and protein kinase C pathways. J Pharmacol Exp Ther 316:789–796

Natusch C, Schwarting RKW (2010) Using bedding in a test environment critically affects 50-kHz ultrasonic vocalizations in laboratory rats. Pharmacol Biochem Behav 96:251–259

Nierenberg AA (2010) A critical appraisal of treatments for bipolar disorder. Prim Care Companion J Clin Psychiatry 12:23–29

Pereira M, Siba IP, Chioca LR, Pizzolatti MG, Correia D, Vital MABF et al (2011) Myricitrin, a nitric oxide and protein kinase C inhibitor, exerts antipsychotic-like effects in animal models. Prog Neuropsychopharmacol Biol Psych 35:1636–1644

Sabioni P, Baretta IP, Ninomiya EM, Gustafson L, Rodrigues ALS, Andreatini R (2008) The antimanic-like effect of tamoxifen: behavioural comparison with other PKC-inhibiting and antiestrogenic drugs. Prog Neuropsychopharmacol Biol Psych 32:1927–1931

Scattoni ML, Crawley J, Ricceri L (2009) Ultrasonic vocalizations: a tool for behavioural phenotyping of mouse models of neurodevelopmental disorders. Neurosci Biobehav Rev 33:508–515

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

Smith R, Daves JM (1977) Comparative effect of d-amphetamine, l-amphetamine and methylphenidate on mood in man. Psychopharmacology (Berl) 53:1–12

Song C, Leonard BE (2005) The olfactory bulbectomised rat as a model of depression. Neurosci Biobehav Rev 29:627–647

Steketee JD (1993) Injection of the protein kinase inhibitor H7 into the A10 dopamine region blocks the acute responses to cocaine: behavioral and in vivo microdialysis studies. Neuropharmacology 32: 1289–1297

Thompson B, Leonard KC, Brudzynski SM (2006) Amphetamine-induced 50 kHz calls from rat nucleus accumbens: a quantitative mapping study and acoustic analysis. Behav Brain Res 168:64–73

Van Kammen DP, Murphy DL (1975) Attenuation of the euphoriant and activating effects of d- and l-amphetamine by lithium carbonate treatment. Psychopharmacologia 44:215–224

Williams SN, Undieh AS (2010) Brain-derived neurotrophic factor signaling modulates cocaine induction of reward-associated ultrasonic vocalization in rats. J Pharmacol Exp Ther 332:463–468

Willson MC, Bell EC, Dave S, Asghar SJ, McGrath BM, Silverstone PH (2005) Valproate attenuates dextroamphetamine-induced subjective changes more than lithium. Eur Neuropsychopharmacol 15:633–639

Wöhr M, Schwarting RKW (2007) Ultrasonic communication in rats: can playback of 50-kHz calls induce approach behavior? PLoS ONE 2: e1365

Wöhr M, Houx B, Schwarting RKW, Spruijt B (2008) Effects of experience and context on 50-kHz vocalizations in rats. Physiol Behav 93:766–776

Wöhr M, Roullet FI, Hung AY, Sheng M, Crawley JN (2011) Communication impairments in mice lacking Shank1: reduced levels of ultrasonic vocalizations and scent marking behavior. PLoS ONE 6:e20631

Wright JM, Gourdon JC, 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 (Berl) 211:1–13

Wright JM, Dobosiewicz MRS, Clarke PBS (2012) α- and β-Adrenergic receptors differentially modulate the emission of spontaneous and amphetamine-induced 50-kHz ultrasonic vocalizations in adult rats. Neuropsychopharmacology 37:808–821

Wright JM, Dobosiewicz MR, Clarke PB (2013) The role of dopaminergic transmission through D1-like and D2-like receptors in amphetamine-induced rat ultrasonic vocalizations. Psychopharmacology (Berl) 225:853–868

Yildiz A, Guleryuz S, Ankerst DP, Ongur D, Renshaw PF (2008) Protein kinase C inhibition in the treatment of mania. A double blind, placebo-controlled trial of tamoxifen. Arch Gen Psychiatry 65: 255–263

Young JW, Minassian A, Paulus MP, Geyer MA, Perry W (2007) A reverse-translational approach to bipolar disorder: rodent and human

studies in the Behavioral Pattern Monitor. Neurosci Biobehav Rev 31:882–896

Young JW, Henry BL, Geyer MA (2011) Predictive animal models of mania: hits, misses and future directions. Br J Pharmacol 164:1263–1284

Zarate CA Jr, Singh JB, Carlson FJ, Quiroz J, Jolkovsky L, Luckenbaugh DA et al (2007) Efficacy of a protein kinase C inhibitor (tamoxifen) in the treatment of acute mania: a pilot study. Bipolar Disord 9:561–570