Identification and pharmacological characterization of multiple call categories within the rich repertoire of adult rat 50-kHz ultrasonic vocalizations

Jennifer M. Wright

Department of Pharmacology and Therapeutics
McGill University, Montreal, Canada

August 2013

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy

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ABSTRACT

Rats communicate extensively in the ultrasonic frequency range; ultrasonic vocalizations (USVs) occurring at 22 kHz serve an alerting function, whereas those occurring around 50 kHz have been proposed to reflect a state of positive affect. However, 50-kHz calls are acoustically heterogeneous and, not surprisingly, they also seem to differ in their behavioural significance.

The first aims of this thesis were to identify subtypes of 50-kHz USVs, and determine how drug or social context alters their absolute and relative prevalence. These aims were addressed in Chapter 2, in which USVs emitted by adult rats were examined following amphetamine (AMPH) administration. A thorough analysis of over 20,000 USVs revealed that 50-kHz calls are comprised of at least 14 distinct call categories. Rat pair-testing and AMPH administration promoted the overall rate of calling in the 50 kHz range, but importantly, certain call subtypes were also preferentially enhanced or suppressed by these experimental conditions. Overall, the findings of Chapter 2 demonstrate that adult rat 50-kHz calls are considerably more heterogeneous than previously described. The first detailed classification of these calls is provided, laying a foundation for future work.

AMPH promotes both dopamine (DA) and noradrenaline (NA) release; however, a possible noradrenergic (NAergic) role in the production of 50-kHz USVs in unstressed adult rats had not been investigated. Therefore, the second aim of this thesis was to test the hypothesis that NA contributes to the emission of 50-kHz USVs, possibly in a call subtype-selective manner. In Chapter 3, spontaneous and AMPH-induced 50-kHz USVs were examined following various systemic pretreatments targeting the NAergic system. The

findings of Chapter 3 provide the first evidence of NAergic involvement in the elicitation of adult rat USVs by AMPH, showing that 50-kHz calls are differentially associated with α1 and β receptor mechanisms. They also highlight the importance of detailed call subtype analysis and suggest that NAergic contributions to psychostimulant euphoria may warrant further investigation.

Opiate agonists form a major class of euphorigenic drugs, but it was unclear whether they affect 50-kHz USVs in a similar way to psychostimulants. Therefore, the third aim of this thesis was to determine the effects of rewarding and non-sedative doses of morphine on the 50-kHz call rate and profile (Chapter 4). Acute morphine administration consistently failed to promote 50-kHz calling or alter the call profile under a variety of experimental conditions. The findings of Chapter 4 show that even if 50-kHz calls, or certain call subtypes in particular, reflect hedonia in some contexts, this is not the case after acute systemic morphine administration. These results encourage caution when appraising the significance of 50-kHz calls.

Finally, while the rate-enhancing and call profile-altering effects of systemic AMPH were found to be critically dependent on NAergic mechanisms (Chapter 3), the contribution of DA remained uninvestigated. Therefore, Chapter 5 tested the hypothesis that the USV response to AMPH depends on DAergic transmission by examining spontaneous and AMPH-induced 50-kHz calls following acute pretreatment with several D1- and D2-like DA receptor antagonists. Additionally, USVs were recorded from rats after acute administration of DA and NA reuptake inhibitors. The findings of Chapter 5 revealed that both the call rate and profile produced by AMPH depend on DA transmission through

both D1- and D2-like receptors. However, inhibition of DA and NA reuptake per se was not sufficient to elicit an AMPH- or cocaine-like USV response.

RESUMÉ

Les rats communiquent considérablement dans la gamme de fréquence ultrasonique; les vocalisations ultrasoniques (VUS) qui survient à 22 kHz reflètent un état d'alerte, tandis que celles produites autour de 50 kHz semblent refléter un affect positif. Cependant, les appels à la fréquence de 50 kHz sont hétérogènes et ils semblent différer aussi au niveau de leur signification.

Les premiers objectifs de cette thèse étaient d'identifier les sous-types de VUS à 50 kHz, et de déterminer si le contexte social et l'administration d'amphétamine (AMPH) peuvent les modifier. Nous avons donc examiné l'effet de l'administration d'AMPH sur les VUS chez les rats adultes dans le chapitre 2. Cette analyse de plus de 20 000 VUS a révélé que la fréquence de 50 kHz est constituée de plus de 14 catégories d'appels. Le taux global de ces appels est augmenté chez les rats en paires et après l'administration d'AMPH mais en plus, certains sous-types d'appels sont également renforcés ou réduits de manière préférentielle par ces conditions. Le chapitre 2 démontre donc que les appels à 50 kHz chez les rats adultes sont beaucoup plus hétérogènes que nous le pensions. Cette étude établit donc la première classification détaillée de ces appels, ce qui servira pour la recherche future.

Nous savons que l'AMPH favorise à la fois la libération de la dopamine (DA) et de la noradrénaline (NA), mais le rôle de la NA dans la production des VUS de 50 kHz chez les rats adultes n'a pas encore été étudié. Par conséquent, le deuxième objectif de cette thèse était de vérifier si la NA contribue à l'émission des VUS de 50 kHz, en modifiant de manière sélective, certains sous-types d'appels. Dans le chapitre 3, nous avons donc examiné les

VUS de 50 kHz qui surviennent spontanément, ainsi que celles qui sont provoqués par l'AMPH, après différents traitements systémiques ciblant le système noradrénergique (NAergique). Le chapitre 3 démontre pour la première fois que le système NAergique est impliquée dans la production des VUS provoqués par l'AMPH. Ainsi, les appels ultrasoniques de 50 kHz sont associés aux récepteurs $\alpha 1$ et β et à leurs mécanismes. On souligne également qu'une analyse détaillée des sous-types d'appels est nécessaire, et que le rôle du système NAergique dans l'euphorie provoquée par les psychostimulants mérite plus d'étude.

Les agonistes des opiacés constituent une classe importante de médicaments qui entraine l'euphorie, mais il reste à clarifier si ils modifient les VUS de 50 kHz de façon similaire aux psychostimulants. Le troisième objectif de cette thèse était donc d'étudier l'effet des doses non sédatives et euphorisantes de morphine sur le profil d'appels ultrasoniques de 50 kHz (chapitre 4). Malgré plusieurs conditions expérimentales, nous n'avons pas réussi à promouvoir ou à modifier les appels de 50 kHz après l'administration de morphine. Le chapitre 4 démontre donc que même si les appels de 50 kHz (et certains sous-types d'appels) reflètent un état hédonique dans certains contextes, ce n'est pas le cas après l'administration de morphine systémique. Ces résultats encouragent donc de la prudence dans l'interprétation des appels de 50 kHz.

Alors que l'AMPH provoque l'augmentation d'appels et la modification de leurs profiles et que ceux-ci dépendent dramatiquement sur les mécanismes NAergique (chapitre 3), la contribution de la dopamine (DA) reste a être élucidé. Par conséquent, le chapitre 5 examine le rôle de la dopamine sur les appels de 50 kHz en analysant si l'effet de

l’AMPH sur les VUS dépend sur la transmission DAergique. C’est après l’administration de plusieurs antagonistes DAergiques aux récepteurs D1 et D2 que les appels de 50 kHz spontanés et provoqués par l’AMPH ont été analysés. De plus, nous avons analysé les VUS chez des rats après l'administration d’inhibiteurs sélectifs de la récapture de la DA et la NA. Le chapitre 5 a donc révélé que le taux d'appel et le profil d’appel provoqué par l’AMPH dépend sur la transmission de la DA et sur l’activation des récepteurs D1 et D2. Cependant, l'inhibition de la recapture de la DA et la NA en soi n'est pas suffisante pour provoquer une réponse de VUS similaire à celle provoquée par l’AMPH ou la cocaïne.

ACKNOWLEDGEMENTS

First and foremost, I would like to express sincere gratitude to my supervisor, Dr. Paul Clarke, for his guidance and mentorship. His support, approachable nature, patience, and dedication towards the next generation of scientists have been truly inspiring.

I would like to thank my advisor, Dr. Terry Hébert, for his advice and encouragement, as well as his helpful comments regarding my work. I would also like to acknowledge the other members of my thesis advisory committee, Dr. Sandra Boye and Dr. Norman White for their guidance as I progressed through each project.

Thank you to all of my laboratory colleagues, past and present, for your support, assistance, and friendship.

Thank you to the support staff of the Department of Pharmacology and the Animal Resource Centre for all your hard work which has made the completion of this degree possible.

I would like to acknowledge the financial support received from the Natural Science and Engineering Research Council of Canada (NSERC) and the Fonds de recherche sur la nature et les technologies, as well as the research funding from NSERC and the Canadian Institutes of Health Research of Canada.

I wish to thank Robert Thomson for his technical help with our earlier experimental set-up.

Finally, to my wonderful parents, Kathryn and Peter Wright, and my brother, David Wright, I sincerely thank you for your love, support, and encouragement.

CONTRIBUTIONS OF AUTHORS

This manuscript-based thesis is comprised of 4 manuscripts. Author contributions are as follows.

CHAPTER 2: 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

All of the experimental work was performed by Jennifer Wright with the exception of the devocalization surgeries, which were performed by Dr. Jim Gourdon, DVM. The first and all subsequent drafts of the manuscript were written by Jennifer Wright and revised with Paul Clarke.

CHAPTER 3: Wright JM, Dobosiewicz MR, 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

Again, all experimental work and data analysis was performed by Jennifer Wright with the exception of Experiment 4 (i.e. Effect of propranolol on AMPH-induced USVs) which was performed and analyzed by May Dobosiewicz. The manuscript was written by Jennifer Wright and revised with Paul Clarke.

CHAPTER 4: Wright JM, Deng L, Clarke PBS (2012) Failure of rewarding and locomotor stimulant doses of morphine to promote adult rat 50-kHz ultrasonic vocalizations. Psychopharmacology (Berl), 224(4): 477-87

Lan Deng conducted and analyzed Experiment 1.1 and 1.2. Jennifer Wright conducted and analyzed Experiments 1.3, 2, and 3. The manuscript was written by Jennifer Wright and revised with Paul Clarke.

CHAPTER 5: Wright JM, Dobosiewicz MR, Clarke PBS (2012) The role of dopaminergic transmission through D1-like and D2-like receptors in amphetamine-induced rat ultrasonic vocalizations. Psychopharmacology (Berl), 225(4): 853-68

All experimental work and data analysis was performed by Jennifer Wright with the exception of Experiments 8 and 9 (i.e. dose-response studies of USVs following GBR 12909 and nisoxetine, respectively), which were performed by May Dobosiewicz. The manuscript was written by Jennifer Wright and revised with Paul Clarke.

STATEMENT OF ORIGINALITY

The original work presented in this thesis is summarized below.

Chapter 2:

- The 50-kHz ultrasonic vocalizations (USVs) emitted by adult laboratory rats are comprised of at least 14 distinct subtypes.
- Systemic administration of the psychostimulant amphetamine (AMPH) dose-dependently increased the overall rate of calling in the 50-kHz range.
- In relative terms, AMPH dose-dependently promoted the "trill" call subtype at the expense of "flat" (i.e. constant frequency) calls.
- Rats produced 50-kHz calls at a higher rate when tested with a cage-mate (on a per rat basis) than when tested individually.
- "Trill" and "flat-trill combination" calls were relatively more prevalent when rats are tested with a cage-mate than when tested individually.
- AMPH did not affect the acoustic parameters (i.e. duration, bandwidth, and mean peak frequency) of any call subtype.
- Marked and stable inter-rat differences were evident with respect to the call profile (i.e. relative prevalence of the different call subtypes) as well as the acoustic parameters (duration, bandwidth, and mean peak frequency) of each call subtype.
- Devocalization surgery, consisting of bilateral transection of the recurrent laryngeal nerve, virtually ablated emission of all 50-kHz USV subtypes.

Chapter 3:

- Systemic pretreatment with the α2 agonist clonidine (0.01-0.1 mg/kg, IP) or the α1 antagonist prazosin (0.3-1 mg/kg, IP) dose-dependently decreased the overall rate of 50-kHz calling under AMPH (1 mg/kg, IP). Prazosin pretreatment also significantly inhibited spontaneous call emission.
- The α2 antagonist atipamezole (0.3 and 1 mg/kg, IP) had no effect on call rate or profile when tested alone or as pretreatment before AMPH challenge.
- The β1/β2 adrenergic receptor antagonist propranolol (1-10 mg/kg, IP) dose-dependently reversed the call profile-altering effect of AMPH, without affecting the call rate.
- Co-administration of β1- and β2-selective antagonists (2.5 mg/kg betaxolol and 1 mg/kg ICI 118,551, respectively), mimicked the effect of propranolol on the call profile after AMPH administration. When administered alone, these antagonists were without significant effect.
- Neither the $5HT_{1A}$ antagonist NAD-299 (0.2 mg/kg, SC) nor the peripheral-only β1/β2 antagonist nadolol (5 mg/kg, IP) altered USV emission under AMPH.
- Passive intravenous cocaine (0.25-1.5 mg/kg, IV) administration promoted the rate of 50-kHz USV calling, but significantly less so than AMPH (0.5 mg/kg, IV). However, all doses of cocaine produced a comparable shift in the call profile.

Chapter 4:

- Acute administration of morphine (1 mg/kg) initially suppressed the rate of 50-kHz calling. Rats became tolerant to this suppression with repeated testing (3x 1 mg/kg, SC). USV emission was significantly reduced when rats were subsequently tested with a higher dose of morphine (i.e. 3 mg/kg, SC).
- Homecage morphine pre-exposure failed to affect calling under systemic morphine in the testing environment.
- Social context (i.e. testing rats with their cage-mate) did not affect USV emission under morphine (1 or 3 mg/kg, SC).
- Rats showed increased locomotor activity despite a concomitant reduction (or no change) in calling after morphine (1 and 3 mg/kg, SC) administration.
- Rats expressed a significant conditioned place preference (CPP) for 1 or 3 mg/kg morphine, but failed to show an increase in USV emission during conditioning, or when on the morphine-paired floor texture on test days (i.e. either on the CPP test day or when subsequently confined to the morphine-paired context). Rats had a normal USV response to systemic AMPH (1 mg/kg, IP) administration.
- Morphine consistently failed to alter the call profile.

Chapter 5:

- The D1-like antagonists SCH 23390 and SCH 39166 dose-dependently reduced the call rate under AMPH and suppressed the proportion of trill calls.
- The D2-like antagonists haloperidol, raclopride, pimozide, clozapine, and risperidone, all markedly decreased the rate of USV emission under saline and

AMPH. The drugs considered classical antipsychotics (i.e. haloperidol, raclopride, and pimozide) also significantly reduced the proportion of trill calls.

- The D2-like antagonist sulpiride failed to affect USV emission significantly, even at high doses that inhibited spontaneous and AMPH-induced locomotor activity.
- The dopamine and noradrenaline reuptake transporters (GBR 12909 and nisoxetine, respectively), given alone or in combination, were not sufficient to mimic the effect of AMPH on the call rate or profile.

LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine (serotonin)
AMPH	amphetamine
EBS	electrical brain stimulation
CPA	conditioned place aversion
CPP	conditioned place preference
DA	dopamine
DAergic	dopaminergic
DAT	dopamine transporter
IP	intraperitoneal
NA	noradrenaline
NAc	nucleus accumbens
NAergic	noradrenergic
NET	noradrenaline transporter
SAL	saline
SC	subcutaneous
USV	ultrasonic vocalization
VEH	vehicle

CHAPTER 1: Introduction and Literature Review

Jennifer M. Wright

General Introduction

Rats are commonly used to study psychological processes underlying motivation and emotional state. Since rats, unlike humans, are unable to self-report about their subjective experience, overt behaviours such as approach or avoidance of a stimulus are typically used for this kind of research. However, there is growing interest in rat ultrasonic communication as a potential index of emotional states such as hedonia and anxiety. Thus, ultrasonic vocalizations (USVs) potentially offer a rich source of unique information, and may contribute significantly to the development of novel animal models. An essential step towards this ultimate goal is to understand the behavioural significance and neurochemical basis of these calls.

Rat ultrasonic vocalizations (USVs)

Sound frequencies that are audible to humans range from 20 Hz – 20 kHz. A number of factors decrease this optimal range however, including age, exposure to loud noise, and disease (Heffner and Heffner 2007). The terms "ultrasonic" and "infrasonic" refer to sounds outside these upper and lower limits of human hearing, respectively.

The range of audible frequencies varies considerably across species. Many animals, especially small mammals such as rodents, have auditory capabilities extending well into the ultrasonic range. The first report that adult rats could hear ultrasound was in 1941 by Gould and Morgan. They used an operant shock avoidance task to show that rats hear very well up to 40 kHz, and suggested that the upper limit of their hearing likely extends to even higher frequencies (Gould and Morgan 1941). It is now well established that rats can hear

sound frequencies ranging from 250 Hz to 65-80 kHz (at 60-70 dB, and depending on the strain) (Heffner et al. 1994; Kelly and Masterton 1977).

In addition to their ability to hear ultrasonic frequencies, most small rodent species are capable of emitting vocalizations composed of pure ultrasound. Rat ultrasonic vocalizations (USVs) were first detected in 1954 by John W. Anderson, who noted the production of ~20-30 kHz sounds by rats in an aversive context (i.e. tail pinch) (Anderson 1954). Importantly, these vocalizations were not correlated with any audible sounds. Further studies have shown that rats emit USVs over a wide range of ultrasonic frequencies (i.e. 20-90 kHz) (Portfors 2007; Sales and Pye 1974). Three main call types have been recognized in laboratory rats:

(1) *40-kHz isolation distress calls* are produced by rat pups when they are separated from their mother and littermates. It appears that a reduction in the ambient temperature triggers their production rather than separation per se, since these calls are dramatically reduced when isolated pups are tested in an elevated temperature environment resembling that of the nest (i.e. 34-36˚C) (Allin and Banks 1972; Blumberg et al. 1992a; Blumberg et al. 1992b; Okon 1971; Oswalt and Meier 1975).

The '40-kHz calls' are approximately 80-150 ms in duration, range from 30-65 kHz, and vary in acoustic structure (Brudzynski et al. 1999; Portfors 2007). The rate of calling is low immediately after birth but rises with the opening of the ears around postnatal day 4 (Noirot 1968). These calls then begin to decline when the eyes open on day 16, falling to zero by day 21 (Noirot 1968).

The USVs emitted by rat pups are critical to their survival. Since pups lack effective insulation (i.e. fur and subcutaneous fat), and have limited locomotor abilities, they are heavily reliant on the presence of their mother and litter for warmth until about day 20, when the pups become capable of homoiothermy (i.e. body temperature homeostasis) (Okon 1971). Rat pup USVs appear to powerfully influence maternal behaviour; these calls reliably cause the mother to search for and retrieve lost pups to the nest (Allin and Banks 1972; Smotherman et al. 1974), decrease the latency to return to the nest area (Jans and Leon 1983), and increase maternal licking (Brouette-Lahlou et al. 1992).

(2) *'22-kHz calls'*, emitted by juvenile and adult rats, were the first USVs detected in rodents (see above - Anderson 1954). These calls are characterized by their low frequency (i.e. typically 18-30 kHz), narrow within-call frequency range (i.e. 1-6 kHz), high amplitude (i.e. 80-85 dB), and relatively long duration (i.e. ~0.3-3 sec) (Barfield and Geyer 1972; Brudzynski et al. 1993; Brudzynski 2005; Brudzynski and Holland 2005; Sales 1979). Most conditions that elicit 22-kHz calls are aversive, e.g. drug withdrawal (Covington and Miczek 2003; Vivian and Miczek 1991), air-puff (Knapp and Pohorecky 1995), predator exposure (Blanchard et al. 1991; Blanchard et al. 1992), acoustic startle (Kaltwasser 1990b; Kaltwasser 1991), inter-male aggression (Corrigan and Flannelly 1979; Sales 1972a; Thomas et al. 1983), and electric shock to the feet or tail (Kaltwasser 1991; van der Poel et al. 1989). These calls are typically accompanied by defensive submissive behaviour, signaling social withdrawal or helplessness (Brudzynski and Ociepa 1992; Hegoburu et al. 2011; Sales and Pye 1974; Wohr et al. 2005). The association between 22-kHz USVs and aversive contexts, together with the ability of anxiolytic drugs to reduce these calls (De Vry

et al. 1993; Naito et al. 2003; Sanchez 2003), has led to the proposal that 22-kHz USVs express alarm, negative affect or anxiety (Jelen et al. 2003; Knutson et al. 2002; Litvin et al. 2007).

(3) *'50-kHz calls'* are also emitted by juvenile and adult rats. They were first detected by Gillian D. Sales (née Sewell), who described the emission of 30-60 ms "short pulses" at about 50 kHz by adult rats in response to handling, aggression, and sexual behaviour (Sewell 1967). In this early paper, she suggested that the short pulses (i.e. 50-kHz calls) may signal aggression, and the 22-kHz calls submission (Sewell 1967). However, subsequent studies have reported the occurrence of 50-kHz USVs in numerous appetitive contexts. The latter include social contact (Brudzynski and Pniak 2002), mating behaviour (Sales 1972b), rough-and-tumble play (Knutson et al. 1998), certain kinds of experimenter-applied tactile stimulation (i.e. "tickling") (Panksepp and Burgdorf 2000), rewarding electrical brain stimulation (EBS)(Burgdorf et al. 2007), as well as systemic or intracerebral amphetamine (AMPH) administration (Ahrens et al. 2009; Burgdorf et al. 2001a; Simola et al. 2009; Thompson et al. 2006; Wintink and Brudzynski 2001). 50-kHz calls are also elicited during *anticipation* of various appetitive stimuli, namely play (Knutson et al. 1998), copulation (Bialy et al. 2000), food and rewarding EBS (Burgdorf et al. 2000). Conversely, the rate of 50-kHz calls is reported to decrease in aversive situations such as presentation of predator (cat) odour or footshock cue (Burgdorf et al. 2000; Panksepp and Burgdorf 1999). Together, these findings have led to the proposal that 50-kHz USVs reflect positive affect (i.e. hedonia) (Brudzynski 2007; Burgdorf and Moskal 2009; Burgdorf et al. 2011;

Knutson et al. 1999) and these calls have even been likened to human joy and laughter (Panksepp and Burgdorf 2000; Panksepp and Burgdorf 2003).

The 50-kHz calls are much shorter than the 22-kHz calls, with an average duration of 30-40 ms (Brudzynski 2009). Despite their denotation, 50-kHz calls encompass a wide frequency range (i.e. 30-90 kHz) (Portfors 2007; Sales and Pye 1974) and display considerable variations in acoustic structure (see "Evidence of 50-kHz call heterogeneity" section below).

Advantages of ultrasonic emission

Communicating in the ultrasonic range might have developed as an evolutionary adaptation to help avoid detection by predators. While some of the many species that feed on rodents (e.g. birds of prey) do not hear ultrasound (Dooling and Popper 2000), many others hear very well at high frequencies. Cats, for example, have one of the broadest hearing ranges among mammals, with the ability to hear sounds up to 85 kHz (Heffner and Heffner 1985). However, ultrasound is absorbed rapidly in air (Lawrence and Simmons 1982) and scattered easily from small objects due to its very short wavelengths (Sales and Pye 1974). Thus, while being effective for intraspecific communication between animals in close proximity, ultrasonic emission impedes detection or location of the source by predators at a distance, especially when emitted underground in burrows (Brudzynski 2009; Sales and Pye 1974).

Mechanism of call production in rodents

In rodents (as in humans), audible vocalizations are produced by passing air through the vocal folds of the larynx, causing them to vibrate. These 'squeals', comprised of a

fundamental and harmonic series, are produced to express physical pain (or anticipation of pain), or as a defensive reaction against predators (Brudzynski 2009; Litvin et al. 2007). The larynx also serves a secondary function in rats, which is to mediate the emission of USVs (Johnson et al. 2010; Roberts 1975a; Roberts 1975b). To produce USVs, the vocal folds tightly constrict and form a small orifice which acts like a whistle, generating pure ultrasound with few or no harmonics when air is forced through (Brudzynski 2009; Johnson et al. 2010; Roberts 1975b). Notably, transection of the inferior (i.e. recurrent) laryngeal nerves, which innervate the lower half of the larynx, abolishes ultrasonic calling (Roberts 1975a). Several investigators have performed laryngeal nerve transections as a means to "devocalize" rats (see below) (Hofer and Shair 1993; Kondo et al. 1999; Thomas et al. 1983; White et al. 1990a; White and Barfield 1987; White and Barfield 1990).

A recent paper used laryngeal muscle electromyographic (EMG) activity to examine the role of two intrinsic and two extrinsic laryngeal muscles in USV production. The extrinsic muscles (sternothyroid and sternohyoid), involved in vocal production in other mammals, were not critical for USV production in rats (Riede 2013). Conversely, EMG activity and intensity of the intrinsic muscles (thyroarytenoid and cricothyroid), were aligned with call duration and fundamental frequency, respectively (Riede 2013). Moreover, phasic activities (i.e. EMG bursts) of the intrinsic muscles corresponded with frequency modulation of the calls (Riede 2013).

Hypotheses of USV function

Since adult rat 50-kHz and 22-kHz calls are associated with various appetitive and aversive contexts, they have been proposed to be an expression of positive and negative affect,

respectively (see above). However, additional hypotheses of USV function have been put forward, which are not necessarily incompatible with the affective expression hypothesis. These mechanisms, which are described in the next section, include USV emission for: (1) intra-specific communication, (2) as a mechanical by-product, and for (3) brain thermoregulation.

Communication

As described above, rat pup isolation-induced distress calls signal the need for maternal care. Evidence of a clear communicative function of USVs was first provided by Gillian D. Sewell in 1970, who demonstrated that playback of isolation-induced distress calls from mouse pups initiated search and retrieval responses from the mother (Sewell 1970). Importantly, USV playback excluded possible confounds of visual or olfactory cues (Beach and Jaynes 1956). Moreover, control signals (i.e. background noise and other sounds) were ineffective, suggesting that the communicative value was restricted to the acoustic qualities of the pup calls (Sewell 1970). Additional playback studies in rats have repeatedly demonstrated the ability of pup calls to elicit maternal search and retrieval (Allin and Banks 1972; Smotherman et al. 1974; Wohr and Schwarting 2008).

USVs also have a communicative role in *adult* rats, as evidenced by the ability of ultrasound to alter behaviour. 22-kHz calls appear to serve as "alarm calls", thus warning conspecifics of potential danger (Blanchard et al. 1991; Blanchard et al. 1992; Litvin et al. 2007; Wohr and Schwarting 2009b). In a naturalistic context (i.e. visible burrow system), emission of 22-kHz calls following cat exposure was greatly facilitated by the presence of conspecifics (Blanchard et al. 1991). In accordance with an alarm function, playback of

natural 22-kHz USVs (or corresponding artificial acoustic stimuli) often elicits defensive behaviours such as freezing or flight in the recipients (Brudzynski and Chiu 1995; Commissaris et al. 2000; Neophytou et al. 2000; Sales 1991; Wohr and Schwarting 2007). Furthermore, 22-kHz calls appear to mollify aggression. Using the resident-intruder paradigm, it was shown that 22-kHz call emission negatively correlated with the extent of aggressive behaviour (Adler and Anisko 1979; Lore et al. 1976; Sales 1972a).

Conversely, 50-kHz calls appear to facilitate social interactions such as copulation, play, and cooperative behaviour. For example, sexual behaviour is disrupted following devocalization or deafening (Thomas et al. 1981; White and Barfield 1987), suggesting that 50-kHz call communication between male and female rats (Sales 1972b; Thomas and Barfield 1985) is required for normal mating activity. Notably, playback of 50-kHz calls can restore normal mating activity (White and Barfield 1987; White and Barfield 1989). Deafening juvenile rats significantly reduced conspecific play, whereas blinding and whisker removal were ineffective (Siviy and Panksepp 1987). Finally, 50-kHz USVs were associated with cooperative behaviour whereby two subjects were required to work jointly on an operant task (Lopuch and Popik 2011).

Emission of 50-kHz USVs by rats may also serve to establish or maintain social contact, as suggested by Wohr and Schwarting (2007). These authors tested the communicative value of 50-kHz calls by measuring behaviour during call playback in a radial arm maze. Playback of 50-kHz calls was found to induce activation (i.e. locomotor activity) in the test subjects, and approach to the loud speaker (Wohr and Schwarting 2007).

Mechanical by-product and thermoregulation

M.S. Blumberg (1992) suggested that at least some rat 50-kHz USVs are merely by-products of thoracic compression during locomotion (Blumberg 1992). The idea was born out of an earlier study in gerbils showing a strong association between body movements and USV production (Thiessen and Kittrell 1979). In reviewing the reports of ultrasound emission during copulation and aggression, Blumberg (1992) noted that 50-kHz calls were often accompanied by biomechanical strain on the thorax and forelimbs. He extended the evidence for his hypothesis by performing a slow-motion analysis of rat copulatory behaviour, observing that USV emission often coincided with thorax compression (Blumberg 1992). However, while some rat ultrasound might simply represent locomotor artifacts, USV production and locomotor activity can be experimentally dissociated (for review, see Knutson et al. 2002), and USV production can and does occur without any physical strain (Blumberg 1992).

Alternative hypotheses for the function of rat pup calls and adult 22-kHz calls have also been described. Blumberg and Sokoloff (2001) suggested that pup calls are an acoustic by-product of an abdominal pressure reaction serving to increase venous return when cardiovascular function is compromised due to cold exposure (Blumberg et al. 1999; Blumberg and Sokoloff 2001). Further support was obtained by showing that pup calls increased following administration of the $\alpha 2$ agonist clonidine, counteracting the drug-induced decrease in cardiac function (Blumberg et al. 2000a; Blumberg et al. 2000b). Blumberg and Moltz (1987) further posited that 22-kHz USV emission by adult rats serves as a thermoregulatory behaviour, since emission of these calls was increased by a brain

warming activity (copulation), and was also modulated by drugs that raise or lower hypothalamic temperature (Blumberg and Moltz 1987).

Methods of ultrasound detection and analysis

Since ultrasound is beyond the human auditory range, rat USV research critically depends on the capacity to acquire and analyze the acoustic signals. Until recently, rodent USVs were typically detected by means of superheterodyne or frequency-division bat detectors, devices that convert the sound signal into the human audible range. While bat detectors offer certain advantages (e.g. relatively cheap and rugged), their signal transformation process introduces the following important drawbacks.

Superheterodyne systems respond only to sounds within a narrow range (i.e. approx. ±5 kHz) of a specified frequency; sound frequencies outside this window are left undetected (Parsons 2000; Sales and Pye 1974). This means that calls may be entirely missed or else only partially detected. Moreover, this method does not preserve important information such as call duration or acoustic 'structure' (i.e. frequency-modulation) (Parsons 2000). The frequency-division bat detector, as its name implies, lowers the frequency of the signal by dividing the waveform by a predetermined factor. Although the latter system monitors all frequencies (i.e. does not require tuning), it distorts the signal (see Figure 1) rendering it impossible to meaningfully analyze the acoustic features of the calls (Parsons 2000).

Thus, many early rat USV studies were limited by these technologies and could only report on the number of calls (e.g. Burgdorf et al. 2001b; Knutson et al. 1998; Panksepp and Burgdorf 2000). In the past ten years or so, more sophisticated equipment has been

developed which uses high sampling rates (e.g. 250 kHz), exceeding the Nyquist rate, which, when combined with higher computing power, has enabled sampling of the ultrasonic signal with much greater fidelity. These "broadband" recording methods differ drastically in the amount and quality of information obtained (see Figure 1).

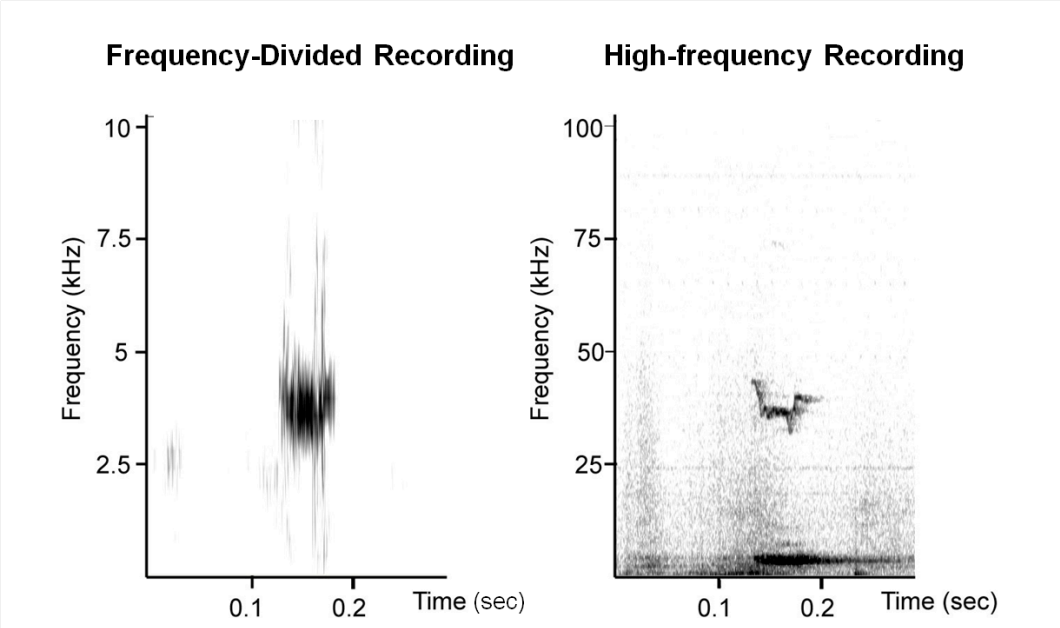

Figure 1: Tickle-induced 50-kHz call simultaneously recorded using a frequency-division (÷10) bat detector and a high-frequency recording system (sampling rate = 200 kHz)

Visualization of rat ultrasonic vocalizations: the spectrogram

A common and useful way of visualizing sound is through the generation of a spectrogram, i.e. a graphical representation of sound frequency *vs.* time. Fourier analysis is a mathematical technique that enables a complex waveform to be decomposed into its frequency components, each having associated amplitude. A spectrogram is essentially the

result of a series of Fourier transformations applied sequentially to a signal over time (Parsons 2000). A fast Fourier transform (FFT) is an efficient algorithm used by many computer software programs to increase calculation speed of the Fourier transform. The time and frequency resolution of the spectrogram depends critically on several factors, including the sampling rate, the FFT length (i.e. the number of values from the signal to be analyzed), and the extent of overlap (i.e. the step width of the sliding time window computing the Fourier transforms).

Evidence of 50-kHz call heterogeneity

Spectrographic analysis has revealed that adult rat 50-kHz calls vary considerably in acoustic 'structure'. On this basis, two main classes of 50-kHz calls have been recognized: (1) "flat" calls (i.e. tones which show little or no change in sound frequency over time), and (2) "frequency-modulated (FM)" calls, which display some degree of pitch fluctuation (Ahrens et al. 2009; Burgdorf et al. 2007; Burgdorf et al. 2008a; Burgdorf and Panksepp 2006; Ciucci et al. 2009; Simola et al. 2009; Wohr and Schwarting 2007).

Importantly, flat and FM calls made by adult rats appear to differ in their behavioral significance and susceptibility to experimental manipulation. For example, playback of FM, but *not* flat calls, is reported to be reinforcing (Burgdorf et al. 2008). Moreover, the emission of FM "trill" calls was sensitized by amphetamine (AMPH), while flat calls were not (Ahrens et al. 2008). FM 50-kHz calls can be selectively induced by experimenter-applied tickling (Burgdorf and Panksepp 2006) and suppressed by manipulations intended to reduce dopaminergic transmission (Burgdorf et al. 2007). These observations have led to the proposal that FM calls may signal a dopamine-dependent reward state (Burgdorf et

al. 2011). Conversely, flat calls appear unassociated with rewarding stimuli and may serve a social-coordinating function. For example, brief separation from a cage-mate primarily increased flat 50-kHz calls (Wohr et al. 2008). According to another report, 95% of the 50-kHz USVs emitted by rat dams upon reuniting with their pups were flat (Stevenson et al. 2009). Finally, the majority of 50-kHz USVs emitted during an aggressive encounter were flat rather than FM (Burgdorf et al. 2008).

The flat *vs.* FM 50 kHz call distinction is useful, but individual spectrograms have hinted at much greater call diversity (e.g. trills, harmonics, flat-step-trill, etc) (Burgdorf et al. 2008a; Ciucci et al. 2009; Fendt et al. 2006; Wohr et al. 2008). Somewhat extended classification schemes have described at most three or four 50-kHz call subtypes, all within the context of sexual or agonistic behaviour (Thomas and Barfield 1985; Vivian and Miczek 1993b; White et al. 1990b). Notably, *nine* USV subtypes were documented in rat pups (Brudzynski et al. 1999) suggesting a larger repertoire might exist in adult rats than has previously been defined.

50-kHz USVs in non-appetitive contexts

Although 50-kHz calls are associated with many rewarding contexts (see above), they are also emitted in situations that are probably not appetitive or even frankly aversive. For example, 50-kHz calls are elicited by brief separation from conspecifics (Wohr et al. 2008) and during agonistic behaviour (Haney and Miczek 1994; Miczek et al. 1995; Takahashi et al. 1983; Thomas et al. 1983; Tornatzky and Miczek 1994; Tornatzky and Miczek 1995; Vivian and Miczek 1993a; Vivian and Miczek 1993b). With regards to the latter, devocalization experiments have demonstrated that the 50-kHz calls emitted during the

aggressive encounter are produced almost entirely by the intruder (Takahashi et al. 1983; Thomas et al. 1983). It is unclear what roles these calls play - whether they are affiliative in nature or perhaps signaling high arousal - but it is highly unlikely they reflect positive affect. 50-kHz calls also occur in other, demonstrably *aversive* contexts such as drug withdrawal (Vivian and Miczek 1991), CO_2 exposure (Niel and Weary 2006), and pain (Dinh et al. 1999).

Importantly, none of above mentioned studies included a detailed examination of the acoustic structure of these calls, leaving open the question of whether 50-kHz calls produced in appetitive *vs.* non-appetitive situations are acoustically comparable.

Call parameters

While the *rate* of calling is typically the focus of most USV studies, accumulating evidence shows that acoustic call parameters such as duration, bandwidth, and mean peak frequency (see Figure 2) are susceptible to experimental manipulation and may potentially contain important information not encoded by the absolute number of calls. For example, the duration of rat pup calls was more sensitive to the effects of anxiolytic pharmacotherapies than the rate of calling (Hodgson et al. 2008). Similarly, the duration of both 50-kHz and 22-kHz USVs during aggressive encounters was dose-dependently decreased by morphine, without any change in the call rate (Vivian and Miczek 1993b). Finally, caffeine (unlike AMPH) dose-dependently affected 50-kHz call bandwidth and peak frequency of both flat and FM calls without altering the number of USVs emitted (Simola et al. 2009).

The ability to selectively alter acoustic parameters suggests two interesting possibilities. First, additional information may be conveyed through these parameters, as

suggested for 22-kHz calls (Brudzynski et al. 1993). A second possibility is that different call subtypes (each with a distinct set of acoustic parameters) emerge under different behavioural contexts and can be preferentially altered by pharmacological manipulations.

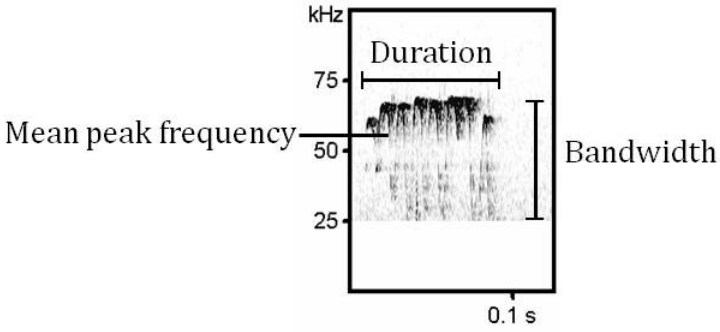

Figure 2: Call parameters of duration, bandwidth, and mean peak frequency are visually depicted for an individual trill call. The bandwidth is the difference between the maximum and minimum peak frequency. The mean peak frequency is the time-averaged peak energy frequency within the call. The duration is the time from the beginning to the end of the call that is spectrographically detectable.

Mouse vs. rat USVs

Infant and adult mice also emit USVs. Mouse pup calls seem to have a similar function as USVs emitted by rat pups; both signal distress and alter maternal behaviour (Wohr and Schwarting 2010; Zippelius and Schleidt 1956). A considerable repertoire of mouse pup calls has been described, with as many as 10 identified subtypes (Scattoni et al. 2008). In contrast, *adult* mouse and rat USVs appear to be functionally and acoustically distinct. First, a mouse call analogous to the 22-kHz 'alarm' call in rats has not been detected. Second, while juvenile mice emit 'interaction-induced USVs' (comprised of at least five subtypes) during social investigation of conspecifics (Moles et al. 2007; Panksepp et al. 2007), these

calls decline rapidly with sexual maturity (Wohr and Schwarting 2010). As adults, male mice produce 'female-induced USVs' in response to females or their odour (Nyby et al. 1977; Sales 1972b). This call type serves to attract females and keep them close, in order to facilitate copulation (Hammerschmidt et al. 2009; Pomerantz et al. 1983). There is however, some recent preliminary evidence that a larger call repertoire exists in adult mice, and that emission of individual call types might be context-dependent (Chabout et al. 2012). Indeed, ten call categories were described in a recent study examining adult mouse USVs; the proportion of each call type emitted differed between behavioural conditions, i.e. social interaction, novelty exploration, and restraint stress (Chabout et al. 2012). However, unlike rat USVs, calls emitted by mice have not been associated with various appetitive or aversive stimuli. Thus, the possible emotional significance of mouse USVs remains unclear.

Inter-individual, sex, and strain differences in USV emission

Inter-individual differences in 50-kHz call rates in response to various stimuli have been widely reported (Burgdorf et al. 2001b; Burgdorf et al. 2005; Burgdorf et al. 2008b; Mallo et al. 2007; Schwarting et al. 2007; Wohr et al. 2008; Wohr et al. 2009; Wohr and Schwarting 2009a). Rats have been successfully bred for high or low levels of tickle-induced 50-kHz calling (Burgdorf et al. 2005; Panksepp and Burgdorf 2000), suggesting that the observed variability may have a genetic basis. Moreover, calling phenotype appears to be correlated with other social and emotional behaviours. For example, rats selectively bred for low levels of 50-kHz USVs emitted more isolation distress calls as pups and failed to show a typical preference for a maternally-associated odor (Harmon et al. 2008). As adults, these rats were more socially withdrawn and produced more fecal boli

during tickling, in the Porsolt swim test, and during the open-field test (Burgdorf et al. 2008b). In contrast, rats bred for high rates of 50-kHz USVs displayed less anxiety-like behaviour and defensive aggression, exhibited more play behaviour with conspecifics, and showed a mild increase in preference for a dilute sucrose solution compared to randomly bred lines (Burgdorf et al. 2008b; Panksepp and Burgdorf 2000).

Few studies have examined sex differences in USV emission in rats, but preliminary evidence suggests that female rats emit considerably more 50-kHz USVs when confronted with an anesthetized conspecific than do males (Blanchard et al. 1993). Female rats also produce more 22-kHz USVs than males in response to tickling and predator exposure (Blanchard et al. 1992; Mallo et al. 2007). Sex differences have also been noted in the relative prevalence of the six 22-kHz call categories defined in the Blanchard et al. (1992) study and in the average sound frequency of 22-kHz calls (Blanchard et al. 1992). Conversely, male rats emitted more 22-kHz calls compared to female rats in response to contextual and auditory conditioned stimuli predictive of footshock (Graham et al. 2009).

There exists some evidence for strain differences in USV production by adult rats. For example, Wistar rats emitted more 50-kHz calls during aggressive encounters than the Listar strain; the opposite was observed for 22-kHz USV emission (Sales 1979). Sprague Dawley rats produced more 22-kHz calls than Long-Evans in response to footshock and associated conditioned stimuli (Graham et al. 2009). Similarly, Sprague Dawley rats emitted more 22-kHz calls compared to Long-Evans and Wistar rats during fear conditioning, despite the Long-Evans rats displaying greater freezing behaviour (Schwarting and Wohr 2012).

CNS mechanisms of 50-kHz USV production and recognition

As noted above, emission of FM 50-kHz USVs has been proposed to reflect a dopamine-dependent reward state. Consistent with this notion, mesocorticolimbic dopamine pathways in the brain appear to play an important role in the production of 50-kHz USVs. For example, electrolytic lesions of the ventral tegmental area (VTA) were reported to decrease FM 50-kHz USVs induced by heterospecific play (Burgdorf et al. 2007). The same study further showed an analogous effect of bilateral 6-OHDA infusions targeting medial forebrain bundle (MFB) neurons (Burgdorf et al. 2007). It was subsequently found that unilateral 6-OHDA lesions of the MFB reduced the ratio of FM to flat 50-kHz calls in response to sex-relevant odours (Ciucci et al. 2007; Ciucci et al. 2009). Moreover, mesolimbic dopamine release is sufficient to *induce* 50-kHz calling. For example, intra-nucleus accumbens (NAc) injections of the indirect dopamine agonist AMPH (see "Psychostimulants: mechanism of action" below) dose-dependently enhanced the 50-kHz call rate (Burgdorf et al. 2001a; Thompson et al. 2006). The latter effect was particularly strong in the shell *vs.* the core region, and was inhibited by pretreatment with a dopamine antagonist injected into the same brain sites (Thompson et al. 2006). However, it is important to also consider the possible contribution of noradrenaline (NA) release by intra-accumbens AMPH in the elicitation of 50-kHz USVs: indeed, the nucleus accumbens (NAc) receives direct noradrenergic input from A1 and A2 brainstem neurons (Weinshenker and Schroeder 2007) and AMPH stimulates NA release as well as DA (see "Psychostimulants: mechanism of action").

Intracerebral injection of glutamate into the anterior hypothalamic-preoptic area has also been shown to induce 50-kHz calling, and this effect was reversible by systemic

pretreatment with the DA receptor antagonist haloperidol (Fu and Brudzynski 1994; Wintink and Brudzynski 2001). These findings are interesting because they suggest that DA may play a more general role in USV production, since a DA antagonist was able to inhibit USVs induced by a non-DA drug.

Finally, 50-kHz USV production can be elicited by directly activating specific brain areas through experimenter-delivered electrical brain stimulation (EBS). Indeed, EBS of the VTA, NAc, prefrontal cortex, ventral pallidum, lateral preoptic area, lateral hypothalamus, and dorsal raphe nucleus unconditionally elicited 50-kHz calling (Burgdorf et al. 2007). Notably, the EBS of those areas also supported self-stimulation behaviour and was therefore reinforcing (Burgdorf et al. 2007).

Some of the brain regions involved in call production may also be important for processing the call as a social signal. Playback of 50-kHz USVs increased neuronal activity in two brain areas, as measured by c-Fos immunolabeling: the secondary motor cortex and the NAc (Sadananda et al. 2008). The former is likely due to the induction of behavioural activation, as playback of 50-kHz calls induced approach towards the sound source (Sadananda et al. 2008; Wohr and Schwarting 2009b). Activation of the NAc, although not statistically significant, was attributed to the appetitive value of 50-kHz USVs (Wohr and Schwarting 2009b). It was therefore suggested that the NAc may provide a functional link between mechanisms of detection and production of these calls (Wohr and Schwarting 2009b).

Conventional measures of drug reward in rats

Various methods have been developed to study drug reward in rats. Since rats are unable to report on their subjective experience, these methods rely on measuring behaviours through which the animal's subjective state can only be inferred. Described below are two of the most widely used behavioural paradigms: the intravenous self-administration procedure and conditioned place preference.

Intravenous self-administration (IVSA)

In the IVSA procedure, rats must perform an operant task (e.g. lever press or nose poke) in order to obtain drug delivery through a surgically implanted intravenous line. The self-administration paradigm is generally regarded as the most relevant model of human drug-taking behaviour, since it not only has high face validity, but also considerable construct validity; for example, drug self-administration by rats is predictive of abuse liability in humans (Collins et al. 1984). Several important parameters can be measured using this procedure, including positive reinforcement (i.e. strengthening of learned stimulus-response tendencies), motivation, and relapse. However, the IVSA paradigm provides no information about the subjective state of the animal. It is well-established that drug 'wanting' (i.e. motivation to obtain the drug) and drug 'liking' (i.e. positive affect or emotion experienced under the drug) are dissociable psychological processes (Berridge and Robinson 2003). Measuring drug taking behaviour through the self-administration procedure only provides information about the former.

Conditioned place preference (CPP)

The conditioned place preference paradigm includes elements of Pavlovian conditioning, whereby drug administration (i.e. the unconditioned stimulus) is repeated paired with previously neutral environmental cues (e.g. tactile, odor, and/or visual cues), causing the latter to acquire secondary motivational properties (i.e. conditioned stimuli)(Bardo et al. 1995; Tzschentke 2007). Thus, following the conditioning period, the conditioned stimuli can elicit approach or avoidance, termed conditioned place preference (CPP) or conditioned place aversion (CPA), respectively (Tzschentke 2007). Drugs that induce CPP are thought to do so because of their positive affective properties (Van Ree et al. 1999). Indeed, many drugs of abuse, such as cocaine, AMPH, and morphine, reliably produce a CPP (Bardo et al. 1995; Tzschentke 1998; Tzschentke 2007). However, it is nevertheless difficult to interpret exactly what is being expressed when the animal approaches the drug-paired environment. For example, the approach towards the drug-paired context presumably reflects a desire to experience the drug effects (Van Ree et al. 1999). It is unclear however, if that desire is driven by memories of past drug euphoria, or simply an attempt to avoid negative affect (e.g. dysphoria, anhedonia, or anxiety) consequential to withdrawal from most drugs of abuse (Weiss 2005). Another potential limitation of the CPP paradigm is that animals are tested in a drug-free state. While the latter avoids drug-induced impairment of performance, state-dependent learning might preclude recollection of the drug-cue associations on test day (Van Ree et al. 1999). Finally, CPP is not easily demonstrable in human subjects, and thus lacks face validity as a model of drug reward (Bardo and Bevins 2000).

USVs as a potential measure of drug reward

As described above, 50-kHz USVs emitted by adult rats have been associated with numerous appetitive stimuli and thus have been proposed to reflect a state of positive affect (Knutson et al. 2002; Panksepp and Burgdorf 2003). From a drug research point of view, the ability to gauge subjective responses in rats by assessing USV emission would be particularly informative and useful. For example, the conventional models described in the previous section use overt behavioural measures (e.g. approach or performing an operant task) to derive conclusions about the animal's subjective state following drug administration. USV emission however, potentially provides a more direct measure of the animal's affective response, in some ways similar to self-report studies in humans. Another potential advantage of USV emission is that, unlike IVSA and CPP, it permits the study of unconditioned drug effects since it does not rely on memory of the stimulus-response or cue associations.

Accordingly, analysis of USV emission by rats has been used increasingly in drug studies. Notably, the psychostimulant amphetamine (AMPH) has been shown repeatedly to elicit high rates of 50-kHz USVs following systemic administration (Ahrens et al. 2009; Simola et al. 2009; Wintink and Brudzynski 2001), and this response has been attributed to the rewarding value of the drug (Burgdorf et al. 2011). Moreover, 50-kHz USVs have also served as potential markers of affective reactions in cocaine self-administration experiments (Barker et al. 2010; Maier et al. 2010). Importantly however, 50-kHz calls vary greatly in acoustic structure (see "Evidence of call heterogeneity" section above). Thus, it is possible that distinct information is conveyed through different subtypes of 50-kHz USVs.

Understanding the potential significance of this call heterogeneity is critical to further develop rat 50-kHz USVs as an animal model of drug reward.

Psychostimulants: mechanism of action

Psychostimulants (e.g. cocaine and amphetamines) encompass a group of drugs that tend to increase alertness, agitation, or excitation. They act through various mechanisms, but generally lead to an increase in extracellular levels of monoamines. For example, cocaine inhibits monoamine transporters (i.e. DAT, NET, and SERT), thus preventing the degradation of the associated neurotransmitters by monoamine oxidase and prolonging their action in the synaptic cleft (Elliot and Beveridge 2005). Conversely, AMPH acts as a substrate for the monoamine transporters, allowing it to enter the synaptic terminal where it can stimulate the release of DA and NA (for reviews, see Fleckenstein et al. 2007; Sulzer et al. 2005). The latter action is mediated through a disruption of vesicular storage. AMPH is a lipophilic weak base that diffuses into and accumulates within catecholaminergic vesicles. The intravesicular environment is relatively acidic and donates protons to AMPH, disrupting the proton electrochemical gradient necessary to concentrate and store neurotransmitter. In addition, AMPH inhibits vesicular monoamine transporter 2 (VMAT-2), further impeding vesicular uptake. The consequent rise in the cytosolic concentration of catecholamines in turn causes their retrograde release through the neuronal membrane monoamine transporters (Fleckenstein et al. 2007; Sulzer et al. 2005).

By increasing extracellular dopamine through the above mechanisms, AMPH activates autoreceptor feedback mechanisms that inhibit DA cell firing (Bunney et al. 1973; Einhorn et al. 1988). However, it was subsequently shown using anesthetized rats that,

under control conditions, this inhibitory effect masks a simultaneous excitatory effect of AMPH (expressed as an increased firing rate and phasic activity) (Shi et al. 2000). The burst-increasing effect of AMPH on DA neurons was blocked by pretreatment with the adrenergic $\alpha 1$ antagonist prazosin; thus, $\alpha 1$ receptors appear to mediate this excitatory effect (Shi et al. 2000). A more recent study using fast-scan cyclic voltammetry in freely moving rats showed that AMPH dose-dependently increases the amplitude, duration, and frequency of spontaneous dopamine transients (Daberkow et al. 2013). Other psychostimulants, such as cocaine, have also been shown to activate phasic dopamine release (Aragona et al. 2008); thus, it has been suggested that this effect may be generalized as an important mechanism of psychostimulants (Daberkow et al. 2013). The enhanced phasic DA signaling by AMPH appears to be mediated by its ability to upregulate the readily releasable pool involved in vesicular release of DA (Covey et al. 2013).

Role of DA and NA in psychostimulant reward: animal and human studies

The reinforcing properties of psychostimulants have been attributed to their capacity to increase levels of DA (*not* NA) in the NAc (Wise and Bozarth 1984). For example, dopaminergic lesions of the NAc reduced cocaine self-administration in rats, while lesions of the dorsal and ventral noradrenergic bundles failed to affect responding (Roberts et al. 1977). Moreover, dopamine receptor blockers affected AMPH and cocaine self-administration, but noradrenergic antagonism was without significant effect (de Wit and Wise 1977; Yokel and Wise 1975). However, while NA may not be critical to psychostimulant *reinforcement*, several studies suggest an important role of NA in mediating other effects of these drugs, namely the positive subjective (rewarding) effects,

behavioural sensitization, and reinstatement (for review, see Sofuoglu and Sewell 2009; Weinshenker and Schroeder 2007).

Since adult rat 50-kHz USV emissions induced by AMPH administration offer a potential gauge of subjective experience, they might shed light on the neurochemical mechanisms that mediate AMPH euphoria in humans. At present, the relative importance of DA *vs.* NA for mediating AMPH euphoria is controversial. Two early studies demonstrated an attenuation of AMPH-induced euphoria following administration of the DA D2 antagonist pimozide (Gunne et al. 1972; Jonsson 1972). However, three subsequent studies failed to show an effect of pimozide on AMPH-induced euphoria in normal, healthy volunteers (Brauer and de Wit 1995; Brauer and de Wit 1996; Brauer and de Wit 1997). This apparent discrepancy could be due to several factors, including drug doses and populations tested. Furthermore, the DA receptor antagonist haloperidol, but not the alpha adrenergic antagonist thymoxamine, prevented the experimenter-rated "excitation and elation" response to AMPH (Nurnberger et al. 1984). In contrast, a more recent study showed that AMPH-induced self-report measures of "stimulated", "high", and "good drug effects" were attenuated by prolonged administration of the NET inhibitor atomoxetine: prolonged (*vs.* acute) treatment with atomoxetine would be expected to attenuate NA responses to AMPH (Sofuoglu et al. 2009). Finally, AMPH-induced positive subjective response measures have been associated with polymorphisms of both the DA transporter gene (DAT1) (Lott et al. 2005) and the NA transporter gene (SLC6A2) (Dlugos et al. 2007), and have been correlated with both DA release (Abi-Dargham et al. 2003) and NA release (Rothman et al. 2001). Clearly, further studies are required in order to gain a comprehensive picture of the role of DA *vs.* NA in AMPH euphoria.

Opioids and USVs

Opioids are psychoactive substances that bind to three main classes of opioid receptors, namely μ, κ, and δ (Chevlen 2003). Opioid agonists, such as morphine, are commonly used clinically as powerful analgesics, but are also well-known for their ability to produce euphoria and other positive subjective effects (Van Ree et al. 1999). The latter properties have motivated their recreational use and have led many people down the path towards opioid dependence and addiction (Van Ree et al. 1999).

Morphine is highly reinforcing and readily produces a CPP in animal studies (Bardo et al. 1995; Stewart et al. 1984). These effects appear to be mediated predominantly through μ opioid receptors, located on GABAergic interneurons in the VTA (Feltenstein and See 2008; Koob et al. 1998). This action in turn leads to disinhibition of dopaminergic neurons in that region and thus increases DA in the NAc, the latter being a common feature of many drugs of abuse (Di Chiara and Imperato 1988; Wise 1996).

To date, the majority of studies examining opiate effects on ultrasonic calling have been in the context of antinociception and social stress on 22-kHz calls (e.g. Oliveira and Barros 2006; Vivian and Miczek 1999), or on isolation-induced pup calls (e.g. Barr and Wang 1992; Shair et al. 2005). Nevertheless, there are several reports on 50-kHz USV production in relation to morphine administration; these studies are reviewed on pages 135-136 of Chapter 4. It is still presently unclear however, whether rewarding morphine administration promotes 50-kHz calling, as would be expected if 50-kHz USVs do indeed reflect positive affect.

Statement of Purpose

As reviewed above, the 50-kHz ultrasonic vocalizations (USVs) emitted by adult rats are suggested to reflect positive affect. However, in-depth examination of these calls reveals remarkable variability in acoustic structure. The issue of call heterogeneity and its potential significance, namely that different call types convey distinct information and differ in their neurochemical basis, has been largely unexplored. The overall aim of this thesis therefore, was to identify subtypes of 50-kHz USVs emitted by adult rats and elucidate the neurochemical substrates underlying their emission using pharmacological means.

The **first specific objective** was to perform thorough analysis of 50-kHz calls produced by adult rats in order to outline and refine criteria for classification. Once a categorization scheme was established, a related aim was to determine whether amphetamine or pair-testing affects the relative prevalence of 50-kHz call subtypes or their acoustic characteristics (i.e. duration, bandwidth, and mean peak frequency). A further objective was to determine the extent of individual differences not only for call rate, but also subtype prevalence and acoustic call parameters.

Amphetamine, which enhances both dopaminergic and noradrenergic transmission, has pronounced effects on the 50-kHz call rate and the relative proportion of call subtypes. While dopaminergic transmission has been linked to 50-kHz USV production, the possible contribution of noradrenaline to the AMPH-induced USV responses remained uninvestigated. Thus, the **second specific objective** was to determine the effects of noradrenergic agonists and antagonists, alone and in combination with AMPH, on the 50-kHz USV rate and subtype profile. An additional objective was to examine whether the

effects of AMPH on USV emission would generalize to the IV route of administration and also to cocaine.

While psychostimulants and non-pharmacological appetitive stimuli increase the rate of 50-kHz calling and the proportion of trill calls, a similar finding had not been reported for another major class of euphoriant drugs, namely opioid agonists. Therefore, the **third specific aim** was to determine if rewarding doses of morphine, as confirmed by conditioned place preference, promote 50-kHz USVs or the trill subtype in particular.

Finally, the effects of AMPH on 50-kHz USVs were found to critically depend on noradrenergic transmission, but the possible contributions of dopamine remained unclear. Therefore, the **fourth specific aim** was to evaluate saline- and AMPH-induced 50-kHz USV emission following DA antagonist pretreatment targeting D1-like *vs.* D2-like DA receptors. A related aim was to determine whether DAT and NET inhibitors, alone or in combination, are sufficient to induce 50-kHz calling.

CHAPTER 2: Identification of multiple call categories within the rich repertoire of adult rat 50-kHz ultrasonic vocalizations: effects of amphetamine and social context

Jennifer M. Wright, Jim C. Gourdon, Paul B.S. Clarke

Psychopharmacology (Berl) (2010), **211**: 1-13

ABSTRACT

Rationale 50-kHz ultrasonic vocalizations (USVs) emitted by adult rats are heterogeneous; they occur over a wide frequency range, show varying degrees of frequency modulation, and appear to differ in their behavioral significance. However, they have not been extensively categorized. *Objectives* The main objective of this study was to identify subtypes of 50-kHz USVs emitted by adult rats and to determine how amphetamine (AMPH) or social testing condition affects their relative and absolute production rate and acoustic characteristics. A second objective was to determine the extent of individual differences in call rate, call subtype profile, and acoustic parameters (i.e., duration, bandwidth, and mean peak frequency). *Methods* Adult male Long–Evans rats were administered systemic amphetamine (0.25–2 mg/kg, IP) and tested individually or with a cage mate for 20 min. Call categories were defined based on visual inspection of over 20,000 USV spectrograms. Surgical devocalization was performed on a subset of AMPH-tested rats in order to confirm the authenticity of call subtypes. *Results* Fourteen categories of 50-kHz USVs were recognized. Call subtypes were differentially affected by social context, AMPH dose, and time within session. In contrast, the acoustic characteristics of call subtypes were notably stable. Marked and stable inter-individual differences occurred with respect to overall 50-kHz call rate, acoustic parameters, and call profile. *Conclusions* The present findings, obtained under saline and amphetamine test conditions, provide the first detailed classification of adult rat 50-kHz USVs. Consideration of 50-kHz USV subtypes may advance our understanding of inter-rat communication and affective state.

Keywords Ultrasonic vocalizations, Amphetamine, Reward, Frequency-modulated, Trill, Dose–response, Individual differences

INTRODUCTION

Ultrasonic vocalizations (USVs) have been observed in a number of rodent species (Sales 1972b). In adult laboratory rats, two main types of USVs have been described: 22-kHz and 50-kHz calls (see Brudzynski 2009 for review). The 22-kHz call type has been termed a distress or "alarm" vocalization (Litvin et al. 2007), as it can be elicited by the presentation of a predator, painful stimuli, startling noises, and intermale aggression (Blanchard et al. 1991; Calvino et al. 1996; Han et al. 2005; Kaltwasser 1991; Thomas et al. 1983). In contrast, calls of the 50-kHz category have been detected in naturalistic appetitive contexts, such as during play, mating behavior, exploratory activity, or in anticipation of food reward (Burgdorf et al. 2000; Knutson et al. 1998; Sales 1972b). 50-kHz calls have also been elicited by several non-natural appetitive stimuli, particularly rewarding electrical brain stimulation and amphetamine (AMPH) administration (Ahrens et al. 2009; Burgdorf et al. 2000, 2001a, 2007; Simola et al. 2009; Thompson et al. 2006; Wintink and Brudzynski 2001). Of note, the 50-kHz class of calls encompasses a wide frequency range (30–90 kHz) (Kaltwasser 1990a; Sales and Pye 1974), and these calls vary considerably in spectrographic structure (see below).

Until recently, rodent USVs were typically detected by means of frequency-division or heterodyne recording devices. These approaches allowed counting of calls but provided little or no information about acoustic parameters (Parsons 2000). In contrast, the use of high-frequency sampling of untransformed microphone signals has shown that adult rat 50-kHz USVs are heterogeneous, comprising "flat" (i.e., constant frequency) and "frequency-modulated" (FM) calls (Ahrens et al. 2009; Burgdorf et al. 2007, 2008a; Burgdorf and Panksepp 2006; Ciucci et al. 2009; Simola et al. 2009; Wohr et al. 2008).

Somewhat more detailed classification schemes have been described, each comprising three or four subtypes of 50-kHz calls (Kaltwasser 1990a; Vivian and Miczek 1993b; White et al. 1990b), but individual spectrograms seem to indicate a much richer diversity (Burgdorf et al. 2008a; Ciucci et al. 2009; Schwarting et al. 2007; Wohr et al. 2008). Recently, five USV subtypes were defined in adult mice (Panksepp et al. 2007) and as many as ten USV subtypes were documented in mouse pups (Scattoni et al. 2008), suggesting that a detailed classification of adult laboratory rat USVs would also be warranted.

Adult rat USVs can play a communicative role, as evidenced by the effects of devocalization and USV playback on rat behavior (Brudzynski and Chiu 1995; Burgdorf et al. 2008a; Thomas et al. 1981; Wohr and Schwarting 2007). Interestingly, FM and flat calls appear to differ in their neurochemical basis and behavioral significance (Ahrens et al. 2009; Burgdorf et al. 2007, 2008a; Burgdorf and Panksepp 2006). For example, FM calls have been suggested to signal a dopamine-dependent reward state (Burgdorf et al. 2008a) and, on preliminary evidence, are increased by the prototypical DA agonist AMPH more than flat calls (Simola et al. 2009). In contrast, flat calls may serve a social-coordinating function (Wohr et al. 2008). Rat 50-kHz USVs have frequently been detected even in the absence of conspecifics (e.g. Burgdorf et al. 2000; Schwarting et al. 2007), but whether rats make the same kinds of 50-kHz USVs when tested singly vs. paired with a conspecific has not, to our knowledge, been investigated. Stable and pronounced inter-individual differences with respect to USV production rates have been noted in several studies (Burgdorf et al. 2001b; Mallo et al. 2007; Schwarting et al. 2007; Wohr et al. 2008, 2009; Wohr and Schwarting 2009a). However, it has not been reported whether individual rats differ in terms of the specific call subtypes that they preferentially emit, that is, whether

each rat possesses a characteristic "call profile". In addition, there have been no reports of stable differences in acoustic parameters of calls.

The present study therefore addressed the following hypotheses: (1) the rich heterogeneity of 50-kHz USVs is captured within a moderate number of discrete call categories, (2) call categories are differentially modulated by AMPH treatment and the presence of conspecifics, and (3) individual differences exist, not only in terms of the overall number of 50-kHz calls emitted, as previously shown, but also in relation to call subtypes and acoustic parameters. A final experiment was performed in order to confirm the authenticity of individual 50-kHz USV subtypes by means of a surgical devocalization procedure (Roberts 1975a; White and Barfield 1990).

METHODS

Subjects

In Experiment 1, subjects were 24 experimentally-naïve male Long-Evans rats (Charles River Laboratories, St. Constant, Quebec, Canada), weighing 319-380 g at the beginning of the experiment. For Experiment 2, subjects were 36 experimentally-naïve male Long-Evans rats (Charles River Laboratories, St. Constant, Quebec, Canada), weighing 267-330 g at the start of the experiment. Subjects were housed three (Experiment 1) or two (Experiment 2) per cage (25 x 48 x 20 cm) in a temperature- and humidity-controlled colony room (19-20°C, 50-60%) at the McGill University Animal Research Center. The rats were maintained on a reverse 12:12 light/dark cycle, with lights off at 0700 h. All behavioral testing took place during the dark phase of the cycle. Food and water were available *ad libitum* except during testing. Before the start of the experiment, animals were handled for approximately

3 min daily for two days. All procedures were approved by the McGill Animal Care Committee in accordance with the guidelines of the Canadian Council on Animal Care.

Acoustic data acquisition, analysis, and classification of ultrasonic vocalizations

Testing took place in clear Plexiglas experimental chambers (ENV-007CT, Med Associates, St. Albans, VT), each of which was enclosed in a melamine compartment lined with sound-attenuating acoustic foam (Primacoustic, Port Coquitlam, British Columbia). Electret microphones with a frequency response range of 15 to 125 kHz (FG-23329-C05, Knowles Acoustic, Itasca, IL) were securely placed through small holes located centrally in the top panels of the experimental chambers. Consequently, the microphones were 15 - 30 cm from the rats during testing. Microphone signals were fed into a preamplifier (QuadMic, RME, Germany) and an anti-aliasing filter (Krohn-Hite 3323, Brockton, MA) before the analog signal was digitized through an A/D card (PCI-6251, National Instruments, Austin, TX) with a sampling rate of 200-kHz and a 16-bit resolution.

Acoustical analysis of the recordings was performed using Avisoft SASLab Pro (Version 4.2, Avisoft Bioacoustics, Berlin, Germany). Spectrograms were generated with a fast Fourier transform (FFT)-length of 512 points and an overlap of 75% (FlatTop window, 100% frame size). Correspondingly, spectrograms had a frequency resolution of 390 Hz and a time resolution of 0.64 ms. Calls were selected manually with section labels, and classified based on pre-defined frequency pattern criteria (see below); call classification was performed masked to the treatment condition. Three acoustic properties of each call were determined by the automatic parameter measurements feature of the software: duration, bandwidth (i.e. difference between the maximum and minimum peak frequency),

and mean peak frequency (i.e. time-averaged peak-energy frequency within each call). In order to improve accuracy of parameter measurements by the software, a threshold between -40 and -50 dB was set (setting: "Reject if peak amplitude <"). If background noise still interfered with proper measurement of acoustic parameters, those calls were discarded from the parameter analysis.

Each ultrasonic "call" had to meet three spectrographic criteria: (1) temporal continuity (i.e. maximal interruption of 20 ms), (2) fundamental frequency between 20 and 95 kHz, and (3) sound intensity and structure that was clearly distinct from background noise when seen under optimal viewing settings. From a visual inspection of over 20,000 USV spectrograms, 15 call categories were recognized (see Fig. 1):

(1) *Complex*: contain two or more directional changes in frequency of at least 3 kHz each

(2) *Upward ramp*: monotonically increasing in frequency, with a mean slope not less than 0.2 kHz/ms

(3) *Downward ramp*: monotonically decreasing in frequency, with a mean negative slope not less than 0.2 kHz/ms

(4) *Flat*: near-constant frequency greater than 30 kHz with a mean slope between -0.2 and 0.2 kHz/ms

(5) *Short*: duration less than 12 ms

(6) *Split*: middle component "jumps" to a lower frequency and contains a harmonic

(7) *Step-up*: instantaneous frequency change to a higher frequency

(8) *Step-down*: instantaneous frequency change to a lower frequency

(9) *Multi-step*: two or more instantaneous frequency changes

(10) *Trill*: rapid frequency oscillations with a period of approximately 15 ms (either sinusoidal or appearing as repeated "inverted-Us").

(11) *Flat/trill combination*: a trill that is flanked on one or both sides by a monotonic portion that is no less than 10 ms

(12) *Trill with jumps*: a trill that contains one or more higher-frequency components

(13) *Inverted-U*: a monotonic increase followed by a monotonic frequency decrease, each of at least 5 kHz

(14) *Composite*: calls (other than flat/trill combinations) that comprise two or more categories

(15) *22-kHz calls*: near-constant frequency calls between 20 and 25 kHz

Finally, a few (1%) other calls were classified as "miscellaneous" because they did not fit any of the above call categories. A proportion of calls (7%) was spectrographically unclear and categorized as “unclassifiable”.

Inter-rater reliability in call category identification was assessed using a subset of 500 calls which was independently rated by the main experimenter (J.M.W.) and a trained student. Reliability was high (Cohen's kappa= 0.95), such that 96% of calls received the same classification from both individuals. Intra-rater reliability, assessed by a repeat scoring of 500 calls by the main experimenter one week apart, was also high (Cohen’s kappa = 0.96), such that 97% of calls were assigned the same classification between scorings.

Drugs

D-amphetamine sulfate (Sigma Aldrich, Oakville, ON) was dissolved in sterile 0.9% saline and administered by IP injection in a volume of 1 ml/kg; doses are expressed as salt.

Experimental protocol

Experiment 1: Systemic AMPH dose-response in singly- and pair-tested rats

The experiment comprised an initial habituation day followed immediately by six test days, spaced two days apart in order to minimize possible carry-over effects of the drug. Rats were housed three per cage: one rat from each home cage (n = 8 rats) was randomly assigned to be tested singly throughout the experiment, and the other two rats from the same home cage were always tested together (n = 8 pairs). On the first day (Habituation), rats were placed in the test chambers for 10 minutes. Over the six test days, each rat or rat pair received two administrations of saline and all four doses of amphetamine (0.25, 0.5, 1, and 2 mg/kg), counterbalanced as fully as possible. Immediately following drug administration, the animals were placed in the test chambers and recorded for 20 min.

Experiment 2: Effects of surgical devocalization

This experiment was conducted in two parts (using 16 and 20 subjects, respectively). Rats were initially tested with AMPH in order to screen out low-rate callers as follows. Every two days over five days, each rat (n=36) received an IP injection of AMPH (1 mg/kg) and was immediately placed in the test chamber alone and recorded for 20 min. The 14 rats with the lowest number of USVs were excluded from the study. The remaining 22 rats were randomly allocated to two groups of comparable pre-surgery call rates (mean ± SEM 73 ± 7 *vs.* 77 ± 6 calls/min). One group received devocalization surgery; the other was sham-

operated (see "Surgery"). Following a 6-day recovery period, rats received one additional test session under AMPH. One sham-operated rat was excluded from analysis since data collection failed in the post-surgery session due to a technical problem.

Surgery

Under general anesthesia, achieved by 2–4 % isoflurane in conjunction with pure oxygen, a 2-cm incision was made on the ventral surface of the neck. Local anesthesia was provided by infiltration of lidocaine. The sternohyoideus muscle was separated to expose the trachea and locate the recurrent laryngeal nerves. Once located, approximately 3 mm of the nerve on both sides (bilateral section) was removed (devocalized), or left intact (sham). The incision was closed with subcutaneous suture and 1-2 staples. Rats were administered the analgesic carprofen (5 mg/kg SC) at surgery and again every 24 h for the following three days. One devocalized rat died the day following surgery. The rats were allowed to recover for 6 days before testing.

Data analysis and statistics

Data were analyzed using commercial software (Systat v11, SPSS Inc., Chicago, Illinois). The number of USVs acquired for the pair-tested rats was divided by two in order to express the results on a per-rat basis. Only USVs that occurred during minutes 3, 8, 13, and 18 (Experiment 1) or during minutes 13 and 18 (Experiment 2) of each 20-min recording were selected. When specifically examining the effect of AMPH on USVs in Experiment 1, only the latter two sampled time intervals were used in order to allow time for AMPH to take effect. 22-kHz calls accounted for less than 1% of USVs in this study and were not analyzed further.

The statistical analysis of acoustic parameters (duration, bandwidth, mean peak frequency) was performed by repeated-measures analysis of variance (ANOVA). The fact that not all subjects emitted every call subtype under every condition posed a challenge, since this type of ANOVA discards subjects with *any* missing values. As a first step, we excluded from analysis four call categories as well as four singly-tested rats which had a large number of missing values. Subsequently, missing values for each remaining rat were replaced by the mean of its other test conditions. In total, 5% of the raw data values were interpolated.

Data from the two saline test sessions were analyzed as follows. For the correlation of the total number of calls between the two saline tests, a Spearman's correlation coefficient was calculated, and unprotected paired *t*-tests were performed for each call category. For the remainder of the analysis, the number of calls was averaged across the first and second saline test. ANOVA was performed to test the effects of "group" (i.e. between-subjects; singly- *vs.* pair-tested), call "category" (within-subjects; 14 call subtypes), "time" (within-subjects; minutes 3, 8, 13, and 18), "dose" (within-subjects; 5 dose levels), and "rat" (between subjects; 8 singly-tested and 8 pair-tested rats), where appropriate, and was performed on each call subtype either in absolute terms, or as a proportion of all calls. Only call categories with a proportion greater than 2% in at least one of the groups were included in this analysis. Two-sample independent *t*-tests or paired *t*-tests were used where appropriate. All ANOVA p values were subject to the Huynh-Feldt correction where applicable. For all tests, a two-tailed *p* value less than 5% was considered significant.

RESULTS

Experiment 1: Systemic AMPH dose-response in singly- and pair-tested rats

(1) *Classification of rat ultrasonic vocalization subtypes*

Over 20,000 calls were examined in this experiment, from which 14 categories of 50-kHz calls were recognized; spectrograms of typical calls of each category are shown in Fig. 1. Three call subtypes were particularly prevalent, together comprising approximately 50% of the observed calls pooled across all experimental conditions: the trill (29% of calls), flat/trill combination (16%) and flat (14%). The least common 50-kHz subtype was the split call, accounting for only 0.5% of calls. Of the 93% of calls that were considered "classifiable" (see Methods), only 1% did not fall into one of the 14 categories. These infrequent miscellaneous calls varied widely in appearance.

(2) *Ultrasonic vocalizations during saline test sessions*

All 50-kHz call categories occurred during saline test sessions. The total number of calls made by each rat during the first and second saline test were significantly correlated (Pearson r = 0.63, df 14, $p<0.01$; Fig. 2a). The mean number of each call subtype did not differ significantly between the two saline tests in either the singly- or pair-tested rats. For all further analysis, the number of USVs was averaged across the two saline sessions.

During saline tests, pair-tested rats emitted approximately twice as many calls as singly-tested rats, on a per-rat basis (main effect of group: $F(1,14) = 4.84$, $p<0.05$; Fig. 2b). The call profile differed markedly between singly- and pair-tested rats (Fig. 3). In particular, pair-tested rats emitted a significantly higher proportion of trills ($t = 2.86$, df 10, $p<0.05$) and flat-trill combinations ($t = 2.24$, df 14, $p<0.05$) than singly-tested rats. The

proportion of flat calls appeared greater in the singly-tested rats, but this difference was not significant, perhaps because this measure was highly variable across singly-tested rats.

(3) *Effect of AMPH on ultrasonic vocalizations*

AMPH dose-dependently increased the total number of 50-kHz USVs per minute ($F(4,56) = 13.30$, $p<0.0001$; Fig. 4). Of note, all doses of AMPH tested, including 0.25 mg/kg, caused a significant increase in calls relative to the saline condition (paired *t*-tests with Bonferroni correction, n=16, $p<0.01$-0.0001). The two groups evinced comparable absolute increases in call rate (per rat) in response to AMPH. In order to assess whether repeated AMPH administration induced behavioural sensitization, we examined whether the overall rate of calling increased across test days; however, no significant change was observed.

AMPH administration did not result in the emergence of new call types, but it clearly altered the call profile (Fig. 5; see Table 1 for statistical analyses). The relative proportion of trill calls dose-dependently increased with AMPH in both singly- and pair-tested rats. The other call types that were preferentially affected by AMPH were the flats and shorts. Similar to the saline condition, pair-tested rats emitted a significantly higher proportion of trills and trills with jumps than singly-tested rats, irrespective of the AMPH dose.

(4) *Effect of time within session*

The overall call rate decreased over the test session ($F(3,42) = 3.320$, $p<0.05$), independently of AMPH dose or social condition (Fig. S1a). However, AMPH-*induced* calling (i.e. calls emitted under AMPH minus those under saline) was most pronounced during the last two sampled time intervals of the 20 min test session (i.e. minutes 13 and 18, Fig. S1b). The call profile also changed over time (category x time interaction, $F(39,507) = 2.63$,

$p<0.05$) irrespective of whether rats were tested singly or in pairs; the only significant time-dependent change was in the proportion of flat calls ($F(3,42) = 5.31$, $p = 0.02$), which became less frequent relative to other calls as the session progressed (i.e. minutes 3 *vs.* 8, 13, or 18: paired *t*-tests, $p<0.05$).

(5) *Correlation between call subtypes*

To test whether rats that preferentially emitted a particular call subtype would also preferentially emit (or avoid emitting) any other call subtypes, we performed an exploratory correlational analysis (Table S1). Among the more frequent call subtypes, there was a significant positive correlation between step-ups and flats, and between step-ups and flat-trill combinations. In contrast, a significant negative correlation occurred between trills and flats, and between trills and step-ups.

(6) *Acoustic parameters (duration, bandwidth, mean peak frequency)*

Acoustic data and related statistical analyses are presented in Tables 2 and S2. Acoustic parameters were largely unaffected by social testing condition and AMPH dose (Table S2). The only exception was a slight (approximately 2 kHz) but significant decrease in bandwidth at the lowest dose of AMPH relative to saline (not shown). There was no difference, in any acoustic parameter examined, between singly-tested and pair-tested rats. Not surprisingly, acoustic parameters differed markedly between 50-kHz call subtypes (main effects of call category: $p<0.0001$). All 50-kHz USV subtypes had a mean peak frequency between 48 and 60 kHz, except split calls, whose mean peak frequency was considerably lower (mean = 40.3 kHz, see Table 2). Split calls were also longer than other

50-kHz USV subtypes, with a mean duration of 105 ms *vs.* 10-90 ms for other call subtypes (Table 2).

(7) *Individual differences in rate of calling, acoustic parameters, and call profile*

Pronounced individual differences occurred in all three respects. First, individual rats differed consistently in terms of rate of calling. This was the case both within sessions and across AMPH doses (Cronbach's alpha = 0.979 and 0.907, respectively; Fig. S2a, b). Analogous differences occurred between rat pairs (Cronbach's alpha = 0.862 and 0.844, respectively; Fig. S2c, d). Second, individual differences in *acoustic parameters* (duration, bandwidth, and mean peak frequency) were found for almost every 50-kHz USV subtype; this is illustrated for trills and flats in Fig. 6. Finally, individual rats differed in their *call profiles (*rat x category interaction, $F(91,377) = 1.68$, $p<0.05$; Fig. S3), and analogous differences were found between dyads of pair-tested rats ($F(91,416) = 2.08$, $p<0.01$).

Experiment 2: Devocalization

Rats that were ultimately selected for surgery emitted 43-113 USVs/min during the pre-surgery AMPH screen. A comparison of the pre-and post-surgery AMPH tests showed that devocalization surgery suppressed USVs (group x time interaction: $F(1,18) = 25.87$, $p<0.001$); the devocalized group emitted significantly fewer calls (paired $t = 6.25$, df 3, $p<0.0001$) following surgery, whereas the rate of USVs emitted by sham-operated rats did not change significantly. Every call subtype was emitted by each group prior to surgery. USVs were completely abolished in 6 out of 10 devocalized rats, and the remaining rats emitted very few calls (mean ± SEM 4 ± 3 USVs/min) when tested under AMPH. Almost

every 50-kHz call subtype was significantly reduced following devocalization surgery (Mann-Whitney U test, $p < 0.05 – 0.001$). The only exceptions were the highly infrequent split and downward ramp calls; here, statistical power was limited. Sham surgery did not appear to alter the spectrographic appearance of 50-kHz calls or the call profile (data not shown); the low number of calls emitted by devocalized rats precluded analysis of these parameters. Finally, devocalized rats tended to gain less weight after surgery than the sham-operated rats (mean ± SEM, respectively: 30.3 ± 17.2 *vs.* 76.8 ± 3.5 g; $t = 2.65$, df 3, $p = 0.07$).

DISCUSSION

The present study provides the first detailed classification of adult rat 50-kHz USVs under saline and amphetamine test conditions. The authenticity of these calls was confirmed through surgical devocalization. Our analysis yielded several novel findings. Systemic amphetamine administration increased 50-kHz calling dose-dependently. More interestingly, call profiles (i.e. relative frequency of each call category) were differentially affected by social context, drug, and time within session. In particular: (1) pair-tested rats produced a higher proportion of trill calls than did singly-tested rats under both drug and saline conditions, (2) amphetamine altered the call profile such that trills became more prominent while flat calls became less so, and (3) flat calls became proportionately less frequent late in the session. In contrast, the acoustic characteristics of individual call subtypes (i.e. duration, bandwidth and mean peak frequency) were notably stable. Stable

inter-individual differences were also found, not only with respect to overall 50 kHz call rate, as previously reported, but also in terms of acoustic parameters and call profile.

All the 14 USV subtypes described here are likely to be authentic rat vocalizations, since they virtually all disappeared after surgical transection of the recurrent laryngeal nerve. The origin of adult rat 50-kHz ultrasounds has been the subject of some debate, with suggestions that some calls might represent a byproduct of locomotion (Blumberg 1992) or serve a thermoregulatory role (Blumberg and Moltz 1987). The locomotor artifact hypothesis, however, has been countered by several lines of evidence (Knutson et al. 2002; Simola et al. 2009). For example, doses of caffeine that would be expected to increase locomotor activity are reported not to increase the rate of 50-kHz calling in adult rats (Simola et al. 2009). A thermoregulatory role of 22-kHz USVs has been proposed in adult rats, in the context of sexual behavior (Blumberg and Moltz 1987). As in the case of copulation, the higher doses of amphetamine used in the present study likely produced mild (1-2 ºC) hyperthermia in the periphery (Lin et al. 1980; Ulus et al. 1975) and therefore quite possibly in the brain as well. However, it seems unlikely that the increased rate of 50-kHz calling seen after amphetamine administration would reflect an attempt at thermoregulation, insomuch as these calls are much briefer and less intense than 22-kHz USVs.

Previous 50-kHz classification schemes have comprised only three or four subtypes (Kaltwasser 1990a; Vivian and Miczek 1993b; White et al. 1990b). We now describe a considerably larger repertoire of calls. We cannot of course exclude the possible occurrence of additional call subtypes in other behavioral contexts, stages of development, or indeed by other rat strains. Importantly, our 14 call categories have nearly all been

depicted previously (see Table 3) and they appear to capture all published spectrographic examples of rat 50-kHz USVs. Interestingly, eight of the ten USV subtypes that were recently described in mice appear to have acoustic counterparts in adult rats (compare Fig. 1 with Fig.2 of Scattoni et al. 2008). This high degree of similarity possibly reflects physical constraints on call production; it remains to be determined whether these seemingly homologous call subtypes signal the same information in rats as in mice.

Automated call categorization has been achieved for a limited number of mouse USV subtypes (Holy and Guo 2005); a similar procedure for categorizing our 14 call subtypes would be highly desirable, for several reasons. First, manual categorization is highly labor-intensive. Second, although categorization was usually unambiguous (as reflected in high inter- and intra-rater reliability), mathematical modeling of 50-kHz calls could potentially eliminate any observer bias. Third, a modeling procedure might reveal further heterogeneity within our existing classification scheme.

In the present study, paired cage-mates called significantly more, on a per-rat basis, than rats tested alone under drug-free conditions. This result is in line with previous findings by Brudzynski and Pniak (2002) using unfamiliar conspecifics, and may be unsurprising given that USVs appear to have a communicative role (see Introduction). It should however be noted that our testing protocol did not distinguish between vocalizations emitted by individual pair-tested rats, and consequently, it is not clear whether each pair-tested rat was similarly affected by AMPH or cage-mate presence.

In our study, AMPH dose-dependently increased the rate of 50-kHz calling, consistent with previous investigations that employed single systemic AMPH doses (Ahrens et al. 2009; Simola et al. 2009; Wintink and Brudzynski 2001). Of note, even the

lowest dose of AMPH tested (0.25 mg/kg), a dose considered to be a "low" behaviorally effective dose in rats (Grilly and Loveland 2001), caused a highly significant increase in USVs, indicating that USVs are a relatively sensitive behavioral measure.

While systemic administration of AMPH has been widely reported to increase 50-kHz calling, effects on 50-kHz *call subtypes* have received less attention. In particular, Ahrens et al. (2009) showed that trill, but not flat, calls increased with repeated AMPH administration. In another study, a single, relatively high dose of AMPH (2 mg/kg) increased the FM-flat ratio (Simola et al. 2009). By examining the effect of AMPH on each of the 14 subtypes of 50-kHz USVs, we found that AMPH dose-dependently increased the proportion of trill calls and decreased the proportion of flat calls. Interestingly, the proportion of the other 12 subtypes of 50-kHz USVs remained stable across AMPH doses, although most 50-kHz subtypes significantly increased in absolute number.

Analysis of rodent 50-kHz USVs has tended to emphasize the calling rate rather than the spectrographic characteristics of calls; however, acoustic call parameters are also susceptible to experimental manipulation, in some cases independently of call rate (Ciucci et al. 2007; Hodgson et al. 2008; Simola et al. 2009; Vivian and Miczek 1993b). It has been reported that neither AMPH administration (Simola et al. 2009; Thompson et al. 2006) nor social testing conditions (Brudzynski and Pniak 2002) alter the acoustic parameters of 50-kHz calls; however, in these studies, little (or no) distinction was made between call subtypes. We now show a similar lack of effect across multiple 50-kHz call categories, the only exception being a modest effect of AMPH on bandwidth.

Inter-rat differences in 50-kHz call rates have been widely reported in response to various stimuli (Burgdorf et al. 2001b; Burgdorf et al. 2008b; Burgdorf et al. 2005; Mallo et

al. 2007; Schwarting et al. 2007; Wohr et al. 2009; Wohr et al. 2008; Wohr and Schwarting 2009a). In the present study, such differences were maintained not only within session, but also across different AMPH doses. To further elucidate the relationship between 50-kHz calls and AMPH's rewarding effects, it would be of interest to determine whether individual differences in AMPH-induced calling rate predicts intravenous self-administration or the magnitude of conditioned place preference.

To our knowledge, this study is the first to report individual differences in *call profiles* (i.e. the proportion of calls in each category). Thus, in addition to differences in absolute call rate, some rats, for example, favored complex calls over trills (or vice-versa). Furthermore, individual differences in *acoustic parameters,* previously reported for 22-kHz calls (van der Poel and Miczek 1991), were evident with respect to 50-kHz calls. Specifically, we found inter-rat differences in the duration, bandwidth, and mean peak frequency of each 50-kHz call subtype. Consequently, we speculate that the combination of call profile and acoustic parameters may be sufficient to allow rats to recognize individual conspecifics, even in the absence of odor cues.

Recent evidence suggests that 50-kHz call subtypes may differ in their behavioral significance; for example, FM calls, but not flat, have been associated with appetitive stimuli (e.g. Ahrens et al. 2009; Burgdorf et al. 2008a; Burgdorf et al. 2007; Burgdorf and Panksepp 2006; Wohr et al. 2008). In the present study, we used two stimuli: AMPH and the opportunity to interact with another similar-aged rat, both of which have been shown to be rewarding (Calcagnetti and Schechter 1992; Spyraki et al. 1982). Only trill calls were proportionally increased by both these conditions (flat-trill combinations increased only when comparing pair- to singly-tested rats). Therefore, we propose that among the 14 call

subtypes, it is specifically trill calls that are reward-associated. Flat calls, in contrast, have been observed in social conditions that are not necessarily appetitive (Burgdorf et al. 2008a; Stevenson et al. 2009; Wohr et al. 2008). In the present study, flat calls tended to be more prevalent in singly-tested rats than pair-tested rats, consistent with the proposal that their purpose is to re-establish social contact (Wohr et al. 2008). We also found a negative correlation between flat calls and trills, further suggesting that they are functionally distinct.

Conclusion

Our new classification scheme represents a significant extension of previous work by others, but it is unlikely to be complete. For example, only future investigation will show whether it generalizes to: adult male rats in other behavioral or pharmacological test conditions; female rats; other rat strains; and other stages of adult development. It is natural to speculate that the various individual call types each communicate unique information. Hence, in the short term, it will be important to try to elucidate the behavioral significance of 50-kHz call subtypes, for example by identifying experimental conditions that preferentially elicit them, and by studying the effects of call subtype playback on rodent behavior. If the meaning of 50-kHz call subtypes can be deciphered, this may offer new avenues for future behavioral and pharmacological studies.

Table 1: ANOVA results for the call profile data (i.e. proportion of calls in each category)

Call Type	S/P F(1,12)	AMPH F(4,48)	S/P x AMPH F(4,48)
Complex	1.75	0.93	0.37
Upward ramp	1.44	0.21	0.10
Downward ramp	0.00	0.69	1.17
Flat	1.34	**4.05***	0.29
Short	1.07	**3.15***	0.74
Split	n/a	n/a	n/a
Step up	0.12	1.34	**2.94***
Step down	1.17	1.33	1.34
Multi-step	1.94	1.56	**4.18****
Trill	**6.26***	**4.63****	0.21
Flat/trill combo	0.32	0.77	0.92
Trill with jumps	**4.91***	0.96	0.40
Inverted-U	3.77	1.25	0.95
Composite	0.99	1.31	1.08

Two-way analyses of variance (ANOVAs) were performed separately for each call subtype, with between-subjects factor S/P (singly- *vs.* pair-tested), and within-subjects factor AMPH (i.e. dose of amphetamine). n/a, split calls were excluded from this analysis (see Methods). n = 8 (rats or rat pairs). Significant F values are shown in bold *p<0.05, **p<0.01.

Corresponding data are shown in Fig. 5

Table 2: Call parameters (duration, bandwidth, and mean peak frequency) of each call category

	Duration (ms)				Bandwidth (kHz)				Mean peak frequency (kHz)			
Category	**n**	**Mean**	**SEM**	**5th and 95th percentile**	**n**	**Mean**	**SEM**	**5th and 95th percentile**	**n**	**Mean**	**SEM**	**5th and 95th percentile**
Complex	1017	28.0	0.32	15.3, 47.3	972	9.1	0.2	3.5, 25.0	996	54.6	0.2	41.1, 64.9
Upward ramp	395	30.3	0.57	15.3, 52.3	378	7.9	0.3	3.5, 19.5	383	52.1	0.3	42.5, 61.8
Downward ramp	88	23.9	1.41	10.2, 55.1	77	8.7	0.8	3.1, 25.5	83	50.4	1.0	37.2, 65.1
Flat	2595	34.8	0.44	13.4, 70.2	2401	3.5	0.0	0.4, 7.5	2460	48.8	0.1	40.2, 59.3
Short	657	9.6	0.07	7.0, 12.1	575	1.7	0.1	0.4, 4.2	624	57.3	0.3	42.2, 67.9
Split	51	105.5	19.26	37.9, 388.4	51	26.5	1.2	14.5, 39.8	51	40.3	0.9	31.2, 51.4
Step up	1649	34.7	0.30	16.6, 55.6	1592	19.0	0.1	12.1, 27.8	1617	52.9	0.1	44.2, 62.9
Step down	222	36.6	1.30	16.0, 71.2	219	16.3	0.5	7.8, 31.9	222	52.0	0.4	41.6, 62.2
Multi-step	439	39.1	0.66	19.2, 66.9	434	19.2	0.3	11.9, 33.1	437	53.4	0.3	41.5, 64.5
Trill	4773	45.7	0.31	20.4, 83.8	4528	15.0	0.2	3.5, 40.2	4644	59.9	0.1	48.2, 68.7
Flat-trill	2923	60.6	0.51	30.0, 114.5	2874	22.2	0.2	12.8, 39.1	2902	55.1	0.1	45.7, 64.0
Trill with jumps	383	67.5	1.62	31.3, 129.5	378	23.9	0.5	13.3, 43.7	382	54.4	0.3	42.6, 64.4
Inverted-U	106	27.1	1.04	15.2, 45.9	99	9.7	0.8	5.1, 29.3	103	51.7	0.7	42.1, 64.6
Composite	191	87.2	3.60	36.4, 192.4	191	25.5	0.8	12.5, 46.1	191	55.3	0.5	42.7, 64.9
22-kHz	117	795.2	49.54	218, 1910	117	9.4	0.7	1.5, 22.3	117	21.9	0.2	18.7, 25.1

Calls are from all testing conditions in Experiment 1. If background noise interfered with accurate measurement of acoustic parameters by the software, those calls were excluded from analysis (see Methods). In some cases, determination of only a subset of parameters was possible for a given call.

Table 3: **The 50-kHz call categories defined in the present study (i.e. call subtype) in relation to published spectrographic evidence from adult rats.**

Call subtype	Sex	Rat Strain	Age/Weight	Condition
Complex	Male[f, i,m]	Sprague-Dawley[f], Wistar[i,m]	192-226 g[i], 220-260 g[f], 280-310 g[m]	intra-NAc carbachol[f], heterospecific play[i], cage exploration[m]
Upward ramp	Male[f,g,k]	Sprague-Dawley[f], Wistar[g], Long-Evans[k]	220-260 g[f], 250-450 g[g], 300-400 g[k]	intra-NAc carbachol[f], intracerebral glutamate[g], aggression[k]
Downward ramp	Male[k]	Long-Evans[k]	300-400 g[k]	aggression[k]
Flat[b,c]	Male[a,e,f,g, i,j,k,m,n]	Long-Evans[a,e,j,k,l], Sprague-Dawley[f], Wistar[g,i,m,n]	380-540 g[a], 9-15 months[e], 220-260 g[f], 250-450 g[g], 192-226 g[i], 6-8 months[j], 300-400 g[k], 280-310 g[m,n]	IV AMPH[a], sexual behavior[e,l], intra-NAc carbachol[f], intracerebral glutamate[g], IP caffeine or AMPH[j], heterospecific play[i], aggression[k], cage exploration[m,n]
Short				
Split	Male[e,f]	Long-Evans[e], Sprague-Dawley[f]	9-15 months[e], 220-260 g[f],	sexual behavior[e], intra-NAc carbachol[f],
Step up	Male[f]	Sprague-Dawley[f]	220-260 g[f]	intra-NAc carbachol[f]
Step down	Male[f,m]	Sprague-Dawley[f], Wistar[m]	220-260 g[f], 280-310 g[m]	intra-NAc carbachol[f], cage exploration[m]
Multi-step	Male[f,h,n]	Black Rat[h], Sprague-Dawley[f], Wistar[n]	220-260 g[f], 280-310 g[n]	aggression[h], intra-NAc carbachol[f], cage exploration[n]
Trill	Male[i,j,n]	Black Rat[h], Long-Evans[j], Wistar[i,n]	192-226 g[i], 6-8 months[j], 280-310 g[n]	IP caffeine or AMPH[j], cage exploration[n], heterospecific play[i], sexual behavior[h]
Flat-trill combination	Male[i]	Long-Evans[l], Wistar[i]	192-226 g[i],	sexual behavior[l], heterospecific play[i]
Trill with jumps	Male[a,d,e]	Long-Evans[a,d,e]	380-540 g[a], 6 months[d], 9-15 months[e]	IV AMPH[a], sexual behavior[d,e]
Inverted-U	Male[f]	Sprague-Dawley[f]	220-260 g[f],	intra-NAc carbachol[f],
Composite[b.c]	Male[m,n]	Wistar[m,n]	280-310 g[m,n]	cage exploration[m,n]

a. Ahrens et al. (2009); **b**. Burgdorf et al. (2007); **c**. Burgdorf et al. (2008); **d**. Ciucci et al. (2007); **e**. Ciucci et al. (2009); **f**. Fendt et al. (2006); **g**. Fu and Brudzynski (1994); **h**. Kaltwasser (1990); **i**. Schwarting et al. (2007); **j**. Simola et al. (2009); **k**. Vivian and Miczek (1993); **l**. White et al. (1990); **m**. Wohr and Schwarting (2007); **n**. Wohr et al. (2008)

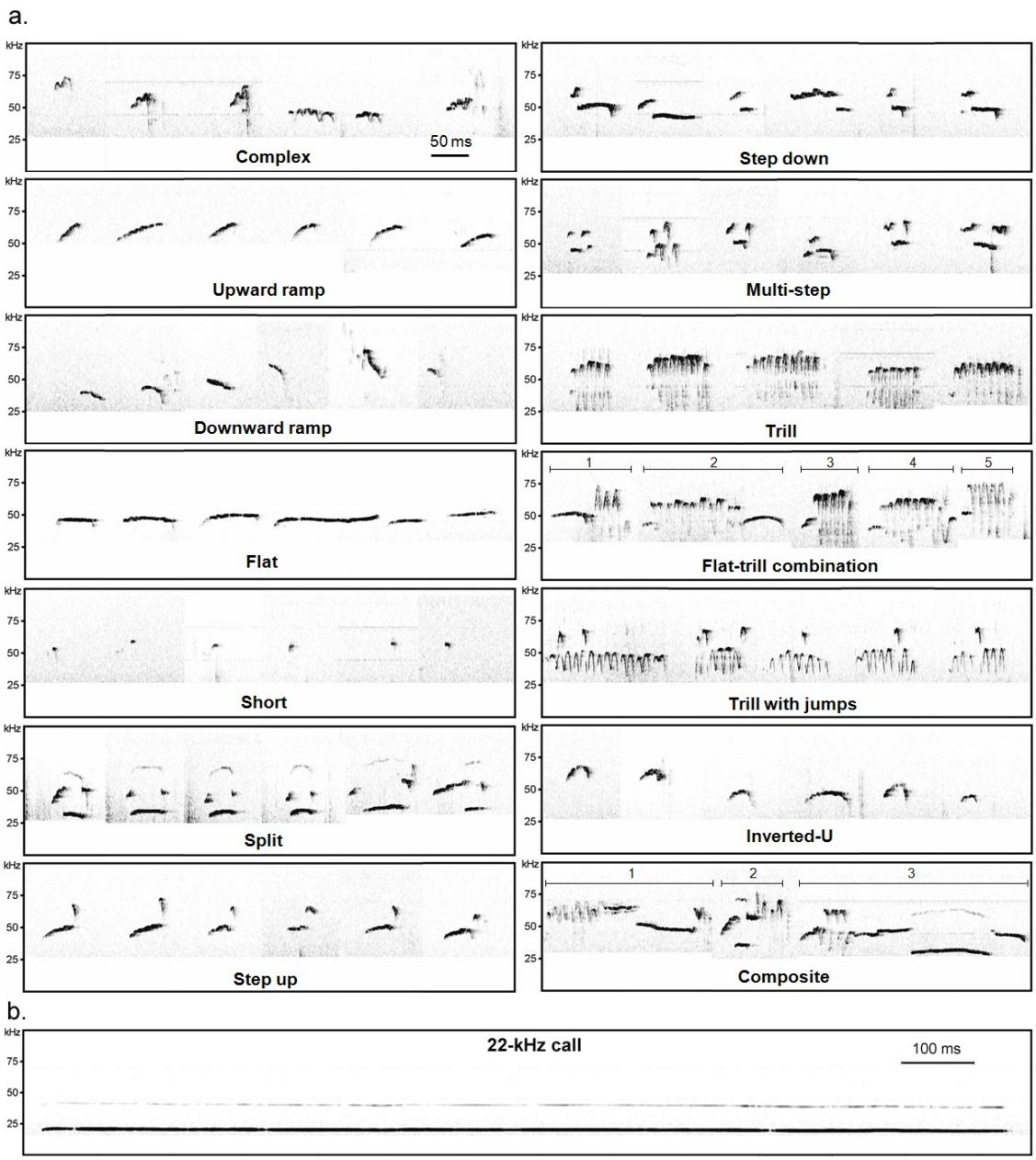

Fig. 1 Representative calls for each of the 14 categories of 50-kHz USVs (**a**) and a 22-kHz USV (**b**). Several exemplar calls are shown for each 50-kHz call category; these examples are not necessarily consecutive nor made by the same rat. Individual "flat-trill combination" and "composite" calls are differentiated by the accompanying line brackets. The time scale for all 50-kHz calls is indicated in the top left panel.

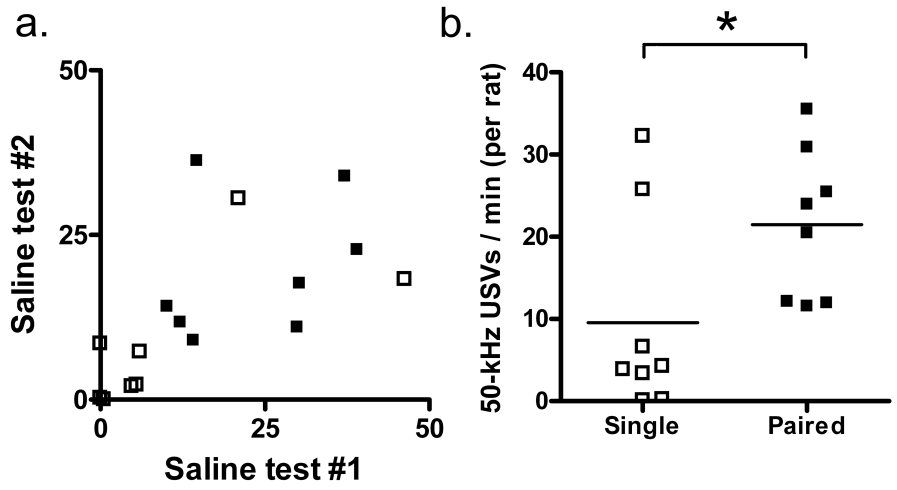

Fig. 2 Rate of 50-kHz calling in the two saline test sessions. Each rat or rat pair is represented by open or closed squares, respectively. The rate of 50-kHz calling was averaged across four time intervals (minutes 3, 8, 13 and 18 post-injection) and expressed as calls/min per rat. The 50-kHz call rate was significantly correlated between the first and second saline test sessions (Spearman r = 0.81, df 16, $p<0.01$) (**a**). Pair-tested rats emitted significantly more 50-kHz USVs (per rat) than singly-tested rats during saline test sessions (**b**). *$p<0.05$, t-test

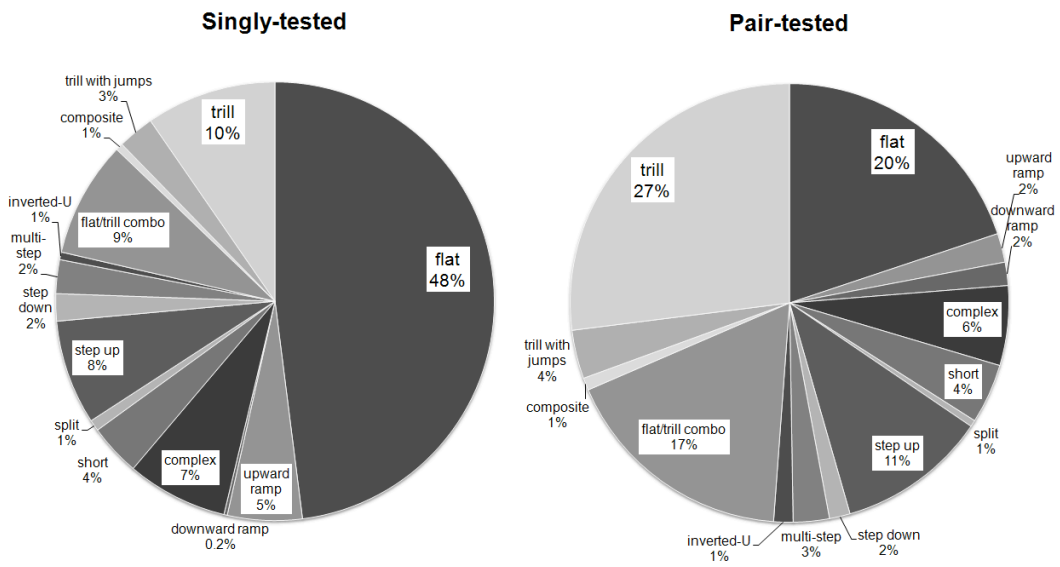

Fig. 3 Call profiles differed between singly-tested and pair-tested rats during saline test sessions. Each sector represents the group mean number of calls in a given category, expressed as a percentage of all classifiable calls (n=8 rats or rat pairs). Pair-tested rats emitted a significantly higher proportion of trills and flat-trill combinations than singly-tested rats (see text), whereas singly-tested rats tended to emit a higher percentage of flat calls.

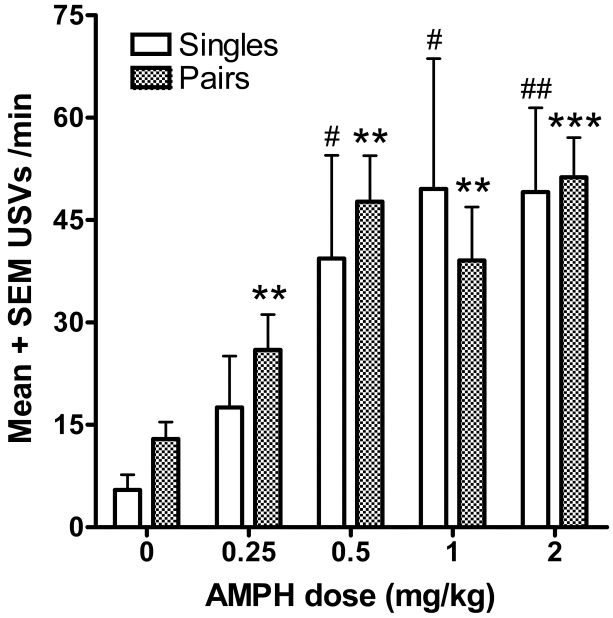

Fig. 4 AMPH dose-dependently increased the number of USVs emitted by singly- and pair-tested rats. The rate of 50-kHz calling was averaged across two time intervals (minutes 13 and 18 post-injection) and expressed as calls/min per rat. #p<0.05, ##p<0.01, **p<0.01, ***p<0.001 *vs.* corresponding saline condition (paired t-tests; n=8 rats or 8 rat pairs, respectively).

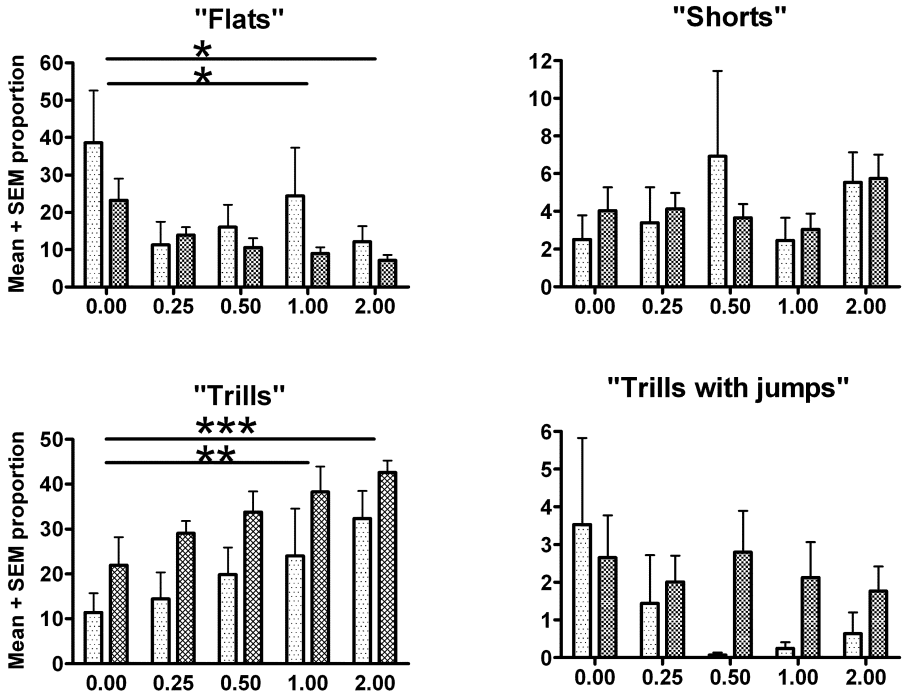

Fig. 5 Amphetamine dose-dependently increased the proportion of trill calls and suppressed flat calls. The *proportion* (mean ± SEM) of each call category for singly- and pair-tested rats is plotted against increasing AMPH dose (only subtypes that exhibited significant changes under AMPH are shown; see supplemental Fig. S4 for *all* call categories). Note also the overall higher proportion of trills and trills with jumps in the pair-tested rats compared to the singly-tested rats. Amphetamine-induced changes with respect to flat, short and trill calls were not significantly group-dependent (Table 1); pooling the two groups (i.e. single and pair-tested), significant differences from the saline condition are indicated by asterisks (*p<0.05, **p<0.01, ***p<0.001, paired t-tests, n = 16).

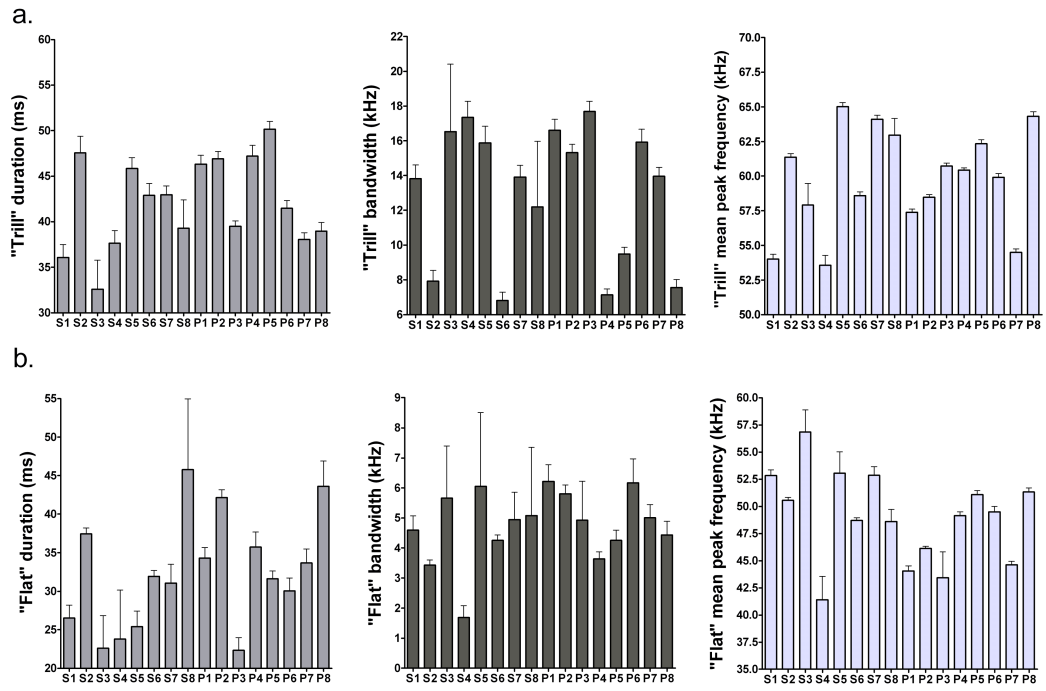

Fig. 6 Individual differences in acoustic parameters of 50-kHz calls. Differences in duration, bandwidth, and mean peak frequency were observed for every call subtype, and are exemplified here for trills (**a**) and flat calls (**b**). The y-axis variables are expressed as mean + SEM. The x axis shows each singly-tested rat (S1-S8) and each pair-tested rat (P1-P8). One-way ANOVAs; n = 9-760 calls; $p<0.0001$

Supplementary Material for Chapter 2

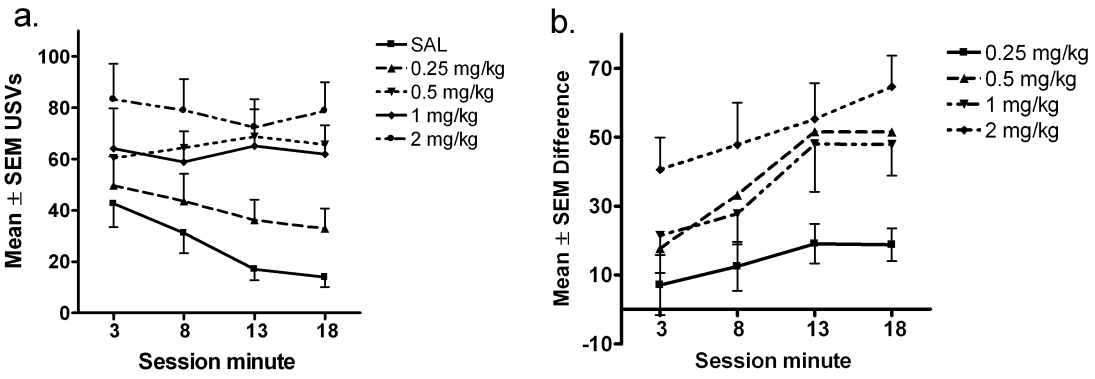

Figure S1: The mean ± SEM number of USVs (per minute) during each of the sampled time intervals during the 20-min session for each of the drug conditions (a), and for the AMPH-SAL difference scores for each AMPH dose (b).

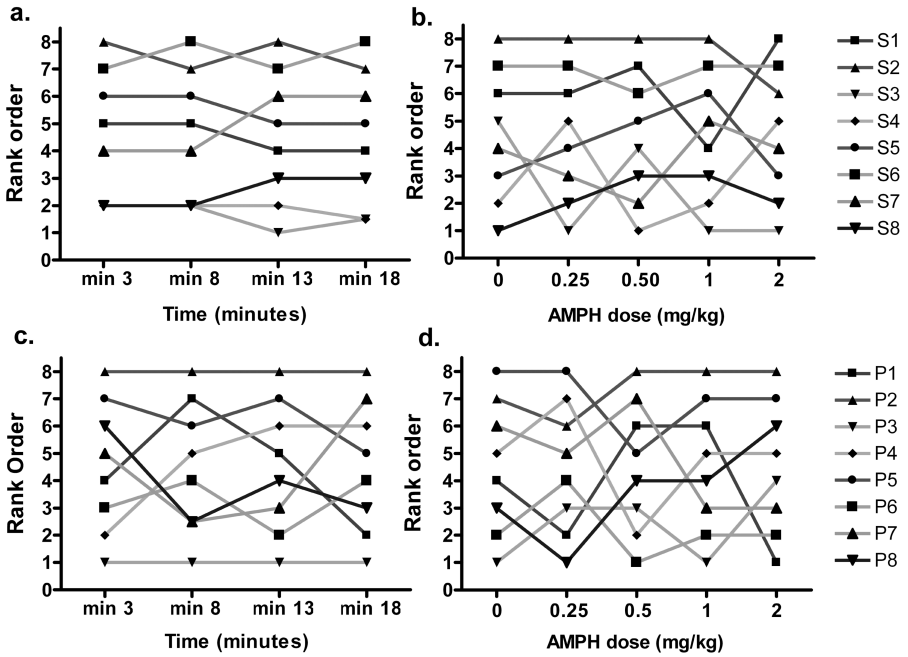

Figure S2: Individual differences in call rate were maintained within test sessions and across AMPH doses. Each rat (panels a and b) or rat pair (panels b and d) was ranked with respect to the total number of USVs emitted, either during particular 1-min time intervals within test sessions (panels a and c) or at different AMPH doses (panels b and d). For the purposes of illustration, the time course data (panels a and c) are from AMPH 1 mg/kg test sessions only. Dose–response data are from numbers of calls averaged across minutes 3, 8, 13, and 18 of the session. Cronbach's alpha = 0.844–0.979 (see text)

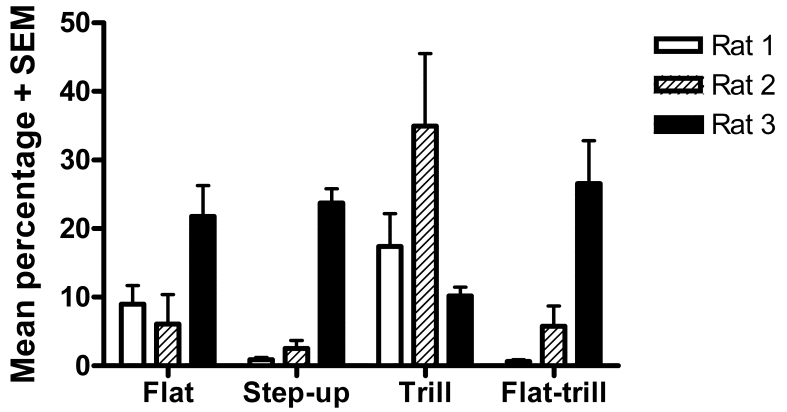

Figure S3: Individual differences in call profile across test sessions. The number of flats, step-ups, trills, and flat–trill combinations as a percentage (+SEM) of the total number of calls (i.e. all subtypes) is shown for three singly-tested rats (n = 5 dose levels). Across test sessions, individual rats exhibit higher or lower proportions of the various subtypes compared to other rats. These rats were chosen to illustrate this phenomenon based on clear differences in tendency to emit the four most frequent call categories.

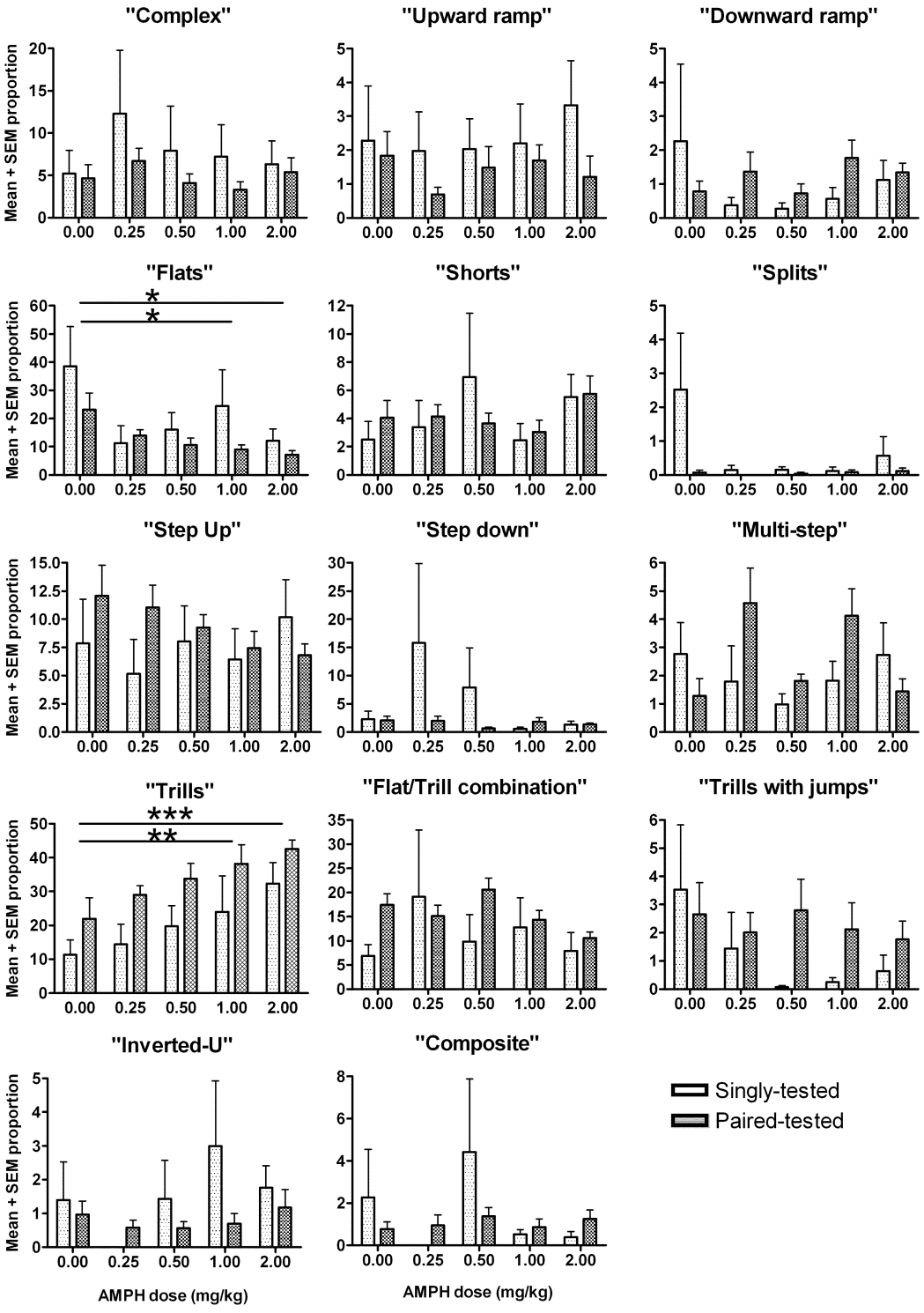
"Complex"
"Upward ramp"
"Downward ramp"
"Flats"
"Shorts"
"Splits"
"Step Up"
"Step down"
"Multi-step"
"Trills"
"Flat/Trill combination"
"Trills with jumps"
"Inverted-U"
"Composite"
Mean + SEM proportion
AMPH dose (mg/kg)
0.00
0.25
0.50
1.00
2.00
*
*
***
**
Singly-tested
Paired-tested

Figure S4: Amphetamine dose-dependently increased the proportion of trill calls and suppressed flat calls. The proportion (mean ± SEM) of each call category for singly- and pair-tested rats is plotted against increasing AMPH dose. Note also the overall higher proportion of trills and trills with jumps in the pair-tested rats compared to the singly-tested rats. Caveat: some call categories were emitted very infrequently, namely the upward and downward ramps, the split, and the inverted U. For example, 1% in the saline condition for singly-tested rats corresponds to approximately three calls. Amphetamine-induced changes with respect to flat, short, and trill calls were not significantly group-dependent (Table 1); pooling the two groups (i.e., single and pair-tested), significant differences from the saline condition are indicated by asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, paired t tests, $n = 16$)

Table S1: Correlation between the average proportion of each call subtype across all test conditions

	CX	UP	DR	FL	SH	SP	SU	SD	MS	TR	FT	TJ	IU
complex													
upward ramp	***0.53**												
downward ramp	-0.03	-0.07											
flat	-0.03	0.49	0.30										
short	0.12	0.10	0.22	-0.02									
split	-0.18	0.25	-0.28	0.23	-0.28								
step up	-0.40	0.07	-0.11	***0.55**	0.03	****0.63**							
step down	0.23	-0.04	0.42	0.17	-0.10	-0.22	-0.35						
multi-step	-0.02	-0.34	-0.27	-0.37	-0.32	-0.16	-0.25	0.37					
trill	-0.31	***-0.59**	0.01	****-0.73**	-0.11	-0.45	****-0.65**	0.05	0.16				
flat-trill	-0.34	0.11	-0.35	0.28	***-0.57**	****0.74**	****0.65**	-0.47	0.00	-0.43			
trill with jumps	-0.15	-0.32	-0.03	-0.21	-0.15	-0.15	-0.17	0.10	*****0.75**	0.05	0.08		
inverted-U	0.42	0.24	0.42	0.18	-0.10	-0.07	-0.37	***0.51**	-0.21	-0.02	-0.27	-0.17	
Composite	0.14	0.27	***0.52**	0.44	-0.35	0.14	0.01	0.20	-0.15	-0.29	0.26	-0.02	0.21

CX = complex; UP = upward ramp; DR = downramp ramp; FL = flat; SH = short; SP = split; SU = step up; SD = step down; MS = multi-step; TR = trill; FT = flat-trill combination; TJ = trill with jumps; IU = inverted-U

Pearson's r values are presented in the table, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Table S2: ANOVA results for the effect of AMPH, social testing condition, and call category on acoustic parameters

		F value		
Comparison	**df values**	**Duration**	**Bandwidth**	**Mean PF**
Group: F(1,10)	1, 10	0.04	1.62	1.75
Dose: F(4,40)	4, 40	1.64	**3.50***	0.88
Category: F(9,90)	9, 90	**88.63*****	**98.17*****	**15.10*****
Dose x Category: F(36,360)	36, 360	1.07	1.57	1.41
Category x Group: F(9,90)	9, 90	1.66	1.28	2.02
Dose x Group: F(4,40)	4, 40	1.87	0.79	1.60
Dose x Category x Group: F(36,360)	36, 360	1.14	1.13	1.12

Three-way ANOVA was performed with factors "group" (single *vs.* pair testing), "dose" (five doses of AMPH), and "category" (fourteen 50-kHz call subtypes). Significant F values are shown in bold.

*p<0.05, ***p<0.00001

Intervening Section 1

In the previous chapter, the 50-kHz USVs produced by adult rats were found to comprise an acoustically discrete set of at least 14 call subtypes. In addition, both social context and AMPH administration not only affected the overall rate of calling, but also the propensity to emit certain call categories in particular. These findings suggested that individual 50-kHz USV subtypes may convey distinct information, and the neurochemical mechanisms underlying the production of 50-kHz USVs might be subtype-specific. Thus, in Chapter 3, the possible contribution of noradrenaline in the emission of 50-kHz USVs was investigated. Several noradrenergic receptor agonists and antagonists were administered alone or in conjunction with AMPH. Moreover, the AMPH-induced USV effects observed in Chapter 2 were obtained using IP injections; Chapter 3 examined whether these results generalize to the IV route, and also to another psychostimulant (i.e. cocaine).

CHAPTER 3: Alpha- and beta-adrenergic receptors differentially modulate the emission of spontaneous and amphetamine-induced 50-kHz ultrasonic vocalizations in adult rats

Jennifer M. Wright, May R.S. Dobosiewicz, Paul B.S. Clarke

Neuropsychopharmacology (2012), **37**: 808-821

ABSTRACT

Amphetamine (AMPH) increases adult rat 50-kHz ultrasonic vocalizations, preferentially promoting frequency-modulated calls that have been proposed to reflect positive affect. The main objective of the present study was to investigate a possible noradrenergic contribution to AMPH-induced calling. Adult male Long-Evans rats were tested with AMPH (1 mg/kg IP) or saline combined with various systemic pretreatments: clonidine (α2 adrenergic agonist), prazosin (α1 antagonist), atipamezole (α2 antagonist), propranolol, betaxolol and/or ICI 118,551 (β1/β2, β1, and β2 antagonists, respectively), nadolol (β1/β2 antagonist, peripheral only) or NAD-299 ($5HT_{1A}$ antagonist). In addition, effects of cirazoline (α1 adrenergic agonist) and cocaine (0.25-1.5 mg/kg IV) were studied alone. AMPH-induced calling was suppressed by low-dose clonidine and prazosin. Cirazoline and atipamezole did not significantly affect calling rate. Propranolol, without affecting the call rate, dose-dependently promoted "flat" calls under AMPH while suppressing "trills", thus reversing the effects of AMPH on the *call subtype profile*. This effect of propranolol appeared to be mediated by simultaneous inhibition of CNS β1 and β2 rather than $5HT_{1A}$ receptors. Finally, cocaine elicited fewer calls than AMPH but produced the same shift in the call subtype profile. Taken together, these results reveal differential drug effects on flat *vs.* trill *vs.* other frequency-modulated 50-kHz calls. These findings highlight the value of detailed call subtype analyses, and show that 50-kHz calls are associated with adrenergic α1 and β receptor mechanisms. These preclinical findings suggest that noradrenergic contributions to psychostimulant subjective effects may warrant further investigation.

Keywords: ultrasonic vocalizations, rat, amphetamine, cocaine, noradrenaline, trills

INTRODUCTION

Adult laboratory rats emit two main types of ultrasonic vocalizations (USVs), commonly termed 22-kHz calls and 50-kHz calls. Evidence suggests that USVs may play a communicative role (Brudzynski 2005; Burgdorf *et al*, 2008a; Wohr and Schwarting 2009a). Vocalizations of the 22-kHz type serve as alarm or distress calls (Covington and Miczek 2003; Litvin *et al*, 2007) , whereas 50-kHz calls are frequently elicited by appetitive stimuli (Burgdorf *et al*, 2010; Knutson *et al*, 2002).

The 50-kHz class of adult rat USVs encompasses a wide frequency range (30-90 kHz) (Kaltwasser 1990a; Sales and Pye 1974; Wright *et al*, 2010) and comprises two main sub-classes: flat (i.e. constant frequency) and frequency-modulated (FM) calls. These two sub-classes appear to differ in their behavioral significance and neurochemical basis (Ahrens *et al*, 2009; Barker *et al*, 2010; Burgdorf *et al*, 2008a; Burgdorf and Panksepp 2006; Burgdorf *et al*, 2007; Ciucci *et al*, 2009; Meyer *et al*, 2011; Simola *et al*, 2009; Wohr *et al*, 2008; Wohr *et al*, 2009). FM 50-kHz USVs are diverse, with at least 13 acoustic subtypes, and the prevalent "trill" call subtype, in particular, consistently occurs in appetitive situations (Burgdorf *et al*, 2008a). On this basis, it has been proposed that FM calls (and especially trill calls) reflect an emotional state homologous to positive affect in humans (Burgdorf and Moskal 2009; Burgdorf *et al*, 2010).

The prototypical euphoriant d-amphetamine (Foltin and Fischman 1991) increases the rate of 50-kHz call production in adult rats, both after systemic and central administration (Ahrens *et al*, 2009; Burgdorf *et al*, 2001; Simola *et al*, 2009; Thompson *et al*, 2006; Wintink and Brudzynski 2001; Wright *et al*, 2010). In addition, AMPH has been shown to modify the 50-kHz call *profile* (i.e. the relative proportion of different call

subtypes), preferentially increasing trills and decreasing flat calls (Wright *et al*, 2010). Cocaine administration is also reported to promote 50-kHz calling (Barker *et al*, 2010; Browning *et al*, 2011; Ma *et al*, 2010; Maier *et al*, 2010; Mu *et al*, 2009; Williams and Undieh 2010), and a recent report shows a preferential increase in *FM* 50-kHz calls in response to IP cocaine (Meyer *et al*, 2011). However, whether intravenous cocaine mimics the amphetamine-induced shift in the call profile has not been reported.

Dopaminergic transmission appears to play a key role in the production of 50-kHz USVs. In particular, dopaminergic manipulations are reported to affect calls elicited by AMPH (Thompson *et al*, 2006), sex-relevant odors (Ciucci *et al*, 2009), tickling (Burgdorf *et al*, 2007), and intracerebral glutamate (Wintink and Brudzynski 2001). However, the observation that dopamine (DA)-depleting lesions inhibited FM but not flat 50-kHz calls (Burgdorf *et al*, 2007; Ciucci *et al*, 2009) indicates that not *all* 50-kHz calls are necessarily dopamine-dependent.

AMPH and cocaine promote noradrenergic as well as dopaminergic neurotransmission (McKittrick and Abercrombie 2007; Segal and Kuczenski 1997). However, a possible noradrenergic role in the production of adult rat 50-kHz USVs has not, to our knowledge, been investigated, except in the context of social stress (Tornatzky and Miczek 1994). This issue is of interest for several reasons. First, recent evidence supports a noradrenergic contribution to conventional reward-related behaviors, notably conditioned place preference and reinstatement of intravenous self-administration (for review, see Weinshenker and Schroeder 2007; see also Discussion). Second, noradrenaline (NA) appears also to contribute to the discriminative stimulus effects of AMPH in several species (Snoddy and Tessel 1983; Snoddy and Tessel 1985); these cues potentially model

subjective drug effects in humans (Stolerman 1992). Third, early studies indicated that AMPH euphoria in human subjects is critically dependent on catecholaminergic transmission (Jonsson *et al*, 1971; Jonsson *et al*, 1969), and in some studies AMPH euphoria appeared to be DA-*independent*, suggesting a possible role for NA (Brauer and de Wit 1997; Rothman *et al*, 2001; Sofuoglu *et al*, 2009).

The main aim of the present study was to test the hypothesis that noradrenaline (or adrenaline) contributes to the emission of spontaneous and AMPH-induced 50-kHz USVs, potentially in a call subtype-selective manner. To this end, we first examined whether 50-kHz USV emission under AMPH was altered by acute pretreatment with the α2 agonist clonidine, given at doses that decrease NA release (Schoffelmeer and Mulder 1984). We then tested the α1 adrenergic antagonist prazosin, the α1 agonist cirazoline, the α2 antagonist atipamezole, and the β1/β2 blocker propranolol. Propranolol produced a dose-dependent shift in the call profile under AMPH and we subsequently investigated the pharmacological mechanism underlying this effect: (1) to test for peripheral mediation, we administered nadolol, a non-selective hydrophilic β blocker which does not readily cross the blood-brain barrier (Schiff and Saxey 1984), (2) we evaluated the contribution of β1 vs. β2 receptor blockade using selective antagonists (betaxolol and ICI 118,551), and (3) since propranolol is a weak $5HT_{1A}$ receptor antagonist, we tested a selective antagonist of this receptor (NAD-299)(Ross *et al*, 1999). In a final experiment, we tested whether the call subtype-dependent effects produced by intraperitoneal (IP) administration of AMPH would generalize to the intravenous (IV) route and also to cocaine.

MATERIALS AND METHODS

Subjects

Subjects were 77 male Long-Evans rats (Charles River Laboratories, St. Constant, Quebec, Canada), weighing 307-425 g (i.e. aged approx. 9-11 wks old) at the start of the experiment. They were housed two per cage (25 x 48 x 20 cm) in a temperature- and humidity-controlled colony room (19-20°C, 50-60%) at the McGill University Animal Research Center. The rats were maintained on a reverse 12:12 light/dark cycle, with lights off at 0700 h. All behavioral testing took place during the dark phase of the cycle. Food and water were available *ad libitum*, except during testing sessions. All procedures were approved by the McGill Animal Care Committee in accordance with the guidelines of the Canadian Council on Animal Care. In all experiments rats were initially drug- and experimentally-naïve; Experiments 3 and 6 were each divided into two parts, with Part b beginning within a week after the end of Part a.

Overview of experiments

Almost all experiments investigated the effects of various drug pretreatments on the USV response (i.e. call rate and acoustic profile) to systemic (IP) AMPH. Exceptionally, Experiment 3a examined the acute USV response to cirazoline and atipamezole alone, and Experiment 7 comprised a dose-response study of IV cocaine given alone. Details of individual experiments are summarized in Table 1.

Protocol for individual experiments

Amphetamine screen

A significant minority of rats emit few USVs in response to systemic amphetamine (Wright et al. 2010). In order to identify and exclude such subjects, rats were initially screened for AMPH-induced calling in three 20-min test sessions spaced two days apart. Immediately before each session, rats were administered AMPH (1 mg/kg, IP) and then placed in a test chamber. On the intervening days, the rats remained in their home cages. Only the third AMPH test session was analyzed because the first two sessions are not necessarily indicative of a rat's subsequent USV response to AMPH (unpublished observation). USVs that were emitted 10-20 min post-injection were counted; the rats with the lowest rate of calling (i.e. 20-43% of rats depending on the experiment) were excluded from subsequent testing. In total, 47 out of 124 rats were excluded on this basis.

Drug testing

Drug testing was initiated 2-5 days after the final amphetamine screening session, with the exception of Experiment 7 (i.e. 11 days) where the rats needed to recover from surgery before drug testing began. All experiments employed a fully parametric within-subject design in which each rat was tested once under each drug condition (see Table 1 for details). Thus, in Experiments 1, 2, 3b, and 4-6, rats received all combinations of pretreatment and treatment drugs including vehicle controls. Similarly, in Experiments 3a and 7, rats received a test with each of the following: vehicle, AMPH, and each dose of the drug(s) being tested. Within each experiment, the order of testing was counterbalanced as far as possible. Test sessions were of 20 min duration except in Experiment 7; here,

subjects were given IV cocaine or AMPH and were tested only 0-10 min post-injection, i.e. during the period of drug onset. Test sessions were spaced two days apart in order to minimize possible carry-over effects of the drugs.

For Experiment 7 (IV cocaine and AMPH), rats first underwent IV catheterization surgery (see below). Following recovery, the experiment comprised an initial habituation day, whereby the rats were placed in the test chambers for 10 min, then removed and immediately injected with 0.1 ml heparin-Baytril™-saline solution in order to maintain catheter patency. On the five test days that followed, each rat received a 10-sec infusion of drug directly after they were placed in the test chamber. Immediately following drug infusion, the tubing was disconnected and the session started.

Intravenous catheterization surgery

General anesthesia was provided by ketamine HCl (80 mg/kg IP) and xylazine HCl (16 mg/kg IP). A 5-mm incision was made on the right ventral surface of the neck. A chronic indwelling silastic catheter (0.5 mm I.D. and 0.9 mm O.D., Fisher Scientific, Montreal, Quebec) was inserted in the right jugular vein and secured with silk sutures. The catheter was passed subcutaneously to a 2-cm incision on the head, where it was connected to a modified plastic cannula (Plastics One, Roanoke, VA), which was then anchored to the top of the skull with stainless steel mounting screws (Plastics One, Roanoke, VA) and dental cement (Stoelting, Wooddale, IL). The cannula was blocked with a plastic stopper made from Tygon tubing (Fisher Scientific, Montreal, Quebec), and shielded with an aluminum cap when not in use. The analgesic carprofen (5 mg/kg SC) was administered during surgery to alleviate post-surgical pain. Four rats out of 19 died from anesthetic overdose

during surgery. In order to verify catheter patency, each rat received an infusion of Na methohexital ("Brevital", 1 mg in 0.1 ml, 2-sec infusion, IV) once in their home cage, 3-5 days post-surgery; three rats failed to show the expected sedative response and were therefore excluded from the experiment. The remaining rats were allowed 7-9 days of recovery before experimental testing began. Immediately following the habituation session and after each test session, the catheters were flushed with 0.1 ml of a sterile 0.9% saline solution containing 0.2 mg/ml heparin (Sigma-Aldrich, Oakville, Ontario) and 17 mg/ml Baytril™ (ICN Biomedicals, Cleveland, OH).

Acquisition and classification of ultrasonic vocalizations

Testing took place in clear Plexiglas experimental chambers (ENV-007CT, Med Associates, St. Albans, VT), each of which was enclosed in a melamine compartment lined with sound-attenuating acoustic foam (Primacoustic, Port Coquitlam, British Columbia). Condenser ultrasound microphones (CM16/CMPA, Avisoft Bioacoustics, Berlin, Germany) were securely inserted through small (5 cm diameter) holes located centrally in the top panels of the experimental chambers. Consequently, the microphones were 15-30 cm from the rats during testing. Microphone signals were fed into an UltraSoundGate 416H data acquisition device (Avisoft Bioacoustics, Berlin, Germany) with a sampling rate of 250-kHz and a 16-bit resolution.

Acoustical analysis was performed using Avisoft SASLab Pro (version 4.2, Avisoft Bioacoustics, Berlin, Germany). Spectrograms were generated with a fast Fourier transform (FFT)-length of 512 points and an overlap of 75% (FlatTop window, 100% frame size). Correspondingly, spectrograms had a frequency resolution of 490 Hz and a time resolution

of 0.5 ms. Calls were selected manually from spectrograms by an individual who was masked to the treatment condition. Each identified 50-kHz call was classified into 1 of 14 distinct categories: complex, upward ramp, downward ramp, flat, short, split, step-up, step-down, multi-step, trill, flat-trill combination, trill with jumps, or composite (see Wright *et al*, 2010 for criteria for call identification and classification, several examples of each call type, as well as descriptive statistics relating to acoustic parameters). This method of manual call selection has been validated by surgical devocalization, and classification is associated with high inter- and intra-rater reliability (Wright *et al*, 2010). Some representative 50-kHz USVs are shown in Figure 1. 22-kHz calls were rarely observed in this study and were not analyzed further.

Drugs

All test drugs, doses and routes of administration are shown in Table 1. Drugs were: d-amphetamine sulfate (Sigma-Aldrich Co Ltd, Poole, UK); cocaine HCl (Medisca, St-Laurent, Quebec); clonidine HCl, prazosin HCl, (±)-propranolol HCl, and nadolol (all from Sigma-Aldrich, Oakville, Ontario); NAD-299 HCl (i.e. Robalzotan), betaxolol HCl, ICI 118,551 HCl, cirazoline HCl, and atipamezole HCl (all from Tocris Bioscience, Ellisville, MO). The doses of prazosin, propranolol, clonidine, and nadolol are expressed as the free base; all other drug doses are expressed as the salt. Drugs were dissolved in sterile 0.9% saline and were administered in a volume of 1 ml/kg body weight with the following exceptions: (1) prazosin was dissolved in distilled water, (2) the combination of betaxolol and ICI 118,551 in Experiment 6 was administered in a volume of 4 ml/kg (divided into 2 separate injections), and (3) nadolol (Experiment 5), as well as betaxolol and ICI 118,551 in

Experiment 5 were administered in a volume of 2 ml/kg. Control injections were of saline (Experiments 1 and 3-7) or water (for prazosin, Experiment 2) and administered in the same volume as the corresponding drug.

Data analysis and statistics

Data were analyzed using commercial software (Systat v11, SPSS Inc., Chicago, IL; GraphPad Prism 4, GraphPad Software Inc., La Jolla, CA). For the IV cocaine dose-response study (Experiment 7), only the USVs emitted during the first 30-sec of each minute were analyzed. For Experiment 3a (effects of cirazoline and atipamezole), minutes 3, 8, 13, and 18 were analyzed. For all other experiments, analysis of USVs was restricted to minutes 12, 14, and 16 of the 20-min session in order to allow time for AMPH to take effect. In the analysis, "call rate" was defined as the total number of 50-kHz calls (i.e. calls of all categories) emitted per minute. ANOVA or Friedman's test was performed, where appropriate, to test the effects of the within-subject factors "pretreatment" and "treatment" (see Table 1), for both the call rate and for each call subtype expressed as a proportion of all calls. Additionally, for Experiments 4, 6a, and 7, a post-hoc analysis was performed on non-trill frequency-modulated calls (i.e. all call subtypes except trills, flats, and shorts). All ANOVA p values were subject to the Huynh-Feldt correction. Multiple comparison tests were performed using Tukey's, Dunnett's, paired t-tests or Wilcoxon tests, depending on the type of comparisons to be made and the distribution of the data. For call rate, the latter two tests were subjected to the Holm-Bonferroni (H-B) correction. However, for call subtype analysis, pair-wise comparisons were performed using unprotected tests in order

to maintain statistical power. For all tests, a two-tailed *p* value less than 5% was considered significant.

RESULTS

Experiments 1 and 2: Effects of clonidine and prazosin

As expected, AMPH given alone (i.e. with vehicle pretreatment) significantly increased the overall rate of calling (i.e. sum of all 50-kHz call categories emitted per minute) (Figure 2a, b). This effect was significantly reduced by the lowest dose of the α2 adrenergic agonist clonidine (i.e. 10 μg/kg) and abolished by the two higher doses (20 or 100 μg/kg; Figure 2a). Clonidine also appeared to decrease calls when given alone (i.e. under saline treatment; Friedman test, $Q_3 = 12.97$, $p<0.01$; Figure 2a), but no individual dose of clonidine exerted a significant effect (Wilcoxon tests with H-B correction, $Z \leq 2.37$, NS). The α1 antagonist prazosin alone significantly inhibited calling (Friedman test, $Q_2 = 18.48$, $p < 0.001$; Figure 2b), and even the lower dose (0.3 mg/kg) of this drug virtually abolished AMPH-induced calling (Wilcoxon test with H-B correction, $Z = 2.59$, $p < 0.01$).

Clonidine and prazosin also modified the call profile. Since many rats failed to make any calls at the higher doses of these drugs, analysis was restricted to the following low-dose conditions: (1) AMPH alone *vs*. clonidine (10 μg/kg)+AMPH, and (2) all four combinations of vehicle or prazosin (0.3 mg/kg) with saline or AMPH. In the presence of AMPH, clonidine increased the proportion of multi-step calls, while decreasing the proportion of flat-trill combination calls (paired t-tests, $t_8 = 2.74$ and 2.83, respectively, both $p < 0.05$; see Supplementary Figure S1). Prazosin (0.3 mg/kg) blocked the AMPH-

induced increase in the proportion of "trills" and "trills with jumps" (Figure 3; ANOVA pretreatment x treatment interactions: trills: $F_{1,7} = 6.74$, $p = 0.036$; trills with jumps: $F_{1,7} = 23.31$, $p < 0.01$).

Experiment 3: Effects of atipamezole and cirazoline

3a: Effect of atipamezole and cirazoline alone

Neither dose of the $\alpha 2$ antagonist atipamezole (0.3 and 1 mg/kg), given alone, significantly altered the call rate (Figure 4a) or altered the call profile (data not shown). The $\alpha 1$ agonist cirazoline at both doses tested (0.5 and 1 mg/kg) produced observable changes in behavior in all rats, such that they disengaged from their cage-mate and reared in one corner of the cage. In the first three test days, two rats died shortly after receiving either 0.5 or 1 mg/kg cirazoline, possibly due to pulmonary edema (Micheletti *et al*, 1987). Consequently, saline injection was substituted for cirazoline for the remainder of the experiment. Among rats that received cirazoline (n=5), there was an apparent but non-significant decrease in the call rate (mean±SEM values for saline and 1 mg/kg cirazoline were, respectively, 10.7±5.4 and 1.0±1.0 calls/min).

3b: Effect of atipamezole in combination with amphetamine

Atipamezole (1 mg/kg) alone tended to increase the call rate in this experiment, but not significantly (Wilcoxon test, $Z = 1.89$, $p = 0.0584$; Figure 4b). Atipamezole did not affect the call rate under AMPH (Wilcoxon test, $Z = 0.41$, NS; Figure 4b), and only had moderate effects on the AMPH call profile. In particular, the percentage of short calls, step-ups, and step-downs was increased by atipamezole (paired t-tests, $t_8 = 2.43$-3.08, each $p < 0.05$). Mean±SEM values in the presence *vs.* absence of atipamezole were, respectively: 17.4 ±

3.1% *vs.* 10.4 ± 1.8% (short calls), 11.5 ± 2.3% *vs.* 6.5 ± 2.3% (step-ups), and 4.1 ± 0.9% *vs.* 2.1 ± 0.7% (step-downs).

Experiment 4: Effect of propranolol

Propranolol failed to change the call rate significantly (Figure 5). Although propranolol appeared to depress calling when given alone, no dose differed significantly from saline in this respect, even before correction for multiple comparisons (Wilcoxon tests, $Z \leq 1.96$, NS for each dose). Propranolol also failed to affect *AMPH-induced* calls (i.e. AMPH minus saline difference score; ANOVA $F_{3,21} = 1.86$, NS; uncorrected paired t-tests, $t_7 = 0.4 – 1.16$, NS). In contrast, propranolol had a striking effect on the *types* of calls emitted (Supplementary Figure S2). Specifically, under AMPH, propranolol dose-dependently promoted flat calls while nearly abolishing trill calls (ANOVA: flat calls $F_{3,21} = 23.9$, $p < 0.0001$; trills $F_{3,21} = 5.66$, $p < 0.05$; see Table 2 for t statistics comparing each propranolol dose to saline; Figure 6a). In contrast, all other non-trill FM calls collectively remained constant across propranolol doses (ANOVA $F_{3,21} = 0.18$, NS; Figure 6b). The absolute number of trills, flats, and non-trill FM calls are provided in Supplementary Table S1.

Experiment 5: Effects of betaxolol, ICI 118,551, and nadolol

In this experiment, the effects of the selective β1 adrenergic antagonist betaxolol, the selective β2 adrenergic antagonist ICI 118,551, and the hydrophilic β1/β2 blocker nadolol were examined. As with propranolol, none of these agents significantly affected the rate of calling after saline or AMPH treatment (Wilcoxon tests; saline treatment: $Z = 0.62 – 0.89$, NS; AMPH treatment: $Z = 0.53$ - 1.95, NS; Figure 7). Analysis of individual call subtypes was

restricted to the AMPH treatment conditions, since saline test session yielded few calls (Figure 8). Propranolol again caused a highly significant shift in the call profile under AMPH (paired t tests: proportion of (1) trills, $t_{10} = 6.54$, $p<0.001$; (2) flat calls, $t_{10} = 4.45$, $p < 0.01$) (Figure 8a, b). Here, propranolol also had effects on other call subtypes: propranolol increased the proportion of flat-trill combinations (paired t test, $t_{10} = 2.4$, $p < 0.05$) and split calls (paired t test, $t_{10} = 2.47$, $p < 0.05$) (Figure 8c, d). However, betaxolol, ICI 118,551, and nadolol were all without effect on call profile (Figure 8). The absolute number of trills, flats, flat-trill combinations, and split calls are provided in Supplementary Table S2.

Given the possible sensitizing effects of AMPH on USVs (Ahrens *et al*, 2009), we assessed order effects by examining the call rate under AMPH as a function of the number of times the rat was exposed to AMPH. The call rate did not change significantly over multiple AMPH exposures in this experiment (Supplemental Figure S3).

Experiment 6: Effects of NAD-299 and higher doses of betaxolol and ICI 118,551

The findings of Experiment 5 indicated that the observed effects of propranolol might require simultaneous β1/β2 receptor blockade, or might result from this drug's ability to antagonize $5HT_{1A}$ receptors; alternatively, our doses of betaxolol and ICI 118,551, chosen to ensure β1 vs. β2 selectivity *in vivo* (see Notes in Supplementary Material), might have been insufficient. Therefore, Experiment 6 examined the effects of (1) higher doses of betaxolol and ICI 118,551 alone or in combination, and (2) the selective 5-HT_{1A} antagonist NAD-299.

6a: Effects of betaxolol and ICI 118,551 in combination and NAD-299

AMPH treatment again produced a highly significant increase in call rate, and this effect was unaltered by pretreatment with either propranolol, the combination of betaxolol and ICI 118,551, or NAD-299 (Tukey's test: AMPH treatment conditions *vs.* saline, q = 9.07-11.22, each $p < 0.001$; AMPH treatment alone *vs.* drug pretreatment + AMPH, q = 0.35 – 1.80, NS; Supplementary Figure S4). As before, propranolol normalized the trill/flat profile shift induced by AMPH (Figure 9a, b; see Table 3 for statistical details), and in addition, it caused a significant decrease in the proportion of short calls (Figure 9c). The betaxolol/ICI 118,551 combination mimicked these effects of propranolol, while NAD-299 was without significant effect (Figure 9a-c). However, propranolol also caused an increase in the proportion of split calls, an effect not observed with the betaxolol/ICI 118,551 combination or with NAD-299 (Figure 9d). There was no significant change in the proportion of non-trill FM calls following any pretreatment in this experiment (Figure 9e).

6b: Effect of betaxolol and ICI 118,551 alone at higher doses

Here, betaxolol or ICI 118,551 were tested individually at the same doses as used in Experiment 6a (2.5 and 1 mg/kg, respectively) in combination with AMPH (1 mg/kg, IP). Neither antagonist affected USV rate or profile (Supplementary Figures S5, S6).

Experiment 7: Effect of IV cocaine and AMPH on 50-kHz ultrasonic vocalizations

The dose of AMPH used in this experiment (0.5 mg/kg IV) was chosen based on a preliminary dose-response study (0.1, 0.5, 1, and 2 mg/kg, IV; Supplementary Figure S7). Only AMPH and the 0.75 mg/kg dose of cocaine significantly increased the call rate compared to saline treatment, and cocaine was less effective than AMPH in this regard

(Tukey's test: AMPH vs. saline, $q = 10.19$, $p < 0.001$; 0.75 mg/kg cocaine vs. saline, $q = 4.08$, $p < 0.05$; AMPH vs. each cocaine dose, $q = 6.11 – 8.66$, all $p < 0.001$; Figure 10a). Analysis restricted to FM calls showed the same pattern of effects (Supplementary Figure S8). Under AMPH, the call rate increased detectably within the first 30 sec following infusion (paired t-test *vs.* saline, $t_{11} = 3.12$, $p < 0.01$), and this drug effect peaked between 180-210 sec (Figure 10b). Cocaine (0.75 mg/kg) produced a significant increase in the call rate 60-90 sec following the infusion (paired t-test *vs.* saline, $t_{11} = 2.27$, $p < 0.05$), and this effect peaked between 120-150 sec (Figure 10b).

Although cocaine only modestly affected call rate, it produced a highly significant shift in the call profile at all doses tested (Figure 11). In this respect, it closely mimicked the effect of AMPH, such that trill calls proportionally increased while flat calls decreased (Dunnett's tests *vs.* saline: trills, $q = 2.67 – 4.35$, $p < 0.01 – 0.05$; flat calls, $q = 3.25 – 4.53$, $p < 0.01$ for each comparison). There was no change in the proportion of non-trill FM calls under AMPH or cocaine (Dunnett's test *vs.* saline: $q = 0.35 – 2.05$, NS).

DISCUSSION

Previous investigations relating noradrenergic mechanisms to rat USV emission have almost exclusively focused on adult 22-kHz USVs (e.g. McIntosh and Barfield 1984) or pup calls (e.g. Blumberg *et al*, 2005); both types of call appear to be functionally distinct from the 50-kHz calls emitted by adult rats (Portfors 2007). To our knowledge, the only previous report of a potential noradrenergic contribution to adult rat 50-kHz USVs was in the context of social stress (Tornatzky and Miczek 1994). Hence, the present study is the first

to examine the association between noradrenaline and 50-kHz USV production in unstressed adult rats.

Pharmacological considerations

As discussed below, the effects of prazosin, clonidine, and propranolol observed in the present study are likely mediated through α1, α2, and β1/β2 adrenergic receptors, respectively. Doses of prazosin were based on the drug's potency in the following *in vivo* assays: α1 radiotracer binding (Couch *et al*, 1988), antagonism of an α1 agonist cue (Schechter 1991), and inhibition the psychomotor stimulant effects of AMPH (Selken and Nichols 2007; Vanderschuren *et al*, 2003). Prazosin, at the doses used in the present study, is highly α1-selective, with negligible affinity for α2 or β adrenergic, dopamine, and serotonin receptors (Balle *et al*, 2003; Clineschmidt *et al*, 1979; Miach *et al*, 1980; Sanger 1989) or for imidazoline sites (Angel *et al*, 1995). However, prazosin also binds to melatonin MT_3 receptors, albeit with significantly lower affinity than to α1 receptors (Doxey *et al*, 1984; Molinari *et al*, 1996; Pickering and Niles 1990). The function of the MT_3 receptor remains poorly characterized, except in the regulation of intraocular pressure (Pintor *et al*, 2001). On present evidence, therefore, it is not clear whether MT_3 antagonism would produce detectable behavioral effects.

Clonidine acts as a potent agonist at both α2 adrenergic and I_1-imidazoline receptors (Edwards *et al*, 2001). In the dose range administered (0.01-0.1 mg/kg), clonidine would be expected to dose-dependently stimulate α2 autoreceptors (Drew *et al*, 1979), thereby inhibiting release and turnover of NA (Anden *et al*, 1970; Sacchetti *et al*, 2001). Also within this dose range, clonidine (0.04 mg/kg) produced an α2 receptor-

mediated drug cue without detectable α1 or β receptor activity (Bennett and Lal 1982). However, clonidine probably also activated I_1-imidazoline receptors. These receptors have been proposed to contribute to the CNS control of blood pressure (Holt 2003) and to modulate aversive effects of opiate withdrawal (Georges *et al*, 2005). Since the neuropharmacological and behavioral consequences of I_1-imidazoline receptor stimulation are largely unknown, we cannot exclude their possible role in the USV inhibition by clonidine.

Propranolol selectively antagonizes β1, β2 and $5HT_{1A}$ receptors (Middlemiss and Tricklebank 1992), while possessing much lower affinity for β3 receptors (Baker 2005). Several observations suggest that $5HT_{1A}$ receptors did not contribute to the call profile-changing effect of propranolol under AMPH. First, the highly selective $5HT_{1A}$ antagonist NAD-299 (Ross *et al*, 1999) failed to affect USVs in the present study, even when given in a dose (0.2 mg/kg) beyond that required to inhibit *in vivo* responses to the $5HT_{1A}$ agonist 8-OH-DPAT (Arborelius *et al*, 1999; Johansson *et al*, 1997). Second, the highest dose of propranolol used here (i.e. 10 mg/kg) did not inhibit 8-OH-DPAT effects on 5-HT release (Sharp *et al*, 1989). Third, the effects of propranolol observed in the present study were mimicked by the co-administration of selective β1 and β2 antagonists (i.e. betaxolol and ICI 118,551), neither of which interact significantly with the $5HT_{1A}$ receptor (Middlemiss *et al*, 1985). Finally, our negative finding with nadolol, a non-CNS penetrant β-adrenergic antagonist (Schiff and Saxey 1984), suggests that propranolol's effects on ultrasonic calling depend on *central* β1 and/or β2 receptors.

Behavioural considerations

Clonidine and prazosin

Both clonidine and prazosin, when given alone, inhibited USV emission. An inhibitory effect of *high-dose* clonidine (i.e. 0.1 mg/kg) is consistent with its known sedative effects (Drew *et al*, 1979). The inhibitory effects of lower doses of clonidine (i.e. 0.01 and 0.02 mg/kg IP) are perhaps attributable to mild sedation, which has been seen in some (Carey *et al*, 2008; Drew *et al*, 1979; Sara *et al*, 1995) but not all (De Luca *et al*, 1999; Skolnick *et al*, 1978) studies. Prazosin, in contrast, inhibited 50-kHz calling at doses that are clearly non-sedative (Drouin *et al*, 2002; Vanderschuren *et al*, 2003).

Both clonidine and prazosin dose-dependently inhibited *amphetamine-induced* calling, with partial-to-complete block even at low doses. It is unlikely that these drugs produced aversive effects, which might have inhibited 50-kHz calling. Clonidine, for example, is self-administered intravenously (Davis and Smith 1977) and induces conditioned place preference (CPP) (Asin and Wirtshafter 1985; Cervo *et al*, 1993) in rats, whereas prazosin appears motivationally neutral (Forget *et al*, 2009; Zarrindast *et al*, 2002). The inhibitory effect of prazosin is potentially interesting in view of its reported failure to block either the discriminative stimulus effects of AMPH in rats (Arnt 1996; West *et al*, 1995) or the acquisition of AMPH CPP (Hoffman and Donovan 1995a).

Although clonidine and prazosin, at doses used here, also suppress AMPH-induced locomotion (Drouin *et al*, 2002; Vanderschuren *et al*, 2003), the act of locomotion *per se* does not appear to cause rats to emit ultrasonic calls (Knutson *et al*, 2002).

Cirazoline and atipamezole

The α1 agonist cirazoline failed to increase the call rate significantly or modify the call profile, when given alone. However, cirazoline (0.5 and 1 mg/kg) produced major adverse side-effects following injection, most likely due to its action on peripheral α1 receptors (Micheletti *et al*, 1987). Thus, it remains unclear whether activation of central α1 receptors without the peripheral side-effects would elicit 50-kHz USVs. Surprisingly, comparable or even higher doses of cirazoline have been used in several other studies of conscious rats (e.g. Alsene *et al*, 2006; Sebban *et al*, 1999; Swerdlow *et al*, 2006).

In contrast, the highly selective α2 antagonist atipamezole (Virtanen *et al*, 1989) did not produce any observable changes in behavior. Doses of atipamezole were chosen based on previous studies showing increased extracellular NA levels in the brain (Bondi *et al*, 2010; Wortley *et al*, 1999). The lack of effect of atipamezole on call rate suggests that increased noradrenaline release resulting from α2 receptor antagonism is not sufficient to elicit USVs. Moreover, the effect of atipamezole on USVs under AMPH suggests that α2 receptor inhibition does not affect AMPH-induced call rate, but may modestly contribute to AMPH's ability to modify the call profile.

Propranolol

The present study reveals potentially novel psychostimulant effects that are mediated by CNS β receptors. Propranolol profoundly altered the call profile in rats that were acutely challenged with AMPH. Thus, propranolol suppressed trill calls and promoted flat calls, effectively countering the profile-altering effects exerted by AMPH alone. Additional tests with betaxolol, ICI 118,551, nadolol, and NAD-299 implicated centrally-located β receptors.

In contrast, propranolol did not inhibit the AMPH-induced enhancement of call rate, a result that may possibly be related to propranolol's inability to inhibit behavioral stimulant effects of AMPH (Simon *et al*, 1972; Vanderschuren *et al*, 2003). Since both USVs and discriminative stimulus (cue) properties have been proposed to model subjective effects of drugs, it is of interest that propranolol antagonized AMPH's effects on call profile (present study) at doses that failed to inhibit the AMPH cue (West *et al*, 1995).

Remarkably, the effects of β blockers on conventional measures of psychostimulant reward or aversion have received little attention in animals. For example, there appear to be no reports of conditioned place preference/aversion testing using propranolol. In an initial study, acute propranolol administration inhibited IV self-administration of AMPH in rats (Yokel and Wise 1976). In contrast, propranolol substantially reduced *cocaine* IVSA (Harris *et al*, 1996). Thus, in light of the present findings, CNS β adrenergic receptors warrant further attention in the context of psychostimulant reward and aversion.

50-kHz ultrasonic vocalizations in relation to subjective drug effects in humans

In human subjects, there is considerable debate as to the relative importance of dopaminergic and noradrenergic mechanisms in the positive subjective effects of AMPH (Abi-Dargham *et al*, 2003; Brauer and de Wit 1997; Dlugos *et al*, 2007; Jonsson 1972; Leyton *et al*, 2007; Lott *et al*, 2005; Nurnberger *et al*, 1984; Rothman *et al*, 2001; Sofuoglu *et al*, 2009). For example, dopaminergic antagonists have failed to reduce psychostimulant euphoria in most studies (Brauer and de Wit 1995; Brauer and de Wit 1996; Brauer and de Wit 1997; Gawin 1986; - but see Gunne *et al*, 1972; Jonsson 1972). Moreover, human and animal studies suggest that dopamine transmission does not contribute to the hedonic

impact of psychostimulants, but rather to the incentive salience of reward-related cues (Berridge and Robinson 1998; Leyton *et al*, 2007; Leyton *et al*, 2005). In contrast, several observations point to possible noradrenergic mediation of AMPH euphoria (Dlugos *et al*, 2007; Rothman *et al*, 2001; Sofuoglu *et al*, 2009); although preliminary studies using α or β receptor antagonists have been largely negative, only low antagonist doses were used (Brauer and de Wit 1995; Jonsson 1972; Nurnberger *et al*, 1984).

Frequency-modulated (FM) 50-kHz calls have been proposed as an index of positive affect in rats (Burgdorf *et al*, 2010). Accordingly, the present study confirmed that AMPH selectively promotes trill calls (present study; Wright *et al*, 2010) at doses that are comparable to euphorigenic doses in human studies (Grilly and Loveland 2001). Propranolol countered this call profile shift. In humans, the impact of β receptor blockade on the euphoric effect of AMPH has been investigated in only two studies (Jonsson 1972; Nurnberger *et al*, 1984), to our knowledge. Both studies used propranolol and were ostensibly negative. However, in the first of these, an unusually high dose of AMPH (200 mg, i.e. approx. 3 mg/kg IV) was combined with only moderate doses of propranolol (20 and 40 mg PO). In the second, the dose of amphetamine was lower (0.3 mg/kg IV), but subjective effects were inferred only from the subjects' behavior; here, too, it is not clear whether propranolol (0.1 mg/kg IV) was given in a sufficiently high dose. Our preclinical findings therefore suggest that CNS β receptor mechanisms would merit further study in humans under AMPH challenge.

Possible (nor)adrenaline-dopamine interactions

USV emission by adult rats is not only influenced by (nor)adrenergic mechanisms (present study) but is also strongly DA-dependent (see Introduction). These neurotransmitter systems are extensively coupled (for review, see Weinshenker and Schroeder 2007); for example, a number of studies have shown a critical role of noradrenergic transmission in AMPH-induced mesoaccumbens dopamine release (e.g. Darracq *et al*, 1998; Pan *et al*, 1996). However, it seems unlikely that clonidine, prazosin, or propranolol interfered with dopaminergic agonist actions of AMPH in the present study. For example, prazosin (0.5 mg/kg IP) did not affect extracellular DA in the nucleus accumbens following systemic AMPH administration (Darracq *et al*, 1998). Similarly, clonidine failed to alter AMPH-induced extracellular dopamine levels (Florin *et al*, 1994; Tanda *et al*, 1996). Finally, propranolol administration did not inhibit several DA-dependent behavioral effects of AMPH, i.e. locomotor stimulation (Simon *et al*, 1972; Vanderschuren *et al*, 2003), stereotypy (Simon *et al*, 1972), and cue properties (West *et al*, 1995).

Generalization to IV cocaine and amphetamine

Acute IP cocaine administration reportedly increases 50-kHz call rate (Williams and Undieh 2010). Previous studies utilizing *intravenous* cocaine have been performed in the context of self-administration (and its anticipation) and sensitization (Barker *et al*, 2010; Browning *et al*, 2011; Ma *et al*, 2010; Maier *et al*, 2010). Here, we provide the first report of the effects of non-contingent IV administration of cocaine on USVs. Cocaine increased the call rate at the 0.75 mg/kg dose, with a rapid onset (peak effect 120-150 sec post-infusion). While this USV rate-enhancing effect of cocaine was less pronounced than that of AMPH,

cocaine nevertheless produced a profound AMPH-like shift in the call profile at all doses tested (i.e. 0.25-1.5 mg/kg). In this dose range, cocaine maintains self-administration (Roberts *et al*, 2007) and induces CPP (Nomikos and Spyraki 1988; Sellings *et al*, 2006), but is also anxiogenic (Ettenberg 2004); how these effects may relate to USV emission merits further investigation.

Information gained from 50-kHz call subtype analysis vs. call rate

Several findings of the present study highlight the importance of detailed call subtype analysis. First, both cocaine and propranolol changed the propensity to emit different call subtypes at doses that did not significantly change the call rate. These results add to evidence that call rate and profile can be manipulated independently by drugs or lesions (Ciucci *et al*, 2009; Ciucci *et al*, 2007). Moreover, while several groups currently distinguish between FM and flat 50-kHz calls (Ahrens *et al*, 2009; Burgdorf *et al*, 2008a; Burgdorf and Panksepp 2006; Burgdorf *et al*, 2007; Simola *et al*, 2009; Wohr *et al*, 2008), only a few investigators have extended their analysis beyond those two classes (Ciucci *et al*, 2009; Kaltwasser 1990a; Takahashi *et al*, 2010; Vivian and Miczek 1993b; White *et al*, 1990; Wright *et al*, 2010). Importantly, our detailed analysis reveals that the prevalent trill call subtype (Wright *et al*, 2010) is not representative of all FM calls.

Human psychostimulant abusers cannot readily discriminate between cocaine and AMPH (Fischman *et al*, 1976). In the present study, cocaine affected the FM call rate less than AMPH, yet produced an equivalent shift in the call profile (i.e. preferentially promoting trills over flat calls). Therefore, insofar as FM 50-kHz calls convey information

about positive affect in rats (as proposed by Burgdorf *et al*, 2010), the call profile might be more pertinent than the absolute FM call rate.

Limitations and methodological considerations

Adult rats vary considerably in their USV response to various stimuli including systemic AMPH (Burgdorf and Panksepp 2006; Schwarting *et al*, 2007; Wohr *et al*, 2008; Wright *et al*, 2010). In order to study the effects of drugs on AMPH-induced calling, it was necessary to exclude low responders based on an initial test screen. However, it is important to bear in mind that low- and high-calling rats may differ in other behavioral or neurochemical respects (Burgdorf *et al*, 2008b). The test screen likely explains why we did not subsequently observe sensitization with repeated exposure to AMPH during the experiment, as USV sensitization seems to occur mainly within the first three exposures to cocaine or AMPH (Ahrens *et al*, 2009; Meyer *et al*, 2011; Mu *et al*, 2009).

The present findings strongly indicate a (nor)adrenergic role in AMPH-induced 50-kHz USVs. However, the evidence for α1 receptor mediation rests on the use of a single drug - prazosin. Although prazosin is a well-characterized and selective α1 receptor antagonist (see above), it would have been desirable to test other drugs of the same class. However, other currently available α1 antagonists are either less α1-selective (e.g. phentolamine), α1 subtype-selective (e.g. tamsulosin), brain-impenetrant (e.g. doxazosin), or little characterized in the rat (e.g. HEAT).

In the present study, only prazosin significantly inhibited 50-kHz calling when given alone. However, rates of spontaneous calling were generally low, making it hard to detect potential suppressive effects of other drugs. In order to determine whether noradrenergic

transmission plays a wider role in USV production, it would be informative to test these drugs in combination with non-pharmacological stimuli that evoke high rates of 50-kHz calling (Ciucci *et al*, 2007; Panksepp and Burgdorf 2000).

Conclusion

These findings provide the first evidence of (nor)adrenergic involvement in the elicitation of adult rat 50-kHz USVs by amphetamine. Furthermore, USV emission appears to be differentially associated with $\alpha 1$ vs. β receptor mechanisms, whereby (nor)adrenergic transmission through $\alpha 1$ receptors principally modulates the call rate, while NA (or adrenaline) acting on β receptors affects the acoustic subtypes of 50-kHz calls emitted.

In the context of drug addiction, psychostimulants reinforce self-administration behavior and acutely promote positive affect. Currently, it is not clear how these two effects are related. Dopaminergic transmission in the brain appears critical to motivation, but has not been convincingly linked to psychostimulant euphoria. Preliminary evidence points to a noradrenergic contribution to euphorigenic effects of amphetamine, but receptor mechanisms have not been identified. The present findings suggest that CNS β adrenergic receptors merit further attention in this regard.

Table 1: Summary of experiments

	Pre-treatment[I]					Treatment[II]				
Expt	Drug		Doses (mg/kg)	Route	Time before treatment (min)	Drug		Doses (mg/kg)	Route	n
1	α2 agonist	Clonidine	0, 0.01, 0.02, 0.1	IP	20		AMPH	0, 1	IP	10
2	α1 antagonist	Prazosin	0, 0.3, 1	IP	30		AMPH	0, 1	IP	12
3a	-	-	-	-	-	α1 agonist	Cirazoline	0.5, 1	IP	12
	-	-	-	-	-	α2 antagonist	Atipamezole	0.3, 1	IP	
	-	-	-	-	-		AMPH	0, 1	IP	
3b	α2 antagonist	Atipamezole	0, 1	IP	20		AMPH	0, 1	IP	9[III]
4	β1/β2 antagonist	Propranolol	0, 1, 3, 10	IP	20		AMPH	0, 1	IP	8
5	β1/β2 antagonist	Propranolol	10	IP	20		AMPH	0, 1	IP	11
	β1 antagonist	Betaxolol	1	IP	20		AMPH	0, 1	IP	
	β2 antagonist	ICI 118,551	0.2	IP	20		AMPH	0, 1	IP	
	β1/β2 antagonist (peripheral)	Nadolol	5	IP	20		AMPH	0, 1	IP	
6a	β1/β2 antagonist	Propranolol	10	IP	20		AMPH	1	IP	12
	β1+β2 antagonist	Betaxolol + ICI 188, 551	2.5 (BET), 1 (ICI)	IP	20		AMPH	1	IP	
	$5HT_{1A}$ antagonist	NAD-299	0.2	SC	20		AMPH	1	IP	
6b	β1 antagonist	Betaxolol	2.5	IP	20		AMPH	1	IP	
	β2 antagonist	ICI 118,551	1	IP	20		AMPH	1	IP	
7		-	-	-	-		Cocaine	0, 0.25, 0.75, 1.5	IV	12
		-	-	-	-		AMPH	0.5	IV	

[I]For experiments 5 and 6, each rat was also tested under vehicle pretreatment combined with saline and AMPH treatment.

[II]In all experiments, treatments were administered immediately before placing the rat in the experimental chambers for recording, with the exception of Experiment 3a where the cirazoline and atipamezole treatments were administered 20 min before. All test session durations were 20 min except for Experiment 7 (i.e. 10 min).

[III]The rats in Experiment 3b were the same as those used in Experiment 3a.

Table 2: Effect of propranolol on percentage of flat calls and trills under AMPH in Experiment 4

Propranolol dose (mg/kg)	Flat calls	Trills
1	-4.51**	1.86
3	-4.22**	2.7*
10	-9.1***	2.55*

Values in the table are the paired t statistics of the propranolol pretreatment conditions *vs.* the saline pretreatment control all under AMPH treatment, df = 7, *p<0.05, **p<0.01,***p<0.001

Table 3: Call profile shifts in Experiment 6a

		AMPH treatment alone				
PRETREATMENT	**TREATMENT**	**Flats**	**Trills**	**Shorts**	**Splits**	**Non-trill FM calls**
Vehicle	Saline	2.48*	2.25*	0.95	1.17	0.2
Propranolol	AMPH	4.85***	4.06**	2.33*	2.41*	2.12
Betaxolol/ICI 118,551	AMPH	4.31**	3.23**	3.05*	1.36	0.29
NAD-299	AMPH	1.31	1.78	0.64	1.11	0.44

Values in the table refer to the paired t statistics comparing the percentage of each call subtype under AMPH treatment alone with percentage under the pretreatment/treatments listed in the first two columns. df = 11, *p<0.05, **p<0.01,***p<0.001

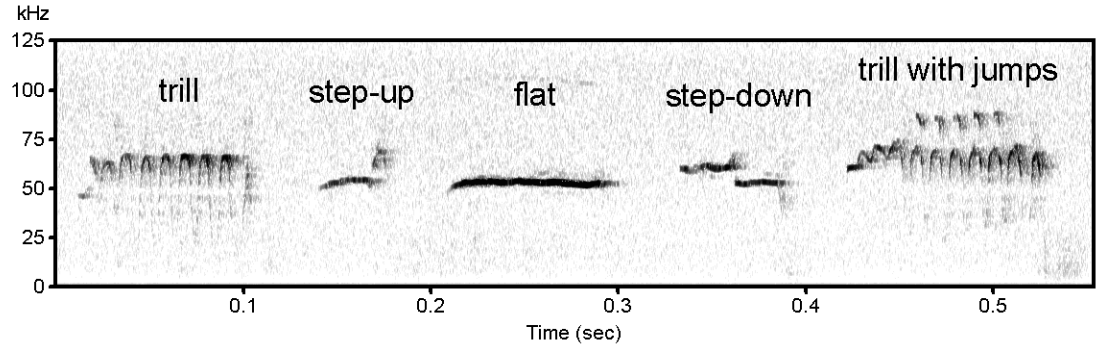

Figure 1 Spectrographic display of individual 50-kHz calls, which are representative of the following subtypes (left to right): trill, step-up, flat, step-down, and trill with jumps. See Wright *et al* (2010) for additional examples of all 14 50-kHz call subtypes so far recognized.

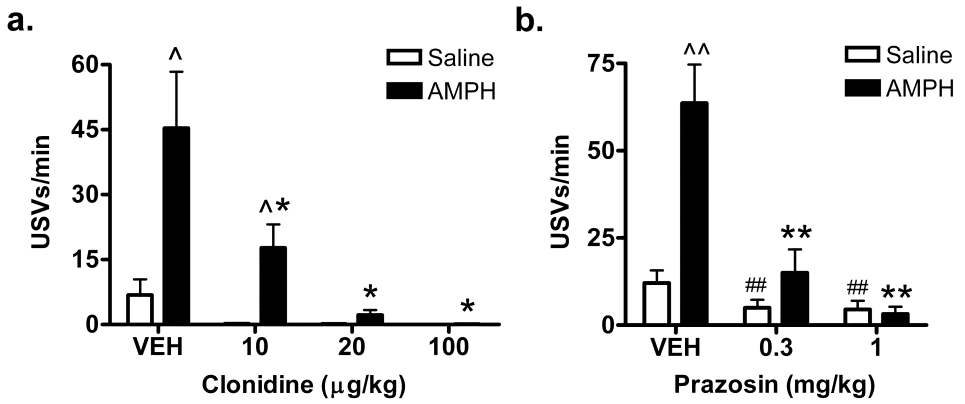

Figure 2 Experiments 1 and 2: Clonidine and prazosin dose-dependently decreased the 50-kHz call rate (i.e. calls of all categories). The y-axis represents mean+SEM calls per minute. Each rat was tested under all conditions (clonidine group n=12, prazosin group n=12). AMPH administration only significantly increased the call rate when rats were pretreated with vehicle (**a, b**) or with the lowest dose of clonidine (**a**). Under AMPH treatment, clonidine (**a**) and prazosin pretreatment (**b**) dose-dependently reduced the call rate. Prazosin alone (i.e. with saline treatment) also decreased the call rate at both doses tested. Clonidine (i.e. saline treatment) appeared to decrease calls when given alone (Friedman test, $p<0.01$), but the trend did not reach statistical significance for any individual dose ($p > 0.05$). All pair-wise comparisons were made by Wilcoxon tests with Holm-Bonferroni (H-B) correction, n = 12 (per experiment). ^$p<0.05$, ^^$p<0.01$ *vs.* the corresponding saline treatment (i.e. same pretreatment), ##$p<0.01$ vs. VEH+saline condition, *$p<0.05$, **$p<0.01$ vs. corresponding VEH pretreatment condition.

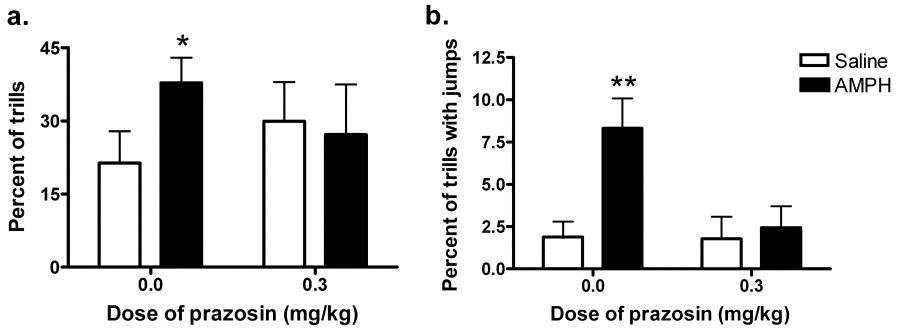

Figure 3 Experiment 2: Prazosin inhibited or blocked the AMPH-induced increase in the percentage of trills (**a**) and trills with jumps (**b**). The y-axes represent mean+SEM percent. Each rat was tested under all conditions (n=12). Both two-way ANOVA interactions were significant (see main text). *p<0.05, **p<0.01 *vs*. corresponding vehicle/saline condition (paired t-tests)

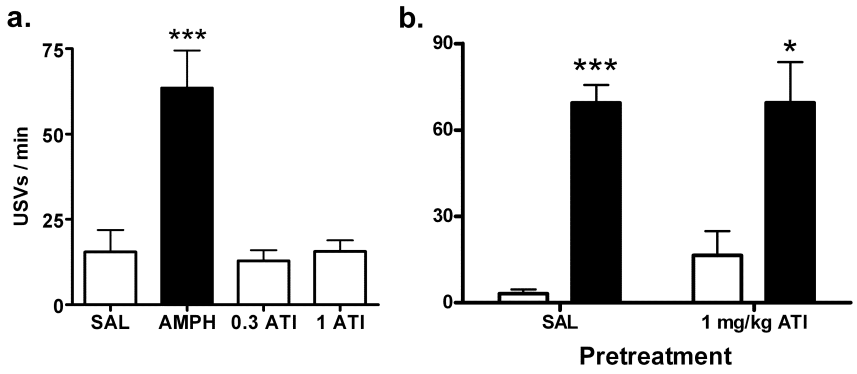

Figure 4 Experiment 3: **a.** AMPH increased the call rate, while atipamezole (0.3 and 1 mg/kg ATI; n=10) given alone had no significant effect. The y-axis represents the mean+SEM call rate per minute. ***p<0.001 *vs.* saline condition (paired *t* test) **b.** AMPH increased the call rate in rats pretreated with saline or atipamezole. Atipamezole alone did not significantly increase the call rate. The y-axis represents the mean+SEM call rate per minute. Filled bars correspond to AMPH treatment and open bars correspond to saline treatment. Each rat was tested under all conditions (n=9). *p<0.05, ***p<0.001 compared to the same pretreatment with saline challenge (paired *t* tests).

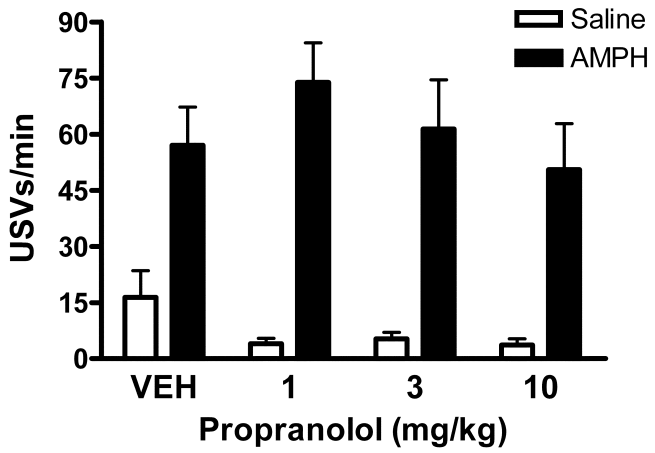

Figure 5 Experiment 4: AMPH-induced 50-kHz call rate was not altered by propranolol. The y-axis shows mean+SEM calls/min (n=8). Each rat was tested under all conditions. AMPH increased the call rate at all doses of propranolol (Wilcoxon tests: $p<0.05$). No dose of propranolol significantly altered the call rate under AMPH (paired t-tests, $p>0.05$) or when given alone (Wilcoxon tests, $p>0.05$). All other pair-wise comparisons were subjected to H-B corrections.

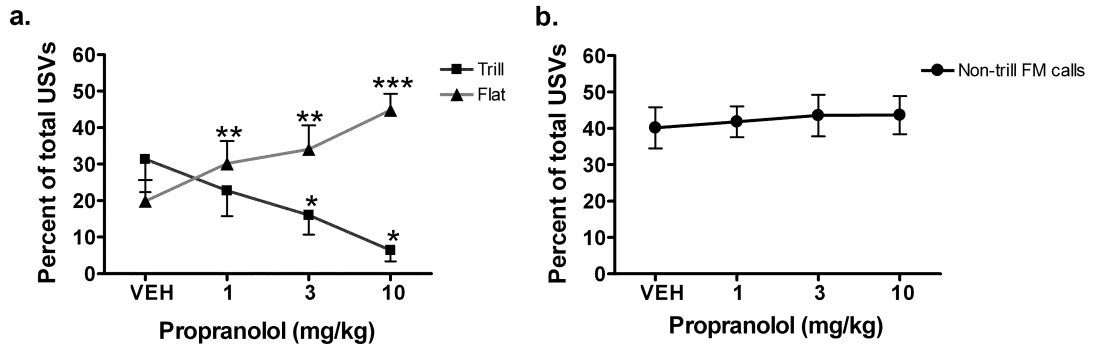

Figure 6 Experiment 4: Propranolol promoted flat calls and inhibited trill calls under AMPH. Line graphs showing (**a**) the dose-dependent increase in flat calls and concomitant decrease in trills, and (**b**) no significant difference in non-trill frequency-modulated calls, expressed as mean ± SEM percent of total calls emitted (i.e. calls of all 50-kHz categories). *p<0.05, **p<0.01, ***p<0.001 compared to vehicle (VEH) pretreatment (paired t-tests, n=8)

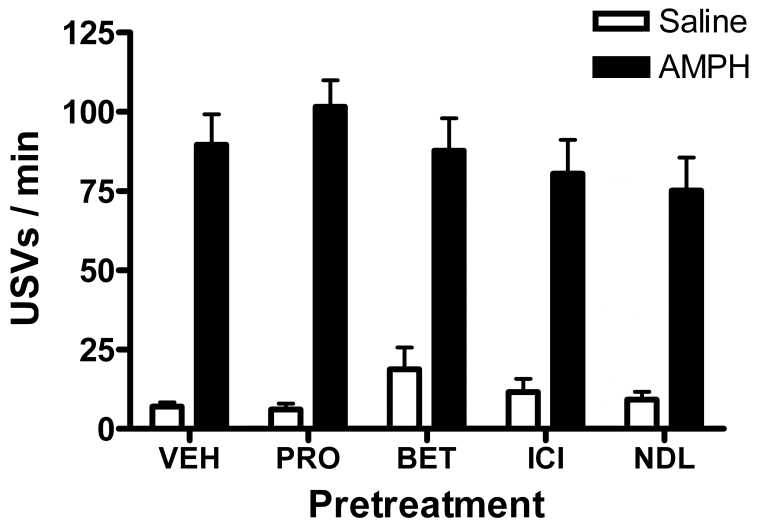

Figure 7 Experiment 5: AMPH-induced 50-kHz calling was not altered by propranolol (PRO; 10 mg/kg, IP), betaxolol (BET; 1 mg/kg, IP), ICI 118,551 (ICI; 0.2 mg/kg, IP) or nadolol (NDL; 5 mg/kg, IP). AMPH robustly increased the call rate under all pretreatment conditions (Wilcoxon tests: $p<0.05$-0.003). No pretreatment affected the call rate when given alone (Wilcoxon tests: $p>0.05$) or when combined with AMPH (Wilcoxon tests: $p>0.05$). The y-axis represents mean+SEM calls/min. Each rat was tested under all conditions (n=11). All pair-wise comparisons were subjected to H-B corrections.

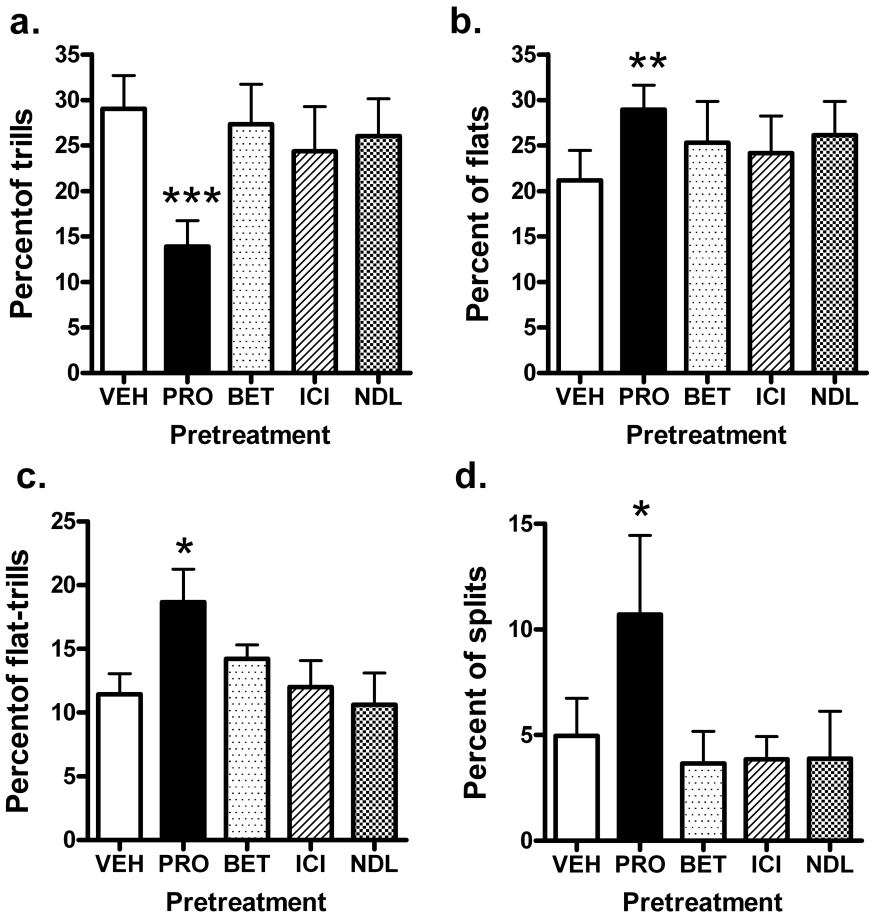

Figure 8 Experiment 5: Propranolol decreased the percentage of trills (**a**) and increased the percentage of flats, flat-trill combinations, and splits (**b-d**) under AMPH. The y-axis represents mean+SEM percent of total calls (i.e. all 50-kHz categories). Each rat was tested under all conditions (n=11). *p<0.05, **p<0.01, ***p<0.001 *vs*. vehicle pretreatment condition (paired t-tests)

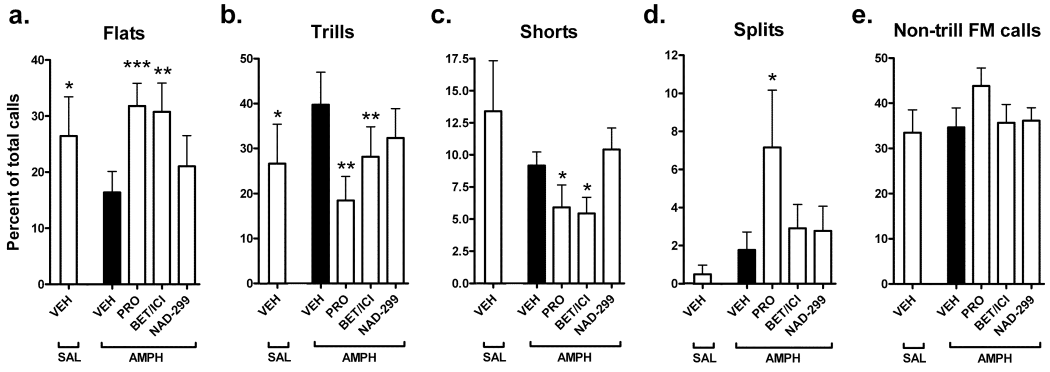

Figure 9 Experiment 6a: Propranolol (PRO; 10 mg/kg, IP) and the combination of betaxolol and ICI 118,551 (BET/ICI; 2.5 and 1 mg/kg IP, respectively) increased the percentage of flat calls under AMPH (**a**) while decreasing the percentage of trills (**b**) and shorts (**c**). In this experiment, propranolol also significantly increased the percentage of split calls (**d**), an effect not observed with the betaxolol/ICI 118,551 combination. There was no significant effect of any pretreatment on non-trill frequency-modulated calls (**e**). All pairwise comparisons between the PRO vs. BET/ICI conditions were non-significant (paired t-tests, p = 0.07-0.81). NAD-299 failed to affect the percentage of any calls emitted. The y-axis shows mean + SEM percent of total calls (i.e. all subtypes) (n=12). Pretreatments are listed immediately below the x-axes, and saline or AMPH treatment conditions are indicated underneath each graph. *p<0.05, **p<0.01, ***p<0.001 compared to the VEH/AMPH condition.

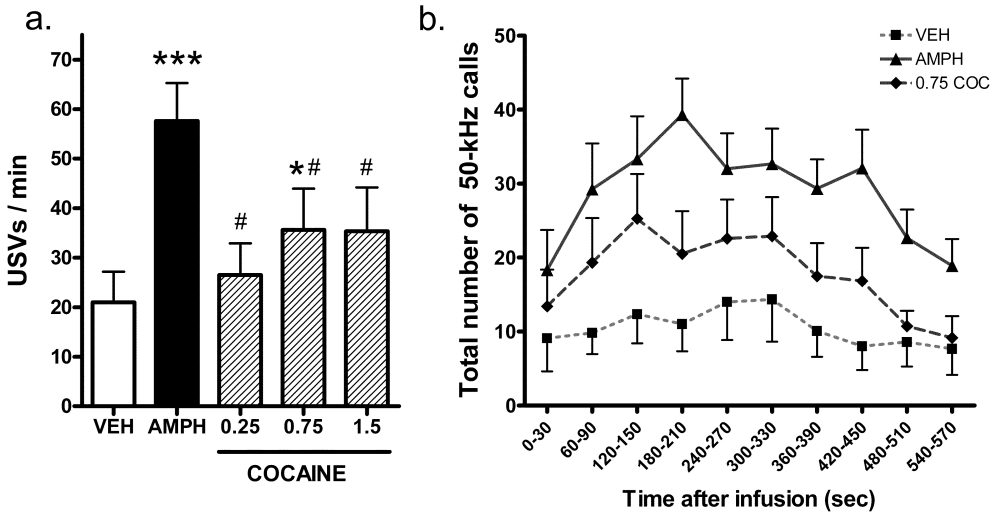

Figure 10 Experiment 7: Cocaine (0.25, 0.75, 1.5 mg/kg, IV) dose-dependently increased the number of USVs emitted by rats, but significantly less so than amphetamine (0.5 mg/kg IV; AMPH). **a.** The rate of 50-kHz calling was averaged 0-10 min post-injection and is expressed as calls per minute (mean+SEM). Each rat was tested under all conditions (n=12). Only AMPH and 0.75 mg/kg cocaine significantly increased the call rate. *p<0.05, ***p<0.001 *vs.* corresponding saline (VEH) condition, #p<0.001 *vs.* the corresponding AMPH condition (Tukey's test). **b.** Time course of the call rate following AMPH (0.5 mg/kg, IV) or cocaine (0.25, 0.75, and 1.5 mg/kg, IV) administration. The x-axis refers to the time after the end of the 10-sec infusion. For visual clarity, only the VEH, AMPH, and the 0.75 mg/kg dose of cocaine (i.e. the most effective dose of cocaine on the call rate) are illustrated.

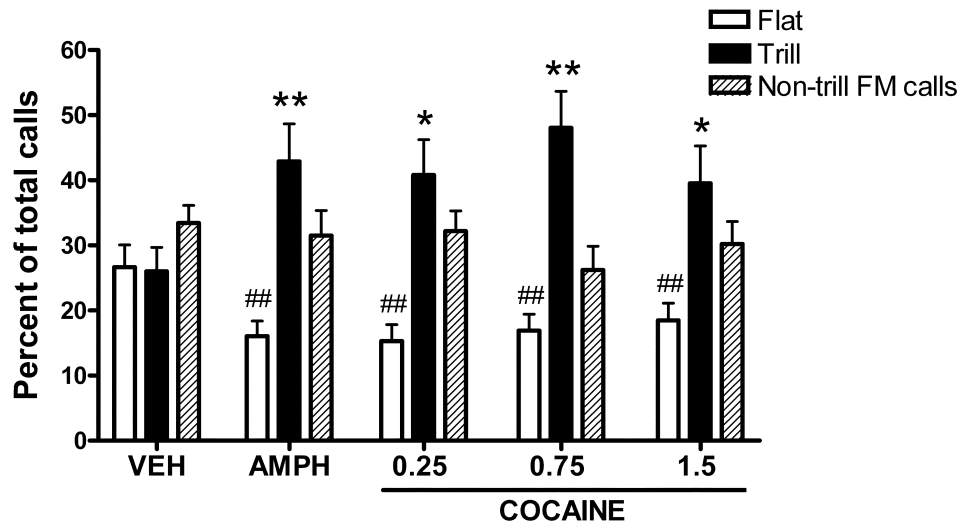

Figure 11 Experiment 7: AMPH (0.5 mg/kg, IV) and all doses of cocaine (0.25, 0.75, and 1.5 mg/kg, IV) promoted trill calls while suppressing flat calls. The y-axis represents the percent of the total calls that were trills, flat calls, and non-trill frequency-modulated calls for each drug/dose condition (mean+SEM, n=12). *p<0.05, **p<0.01, ##p<0.01 *vs.* corresponding VEH (i.e. saline) condition (Dunnett's tests)

Supplementary Material for Chapter 3

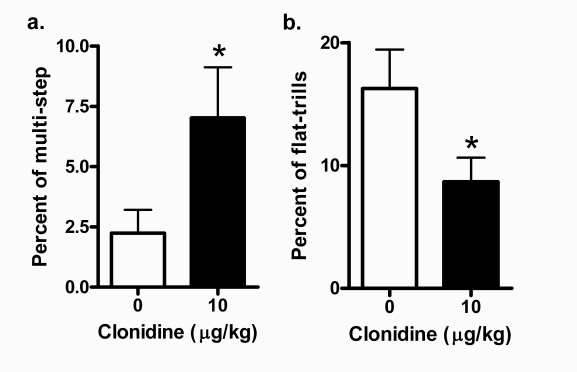

Figure S1 Experiment 1: In the presence of AMPH, low-dose clonidine increased the proportion of multi-step calls (**a**), while decreasing the proportion of flat-trill combination calls (**b**) *p<0.05 (paired t-tests, n=12).

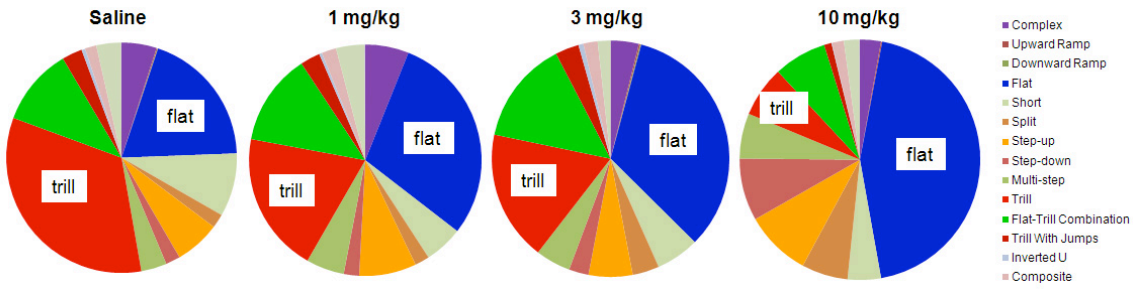

Figure S2 Experiment 3: Propranolol promoted flat calls and inhibited trill calls under AMPH. Pie chart showing the mean (n=8 rats) proportional contribution of the 14 subtypes of 50-kHz USVs following AMPH administration combined with different doses of propranolol as shown.

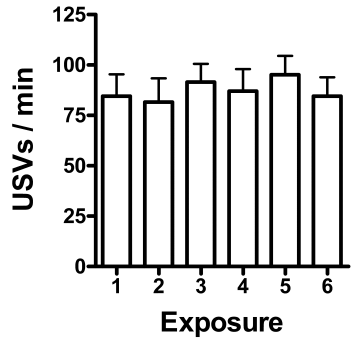

Figure S3 Experiment 5: The call rate in response to AMPH treatment did not increase over successive exposures (i.e. 1-6) throughout the experiment (ANOVA linear trend; $F_{1,10}$ = 0.67, p = 0.43). The y-axis represents the call rate per minute.

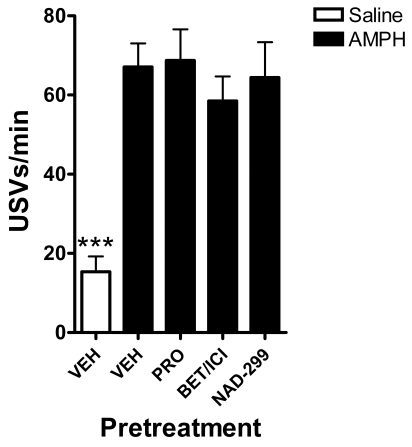

Figure S4 Experiment 6a: The call rate increase in response to AMPH treatment (dark bars) was unaffected by pretreatment with propranolol (PRO; 10 mg/kg, IP), betaxolol + ICI 118,551 (BET/ICI; 2.5 and 1 mg/kg IP, respectively), or NAD-299 (0.2 mg/kg, SC). The y-axis represents the mean+SEM call rate per minute. Each rat was tested under all conditions (n=12). ***p<0.001 *vs.* each AMPH treated condition (Tukey's test). All other comparisons were non-significant.

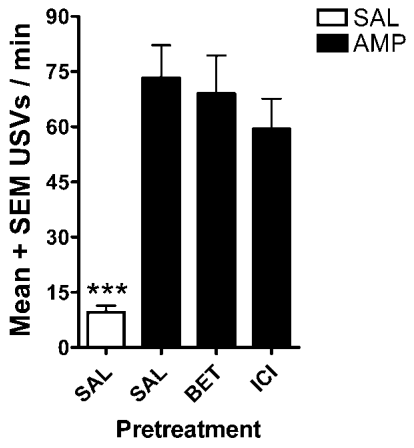

Figure S5 Experiment 6b: The call rate in response to AMPH treatment (dark bars) was unaffected by pretreatment with betaxolol (2.5 mg/kg) or ICI 118,551 (1 mg/kg). The y-axis represents the call rate per minute. Each rat was tested under all conditions (n=12). ***p<0.001 *vs.* each AMPH treated condition (Tukey's test; q = 8.32 – 10.63). All other comparisons were non-significant (Tukey's test; q = 0.71 – 2.3, NS).

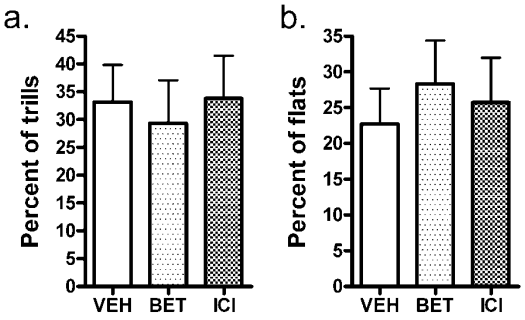

Figure S6 Experiment 6b: Pretreatment with betaxolol (BET; 2.5 mg/kg) or ICI 118,551 (ICI; 1 mg/kg) failed to affect the percentage of any call subtype under AMPH, including trills (**a**) and flat calls (**b**). The y-axis shows mean + SEM percent of total calls (i.e. all subtypes) (n=12)

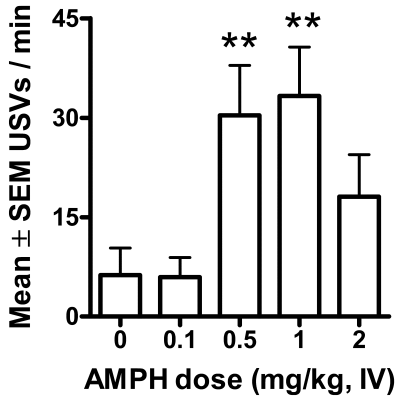

Figure S7 AMPH (0.1, 0.5, 1, and 2 mg/kg, IV) dose-dependently increased the number of USVs emitted by rats. The rate of 50-kHz calling was averaged 0-10 min post-injection and is expressed as calls per minute. Each rat was tested under all conditions (n=8). **p<0.01 *vs.* corresponding saline condition (paired t-tests with Holm-Bonferroni correction)

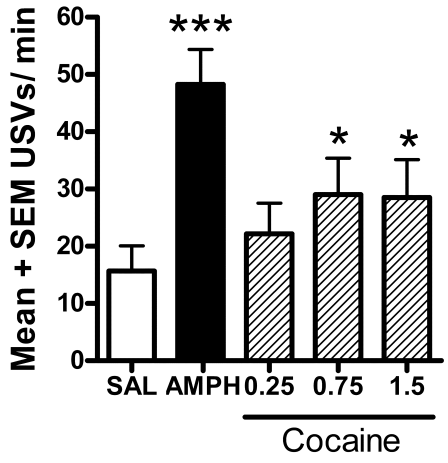

Figure S8 AMPH as well as 0.75 and 1.5 mg/kg of cocaine significantly increased the FM call rate compared to saline treatment; however, cocaine at all doses tested elicited fewer FM calls than AMPH (Tukey's test: AMPH vs. each cocaine dose, q = 6.47 – 8.76, all p < 0.001). *p<0.05, ***p<0.001 *vs.* saline

Table S1: Absolute number of trills and flat calls under AMPH treatment following pretreatment with propranolol (Experiment 4)

	Dose of propranolol (mg/kg)							
	0		1		3		10	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Trills	19.0	5.2	14.5	3.8	11.0	4.6	3.5	1.9
Flats	11.0	3.7	21.8	3.8	20.6	5.6	22.3	5.5
NTFM	21.9	5.1	33.6	7.7	26.0	7.1	22.5	6.3

Values in the table are mean and SEM values for trill, flat, and non-trill frequency-modulated (NTFM) calls emitted per minute.

Table S2: Absolute number of trills, flat calls, flat-trill combinations, and split calls under AMPH (Experiment 5)

	Pretreatment									
	VEH		PRO		BET		ICI		NDL	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
trills	11.9	1.8	6.5	1.2	11.6	2.4	10.5	2.4	8.5	1.4
flats	9.2	1.6	14.0	1.6	10.0	1.7	8.4	1.7	8.9	1.5
flat-trills	5.4	1.3	9.1	1.6	6.2	1.2	4.5	0.9	4.2	1.3
splits	2.2	0.9	5.2	1.9	1.5	0.6	1.5	0.5	1.9	1.2

Values in the table are mean and SEM values for trill, flat, flat-trill combination and split calls emitted per minute under AMPH following pretreatment with vehicle (VEH), propranolol (PRO), betaxolol (BET), and nadolol (NDL)

NOTES

- Propranolol is a $5HT_{1A}$ receptor antagonist (Middlemiss 1984; Sprouse and Aghajanian 1986; Tricklebank *et al*, 1984).
- Doses of betaxolol and ICI 118,551 in Experiment 4 were chosen to ensure β1 *vs.* β2 selectivity (Crissman *et al*, 2001; Crissman and O'Donnell 2002; Edwards *et al*, 1989; O'Donnell 1990; Zhang *et al*, 2001).
- Neither betaxolol nor ICI 118,551 interacts significantly with the $5\text{-}HT_{1A}$ receptor (Langlois *et al*, 1993; Middlemiss *et al*, 1985; Sanchez *et al*, 1996).

Intervening Section 2

Chapter 3 showed that both psychostimulants tested (i.e. AMPH and cocaine) significantly increased the call rate and produced an analogous shift in the proportional emission of call subtypes. Whether these USV-altering effects occur following administration of another class of euphoriant drugs (i.e. opioid agonists) was unclear. Therefore, Chapter 4 examined the effects of morphine administration on 50-kHz USV production in adult rats. USVs were measured in conjunction with tests of locomotor activity and CPP; these additional behavioural measures were performed in order to confirm the well-established psychomotor activating and rewarding properties of morphine administration.

CHAPTER 4: Failure of rewarding and locomotor stimulant doses of morphine to promote adult rat 50-kHz ultrasonic vocalizations

Jennifer M. Wright, Lan Deng, Paul B.S. Clarke

Psychopharmacology (Berl) (2012), **224**(4): 477-87

ABSTRACT

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

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

INTRODUCTION

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

Whereas the 22-kHz calls appear to express distress or alarm (Covington and Miczek 2003; Litvin et al. 2007), 50-kHz calls are elicited by a number of appetitive stimuli (Burgdorf et al. 2011; Knutson et al. 2002). Consequently, adult rat 50-kHz calls (and FM calls in particular) have been proposed to be a measure of hedonia (Burgdorf and Moskal 2009), and have been described as a reliable indicator (Brudzynski 2007) and validated model (Burgdorf et al. 2011) of positive affective states. Consistent with this notion, the psychomotor stimulants amphetamine (AMPH) and cocaine both increase the 50-kHz call rate and promote FM calls after systemic injection (e.g. Ahrens et al. 2009; Williams and Undieh 2010; Wright et al. 2010; Wright et al. 2012b). Among the FM calls, it is the trill calls in particular that appear to be preferentially increased by these drugs (Wright et al. 2010; Wright et al. 2012b). Psychostimulant drugs, however, are not only euphorigenic (Foltin and Fischman 1991) but are also anxiogenic in rodents and humans (Biala and Kruk 2007;

File and Hyde 1979; Pellow et al. 1985); in high doses, these drugs can even produce acute psychosis (Angrist et al. 1974; Robinson and Becker 1986). Hence, it is important to note that trill calls are also affected by appetitive nonpharmacological manipulations. In particular, trill calls are also enhanced by testing rats in pairs (Wright et al. 2010), which itself is reported to be rewarding (Calcagnetti and Schechter 1992), whereas they appear less prevalent upon social separation (Wohr et al. 2008). Hence, on present evidence, the trill call subtype appears most closely associated with positive stimuli (Wright et al. 2010).

Opiate agonists form a second major class of euphorigenic drugs (Jasinski and Preston 1986; Zacny et al. 1994), but it is unclear whether they affect 50-kHz USVs in a similar way to psychostimulants. Systemic morphine administration was initially reported to inhibit 50-kHz calling (Haney and Miczek 1994; Haney and Miczek 1995; Vivian and Miczek 1993b); however, these experiments were conducted in an aversive context (social stress) in which the vocalizations were probably acoustically distinct from those proposed to reflect positive affect (Wohr et al. 2008). Acute systemic morphine has also been combined with experimenter-applied tickling-like tactile stimulation, with no apparent drug effect on call rate (Panksepp and Burgdorf 2000). Acute morphine also failed to affect 50-kHz calling rate in a novel testing environment; however, call rates were uniformly very low (i.e. ≤1 call per minute) even in saline-challenged rats (Wohr and Schwarting 2009a), suggesting that the environment itself may have generally inhibited USV production. In contrast, morphine reportedly induced 50-kHz USVs in a reward context, i.e. conditioned place preference (CPP) (Burgdorf et al. 2001b; Knutson et al. 1999). The latter studies, however, demonstrated only morphine-*conditioned* USVs and, importantly, the

unconditioned effects of morphine (i.e. occurring during the CPP acquisition phase) were not reported.

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

Since FM 50-kHz calls (and trill calls in particular) have been proposed to reflect positive affect in rats (see above), we hypothesized that after systemic administration, morphine would promote trill calls and possibly increase the overall rate of 50-kHz FM calls. This question was addressed in three experiments, using doses of morphine that are reportedly rewarding in CPP tests (Bardo et al. 1995). In Experiment 1, we initially tested individual rats with repeated morphine challenge; a low dose (1 mg/kg SC) was chosen to avoid catalepsy, which tends to occur above 5 mg/kg in drug-naïve rats (Fernandes et al. 1977; Fog 1970). We then tested the USV response to different doses of morphine (1 and 3 mg/kg) and also to AMPH (1 mg/kg). Here, rats were tested both individually and with a cage-mate. The latter condition served as a test of generalizability, since rats call more frequently and with a distinct acoustic profile in the presence of conspecifics (Brudzynski

and Pniak 2002; Wright et al. 2010). Having noted a USV-depressant effect of morphine, we next studied ultrasonic calling in parallel with locomotor activity in order to test for sedation. Next, in Experiment 2, the rewarding and locomotor effects of morphine were examined using a conditioned place preference (CPP) procedure; here, USVs were recorded during the acquisition (conditioning) phase, and during subsequent drug-free sessions where rats were restricted to either the saline- or morphine-paired floor texture. Finally, in Experiment 3, USV recording was performed during the acquisition phase and during the free-choice CPP test session.

METHODS

Subjects

Subjects were 64 experimentally-naïve male Long Evans rats (Charles River Laboratories, St. Constant, Quebec, Canada) weighing 339±14 g (mean±SD) (i.e. aged approximately 9 weeks) at the start of the experiment. Subjects were housed 2 per cage (25 x 48 x 20 cm^3) in a temperature- and humidity-controlled colony room (20-22˚C, 50-60%) at the McGill University Animal Resources Centre. The rats were maintained on a reverse 12:12 light/dark cycle, with lights off at 0730 h. All behavioural testing took place during the dark phase of the cycle. Food and water were available ad libitum, except during testing. Before the start of the experiment, each rat was handled by the experimenter for approximately 3 min per day on 1-2 days (Experiments 1 and 2) or 5 min per day over 6 days (Experiment 3). All procedures were approved by the McGill Animal Care Committee in accordance with the guidelines of the Canadian Council on Animal Care.

Locomotor activity and CPP apparatus

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

Acoustic data acquisition

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

(Primacoustic, Port Coquitlam, British Columbia) enveloped the outside of the CPP chambers, and extended 20 cm above the top of each chamber.

Drugs

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

Experimental protocol

Experiment 1

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

Part 1.1: Effects of repeated morphine administration on 50-kHz ultrasonic vocalizations

Repeated morphine exposure can produce sensitization to its rewarding effects (Lett 1989), and we anticipated that an analogous effect might occur with ultrasonic calling. Accordingly, the 24 subjects were randomly allocated to receive either morphine (1 mg/kg SC, n=12) or saline (n=12) daily over three days in the home cage prior to USV testing. On each of the following six test days, rats received an injection of morphine (1 mg/kg SC) or saline (presented in alternating sequence), and were placed in a recording test chamber from 30-60 min post-injection. The order of testing was counterbalanced such that within each group of 12 rats, 6 rats on each test day received morphine and 6 rats received saline.

Part 1.2: Effects of morphine and amphetamine on 50-kHz ultrasonic vocalizations in rats tested individually or in pairs

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

Part 1.3: Effect of morphine on 50-kHz ultrasonic vocalizations and locomotor activity

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

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

Subjects (n=24) were not pre-exposed to morphine in the home cage since this had had no detectable effect on the USV responses in Experiment 1. Behavioural testing consisted of four main phases, extending over 12 consecutive days in total: Habituation (Day 1), Conditioning (Days 2-9), CPP test (Day 10), and Conditioned USV tests (Days 11-12). On the first day (Day 1), the 24 rats, which were all drug- and experimentally-naïve, were habituated to the CPP chambers for 20 min on a layer of wood-chip bedding. Rats then underwent eight once-daily conditioning trials (Days 2-9), whereby morphine (1 or 3 mg/kg, depending on group, n=12) and saline were repeatedly paired (on alternating days)

with a distinct floor texture which served as a tactile cue. Immediately following injection, half the rats (n=12) were conditioned in the CPP cages for 20 min, and then promptly transferred to the USV recording chambers where they received an additional 20 min of contact with the same drug- or saline-paired floor texture; this order of conditioning was reversed for the other rats (n=12). On the CPP test day (Day 10), rats were placed drug-free in the middle of the CPP cage, straddling the two floor tiles, and for the next 20 min were free to choose between the two floor textures. On the conditioned USV test days (Days 11-12), each rat was recorded in the USV test chamber while being exposed for 20 min to the saline- or morphine-paired floor texture. This entire experiment was counterbalanced as far as possible in all respects, i.e. drug/floor-texture pairing, the order of drug *vs.* saline administration, position of morphine-paired floor texture within the test cage, and order of floor texture presentation during the two USV test days. This experiment concluded one week later with two additional USV test sessions which served to demonstrate that the rats were capable of emitting high rates of calling in response to a drug. Here, rats were tested for 20 min immediately after AMPH (1 mg/kg, IP) or saline, given in counterbalanced order.

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

Experiment 3 differed from Experiment 2 in the following respects. (1) All behavioural measures (i.e. USVs, locomotor activity and place preference) were collected simultaneously in the same (CPP) apparatus. (2) In order to minimize possible experimenter-induced stress or anxiety, rats were handled more (see Subjects above) and underwent two (*vs.* one) habituation sessions to the CPP apparatus before the conditioning

phase. (3) All rats (n=16) were conditioned with only the lower morphine dose (i.e. 1 mg/kg). (4) Testing for conditioned USVs was conducted during a single CPP test session during which the rats were free to choose between the drug- and saline-paired floor textures.

Analysis and classification of ultrasonic vocalizations

Acoustical analysis was performed as previously described (Wright et al. 2012b) using Avisoft SASLab Pro (version 4.2, Avisoft Bioacoustics). Calls were selected manually from spectrograms by an individual who was masked to the treatment condition. Each identified 50-kHz call was classified into 1 of 14 distinct categories: complex, upward ramp, downward ramp, flat, short, split, step-up, step-down, multi-step, trill, flat–trill combination, trill with jumps, inverted-U or composite (see Wright et al. 2010 for criteria for call identification and classification, several examples of each call type, as well as descriptive statistics relating to acoustic parameters). This method of manual call selection has been validated by surgical devocalization, and our method of classification is associated with high inter- and intra-rater reliability (Wright et al. 2010). 22-kHz calls were rarely observed in the present study; only a small minority of rats in Experiment 1.2 (mostly in the pair-tested condition) emitted any 22-kHz USVs. Therefore these calls were not analyzed further.

Data analysis and statistics

Data were analyzed using commercial software (Systat v11, SPSS, Chicago, IL; GraphPad Prism 4, GraphPad Software, La Jolla, CA). In Experiment 1.1 and 1.2, USVs that occurred during min 1, 6, 11, 16, 21, and 26 of the 30 min session were counted and classified. For Experiment 1.3, USV analysis was performed for min 1, 11, 20 of the 20 min session. In

Experiment 1.1, one rat was a serious outlier (i.e. >3 standard deviations from the mean) rendering the use of ANOVA invalid and was therefore excluded from the statistical analysis. In Experiment 1.2, rats were tested both singly and with their cage-mate. When rats were tested in pairs, the call rate was divided by two. Therefore, there were n=12 pairs for Part 2 and the data are expressed as calls per min per rat. One rat pair was a serious outlier and was therefore excluded from analysis. For Experiment 2 and 3, USV analysis was performed for min 3, 8, 13, and 18 of the 20 min session, and 3, 8, 13, 18, 23, 28, 33, and 38 of the 40 min session, respectively. However, all USVs that occurred during the entire 20 min CPP test were counted and classified. Due to a technical problem, locomotor data collection failed for four out of the 24 rats during the third conditioning trial in Experiment 2. Therefore, these rats were excluded from the locomotor activity analysis for that trial. Repeated measures ANOVA or Friedman's test was performed, where appropriate, and pairwise comparisons were performed using paired t-tests or Wilcoxon tests. The choice of test depended on the distribution of the raw data. ANOVA determined the effect of TESTPAIR (i.e. morphine minus saline difference scores for USV rate for each of the 3 morphine/saline tests in Experiment 1.1), GROUP (i.e. homecage morphine pre-exposure), and DRUG (i.e. morphine dose in Experiment 1.3). ANOVA p values were subject to the Huynh-Feldt correction, where appropriate, and multiple comparisons were subject to Holm-Bonferroni correction. For all analyses, a two-tailed p-value < 5% (after any correction) was considered significant.

RESULTS

Experiment 1

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

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

Part 1.3: USVs were tested either before or after locomotor activity, depending on group (n=12 rats per group). Data from these two groups were pooled, since neither the locomotor activity nor the USV rate revealed a significant between-group difference. Both doses of morphine (1 and 3 mg/kg) increased total locomotor activity per 20-min session (DRUG: $F_{2,44}=7.68$, $p<0.01$; 1 mg/kg vs. saline, $t_{23}=4.5$, $p<0.001$; 3 mg/kg vs. saline, $t_{23}=3.0$, $p<0.01$; Fig. 4a) but decreased the call rate (Friedman $Q_2=25.97$, $p<0.0001$; Wilcoxon tests:

saline *vs.* 1 mg/kg, Z=3.67, p<0.001; saline *vs.* 3 mg/kg, Z=3.81, p<0.001; Fig. 4b). The higher dose of morphine virtually abolished calling (Fig. 4b).

Experiment 2

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

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

Experiment 3

USV call rate was markedly inhibited by morphine (1 mg/kg) during the first two conditioning trials (saline *vs.* morphine on trials 1 and 2: Wilcoxon Z=2.71 and 2.63, both

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

Analysis of USV subtypes

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

DISCUSSION

The present study yielded several novel findings. First, morphine failed to increase the 50-kHz call rate under a variety of experimental conditions, i.e. after morphine pre-exposure, with repeated drug testing, or in different social contexts. Second, low doses of morphine that failed to increase 50-kHz call rates were nevertheless rewarding and non-sedative.

Third, detailed call subtype analysis indicated that, contrary to our hypothesis, morphine did not preferentially promote trill calls.

The USV-suppressant effect of morphine, which generalized to pair-tested subjects, did not appear to reflect general behavioral inhibition since it occurred at sub-cataleptic doses (Fernandes et al. 1977; Fog 1970) and was associated with locomotor stimulation rather than sedation (Experiments 1.3 and 3). This USV-suppressant effect was unaffected by prior administration of morphine in the home cage (Experiment 1.1), whereas it waned rapidly when the drug was later tested repeatedly in the same environment (Experiment 1.1 and Experiment 3); tolerance to this USV-inhibitory effect was therefore likely context-specific (Siegel and MacRae 1984). The observed tolerance appeared reversible upon drug withdrawal, as evidenced by an inhibition of USVs with morphine after 8 days of abstinence (1.2 *vs.* 1.3).

Horizontal locomotor activity is inhibited or stimulated by acute systemic administration of morphine, depending on dose and time after injection, with a shift towards locomotor stimulation upon repeated drug testing (Babbini and Davis 1972; Nakamura et al. 1978; Shoaib et al. 1994; Vasko and Domino 1978). Consistent with this literature, a locomotor stimulant effect was immediately apparent in subjects that had already received 7-10 morphine injections (Experiment 1.3) whereas such an effect emerged gradually in subjects that were initially drug naïve (Experiment 2 and 3).

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

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

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

Although morphine administration produced a robust CPP, it failed to produce *conditioned ultrasonic calling* when the rat was exposed to the drug-paired context, either though passive confinement (Experiment 2) or during free choice (Experiment 3). In Experiment 2 (but not Experiment 3), the tests of conditioned USVs occurred after the CPP test session, and so it is possible that the reward-cue associations had already extinguished. However, this is unlikely for two reasons. First, CPP is reported to take several drug-free sessions to extinguish after a similar morphine conditioning regime (Mueller et al. 2002; Parker and Mcdonald 2000; Rutten et al. 2011). Second, extinction of morphine CPP is typically context-dependent (Parker et al. 2006), and the USV tests were performed in a separate room and in a distinct apparatus from the CPP test.

The non-occurrence of morphine-conditioned 50-kHz USVs in Experiments 2 and 3 stands in contrast to two positive reports of conditioned calls occurring either during the expression of morphine CPP (Knutson et al. 1999) or in rats passively exposed to drug-paired CPP cues (Burgdorf et al. 2001b). Several methodological factors could potentially help to explain these disparate findings, for example: the dose of morphine (i.e. 5 mg/kg) (Burgdorf et al. 2001b), use of visual as well as tactile cues (Burgdorf et al. 2001b; Knutson et al. 1999), biased CPP procedure (Knutson et al. 1999), conditioning session duration and number (Knutson et al. 1999), and rat strain (Burgdorf et al. 2001b). Perhaps most significantly, however, call counting in these earlier reports was based on heterodyne and/or frequency-divided acoustic signals, which is less reliable than spectrographic analysis of broadband signals (Hamdani and White 2011; Parsons 2000).

Limitations

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

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

Conclusion

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

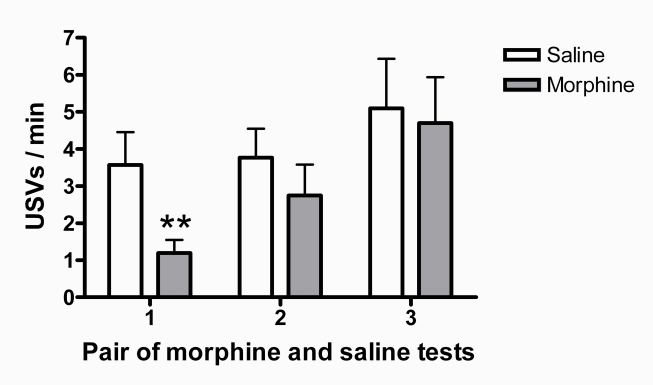

Fig. 1 Experiment 1.1: Morphine (1 mg/kg, SC) initially suppressed 50-kHz calling, but rats became tolerant to this effect with repeated testing. The y-axis represents mean+SEM 50-kHz USVs per minute under saline (open bars) and 1 mg/kg morphine (grey bars). **p<0.01 (n=24)

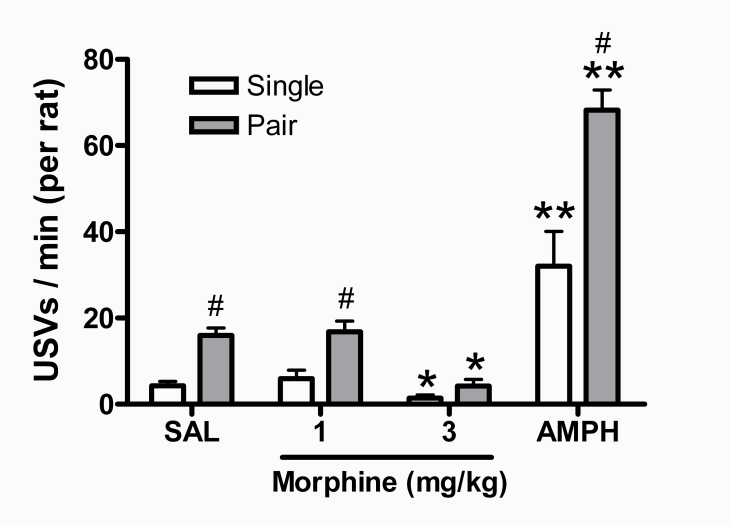

Fig. 2 Experiment 1.2: Morphine dose-dependently inhibited calling in both singly- (open bars) and pair-tested (grey bars) rats. The y-axis represents mean+SEM USVs per minute (on a per rat basis). Each rat was tested under all eight conditions (n=11 rat pairs). *p<0.05, **p<0.01 *vs.* respective saline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

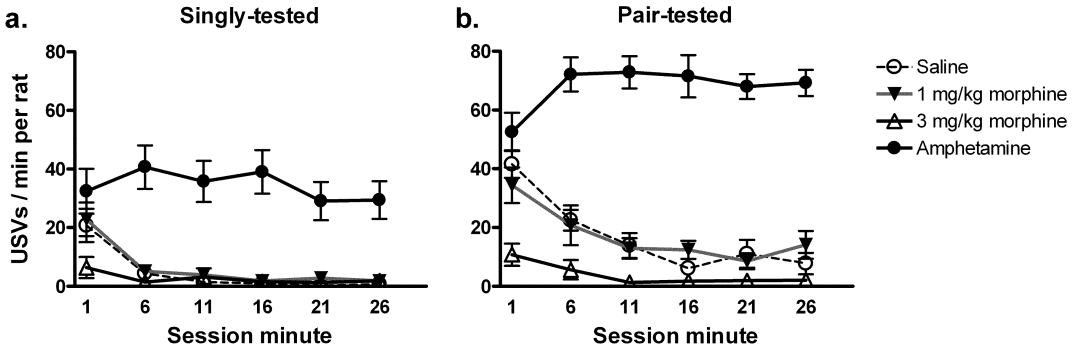

Fig. 3 Experiment 1.2: Time-course of USV emission following saline (open circles), morphine (1 or 3 mg/kg, SC; downward/solid and upward/open triangles, respectively) or AMPH (1 mg/kg, IP; solid circle) when rats were singly-tested (**a**) and pair-tested (**b**). The y-axes represent mean+SEM USVs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

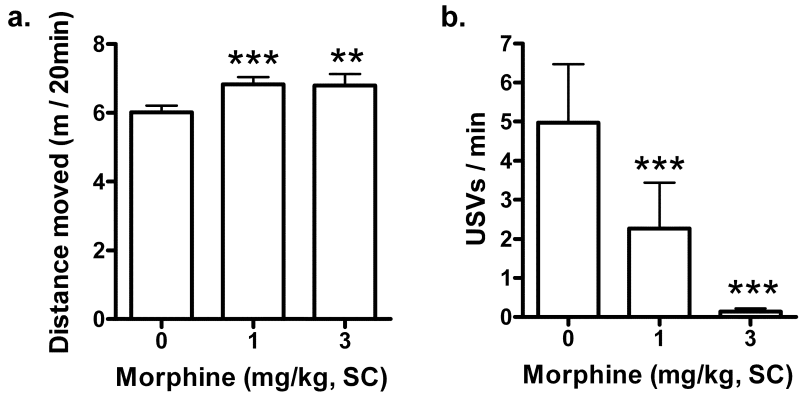

Fig. 4 Experiment 1.3: Morphine (1 and 3 mg/kg, SC) significantly increased locomotor activity (**a**), while dose-dependently decreasing emission of 50-kHz calls (**b**). Each rat was tested under all conditions (n=24). **p<0.01, ***p<0.001 vs. saline condition

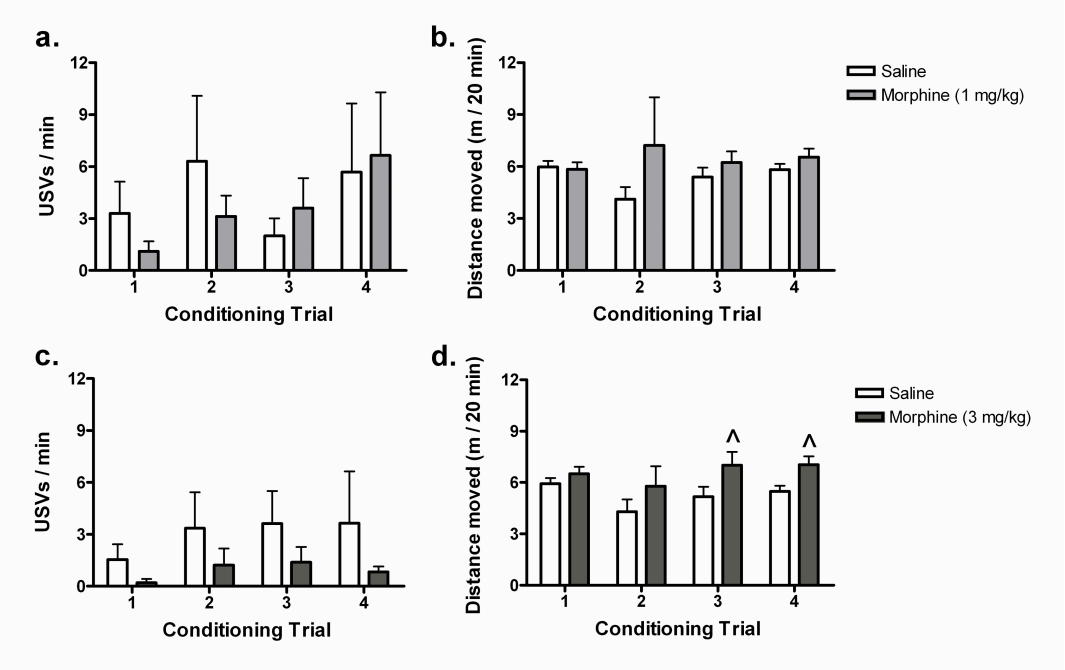

Fig. 5 Experiment 2: Call rate was unaffected during morphine conditioning. Each rat received saline (open bars) and morphine, given at 1 mg/kg (panels **a** and **b**, n=12, light grey bars) or 3 mg/kg (panels **c** and **d**, n=12, dark grey bars). Locomotor activity during conditioning with 1 mg/kg (**b**) and 3 mg/kg (**d**) morphine was unaltered during the four conditioning trials, except for an apparent stimulatory effect at the higher dose (3 mg/kg) on the 3rd and 4th conditioning trial, ^$p<0.06$

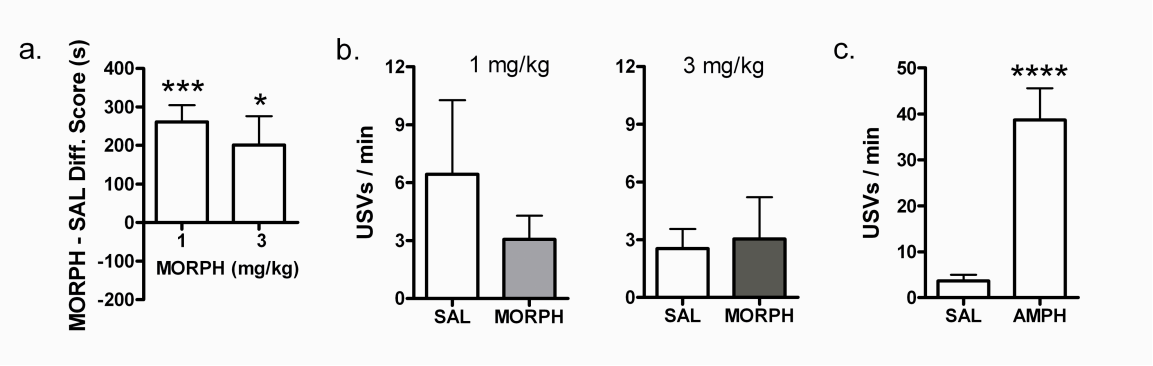

Fig. 6 Experiment 2: Post-conditioning and AMPH tests. **a.** On the CPP test day (Day 10), both doses of morphine produced a significant place preference. **b**. On Days 11 and 12, there was no significant difference in USV emission when rats were confined when drug-free to the morphine-paired *vs.*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

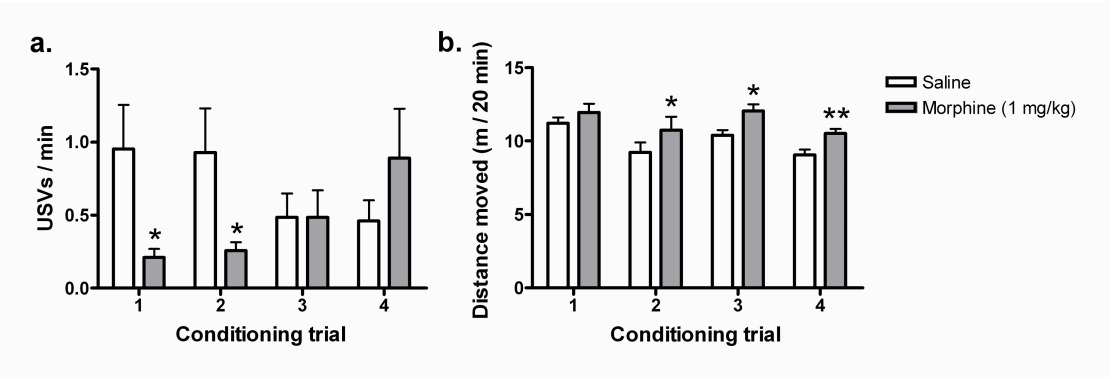

Fig. 7 Experiment 3: During conditioning trials, morphine initially decreased the call rate (**a**). However, morphine significantly increased locomotor activity during the last three trials (**b**). *p<0.05, **p<0.01 (n=16)

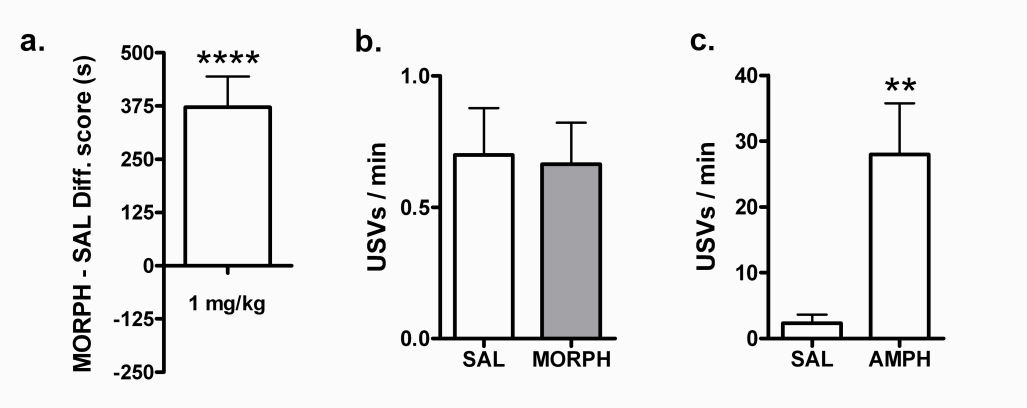

Fig. 8 Experiment 3: **a.** On the CPP test day, morphine produced a significant place preference. **b**. There was no significant difference in USV emission when rats were on the morphine- *vs.* saline-paired floor textures during the CPP test (open and grey bars, respectively, mean+SEM USVs per minute).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

Supplementary Material for Chapter 5

Table S1: Experiment 1.1: Number of calls of each category

	TRIAL 1		TRIAL 2		TRIAL 3	
USV subtype	**SAL**	**MORPH**	**SAL**	**MORPH**	**SAL**	**MORPH**
Complex	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Upward ramp	0.2 ± 0.1	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.1
Downward ramp	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Flat	3.5 ± 1.1	1.6 ± 0.5	2.6 ± 0.6	1.5 ± 0.5	5.2 ± 2.6	2.1 ± 0.6
Short	2.5 ± 0.5	0.7 ± 0.2	3.4 ± 0.7	1.6 ± 0.4	4.4 ± 1.1	3.4 ± 0.9
Split	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Step up	0.2 ± 0.1	0.1 ± 0.1	0.4 ± 0.2	0.4 ± 0.2	0.5 ± 0.2	0.1 ± 0.1
Step down	0.0 ± 0.0	0.1 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
Multi-step	0.3 ± 0.2	0.0 ± 0.0	0.2 ± 0.1	0.4 ± 0.2	1.4 ± 1.1	0.4 ± 0.2
Trill	7.0 ± 1.9	2.2 ± 0.7	7.3 ± 1.8	6.6 ± 2.5	9.3 ± 2.2	12.4 ± 3.4
Flat-trill combination	3.3 ± 1.3	1.0 ± 0.4	4.3 ± 1.3	1.7 ± 0.5	5.5 ± 1.9	4.3 ± 1.4
Trill with jumps	3.4 ± 1.4	1.2 ± 0.5	3.1 ± 1.3	3.2 ± 1.3	2.7 ± 1.0	4.0 ± 1.4
Inverted-U	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Composite	1.0 ± 0.4	0.3 ± 0.1	1.1 ± 0.4	0.9 ± 0.4	1.3 ± 0.5	1.3 ± 0.5

Data in the table refer to the mean ± SEM calls per 6 min of each call category (i.e. USV subtype) during trials 1-3 (n = 24 rats). The three trials occurred over 6 consecutive days, and each trial comprised one test with saline (SAL) and one test with morphine 1 mg/kg (MORPH), given in counterbalanced order.

Table S2: Experiment 1.1: Percentage of calls of each category

	TRIAL 1		TRIAL 2		TRIAL 3	
USV subtype	**SAL (22)**	**MORPH (15)**	**SAL (22)**	**MORPH (21)**	**SAL (24)**	**MORPH (22)**
Complex	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Upward ramp	0.3 ± 0.2	0.9 ± 0.9	0.2 ± 0.2	0.0 ± 0.0	0.1 ± 0.1	2.3 ± 2.3
Downward ramp	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.3	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0
Flat	23.9 ± 5.7	25.1 ± 8.6	18.9 ± 5.4	11.0 ± 3.1	16.8 ± 4.1	14.3 ± 4.1
Short	24.0 ± 6.8	18.8 ± 7.7	25.7 ± 7.1	21.2 ± 6.6	20.2 ± 4.0	16.5 ± 4.1
Split	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Step up	0.9 ± 0.6	2.4 ± 2.2	1.3 ± 0.6	1.3 ± 0.6	2.9 ± 1.7	0.3 ± 0.3
Step down	2.3 ± 2.3	0.8 ± 0.6	2.9 ± 2.3	0.8 ± 0.5	1.1 ± 0.8	3.1 ± 2.4
Multi-step	0.5 ± 0.3	0.4 ± 0.4	1.7 ± 1.2	1.3 ± 0.9	1.5 ± 0.8	1.5 ± 0.7
Trill	24.9 ± 3.9	33.4 ± 8.5	26.4 ± 4.4	37.8 ± 5.5	31.7 ± 5.4	38.0 ± 5.2
Flat-trill combination	10.9 ± 3.6	6.4 ± 2.2	12.1 ± 2.7	9.4 ± 2.3	17.3 ± 4.4	9.5 ± 2.3
Trill with jumps	.	9.9 ± 3.3	7.7 ± 3.1	14.1 ± 3.4	6.4 ± 1.8	11.9 ± 2.9
Inverted-U	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.1 ± 0.1
Composite	2.0 ± 0.7	1.8 ± 0.8	3.0 ± 0.8	3.1 ± 1.7	2.0 ± 0.6	2.5 ± 0.8

Data in the table refer to the mean ± SEM percent of each call category (i.e. USV subtype) per 6 min during trials 1-3 where rats were administered repeated saline (SAL) and morphine 1 mg/kg (MORPH). The number in brackets in a given drug condition corresponds to the number of subjects with non-missing values (i.e. rats that failed to emit any calls did not have calculable percent values and therefore did not contribute to the mean).

Table S3: Experiment 1.2: Number of calls of each category

	SINGLES				PAIRS			
USV subtype	**SAL (24)**	**MOR 1 (24)**	**MOR 3 (24)**	**AMPH (24)**	**SAL (12)**	**MOR 1 (12)**	**MOR 3 (12)**	**AMPH (12)**
Complex	3.1 ± 1.0	5.0 ± 1.5	1.9 ± 1.3	10.0 ± 2.4	13.8 ± 2.3	12.3 ± 2.8	4.4 ± 1.6	35.3 ± 4.8
Upward ramp	0.3 ± 0.2	0.1 ± 0.1	0.0 ± 0.0	0.3 ± 0.1	0.8 ± 0.5	0.3 ± 0.1	0.1 ± 0.1	2.5 ± 0.8
Downward ramp	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.1 ± 0.1	0.6 ± 0.3
Flat	1.8 ± 0.6	2.6 ± 0.7	0.8 ± 0.5	7.8 ± 2.2	7.2 ± 1.1	6.8 ± 1.9	3.1 ± 1.4	25.8 ± 5.3
Short	4.0 ± 0.8	4.7 ± 1.2	1.5 ± 0.8	22.3 ± 3.2	29.5 ± 3.6	25.0 ± 3.0	5.8 ± 2.1	94.1 ± 8.1
Split	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.2	0.2 ± 0.2	0.0 ± 0.0
Step up	2.8 ± 0.8	4.2 ± 1.3	1.3 ± 0.7	15.3 ± 3.1	19.7 ± 5.0	12.7 ± 2.2	4.3 ± 1.7	58.3 ± 7.6
Step down	0.4 ± 0.1	0.5 ± 0.2	0.0 ± 0.0	1.6 ± 0.6	1.5 ± 0.3	1.5 ± 0.5	0.8 ± 0.5	4.8 ± 0.9
Multi-step	0.8 ± 0.3	1.3 ± 0.4	0.8 ± 0.5	4.6 ± 1.1	4.1 ± 0.8	3.7 ± 0.8	1.5 ± 1.1	11.7 ± 1.0
Trill	9.1 ± 2.7	11.3 ± 3.7	3.7 ± 2.1	76.1 ± 13.8	73.2 ± 8.5	80.2 ± 13.1	14.3 ± 6.2	278.5 ± 23.0
Flat-trill combination	3.5 ± 1.2	3.7 ± 1.0	2.8 ± 1.6	33.8 ± 7.5	32.3 ± 6.9	28.2 ± 7.3	6.3 ± 3.5	133.0 ± 15.1
Trill with jumps	1.6 ± 0.7	2.4 ± 1.0	1.0 ± 0.5	20.1 ± 10.7	9.0 ± 1.8	11.6 ± 3.4	2.3 ± 1.1	89.8 ± 20.7
Inverted-U	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.7 ± 0.3	0.5 ± 0.2	0.8 ± 0.3	0.3 ± 0.1	2.2 ± 0.6
Composite	1.0 ± 0.4	2.3 ± 0.9	1.7 ± 1.1	13.8 ± 3.1	14.1 ± 2.7	22.9 ± 4.5	3.2 ± 2.1	75.8 ± 17.1

Data in the table refer to the mean ± SEM calls / 6 min of each call category (i.e. USV subtype) under saline (SAL), 1 mg/kg morphine (MOR 1), 3 mg/kg morphine (MOR 3), and 1 mg/kg amphetamine (AMPH) when tested singly (n=24 rats) and with a cagemate (n=12 pairs of rats).

Table S4: Experiment 1.2: Percentage of calls of each category

	SINGLES				PAIRS			
USV subtype	**SAL (21)**	**MOR 1 (21)**	**MOR 3 (10)**	**AMPH (24)**	**SAL (12)**	**MOR 1 (12)**	**MOR 3 (10)**	**AMPH (12)**
Complex	14.7 ± 5.5	15.3 ± 4.8	9.8 ± 4.0	4.6 ± 0.8	7.0 ± 1.1	7.7 ± 1.8	11.8 ± 6.3	4.6 ± 0.7
Upward ramp	1.0 ± 0.5	0.6 ± 0.4	10.0 ± 10.0	1.1 ± 1.0	0.5 ± 0.3	0.1 ± 0.1	0.1 ± 0.1	0.3 ± 0.1
Downward ramp	0.0 ± 0.0	0.0 ± 0.0	5.1 ± 5.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.0
Flat	8.7 ± 3.9	18.9 ± 6.4	13.1 ± 9.8	4.2 ± 0.8	3.8 ± 0.5	3.8 ± 1.0	19.3 ± 9.9	3.2 ± 0.7
Short	24.0 ± 5.5	10.6 ± 1.9	8.6 ± 3.3	14.9 ± 1.9	14.5 ± 1.2	13.7 ± 1.5	19.3 ± 9.5	11.8 ± 0.8
Split	0.0 ± 0.0	0.4 ± 0.4	0.0 ± 0.0	1.1 ± 1.0	0.0 ± 0.0	0.2 ± 0.2	0.9 ± 0.9	0.0 ± 0.0
Step up	6.9 ± 1.5	7.6 ± 1.4	4.4 ± 1.8	6.2 ± 0.9	9.3 ± 1.3	6.0 ± 0.8	10.6 ± 4.7	7.5 ± 1.0
Step down	1.1 ± 0.4	1.2 ± 0.5	0.2 ± 0.2	0.5 ± 0.2	0.7 ± 0.1	0.9 ± 0.3	3.0 ± 2.7	0.6 ± 0.1
Multi-step	2.2 ± 0.8	3.0 ± 0.8	5.3 ± 3.3	2.0 ± 0.3	2.0 ± 0.4	2.0 ± 0.4	6.2 ± 4.9	1.5 ± 0.2
Trill	27.6 ± 4.4	25.3 ± 5.0	17.0 ± 6.6	40.5 ± 2.9	36.5 ± 2.3	38.9 ± 2.4	16.3 ± 6.2	34.6 ± 2.1
Flat-trill combination	7.3 ± 1.6	8.8 ± 1.9	8.7 ± 2.9	12.8 ± 1.6	14.7 ± 1.9	11.6 ± 2.2	6.5 ± 3.5	16.7 ± 1.6
Trill with jumps	3.1 ± 1.0	4.3 ± 1.3	14.4 ± 9.8	6.0 ± 1.5	4.4 ± 0.6	5.6 ± 1.3	3.0 ± 1.4	10.2 ± 2.1
Inverted-U	0.1 ± 0.1	0.0 ± 0.0	0.1 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.2	0.3 ± 0.1
Composite	3.4 ± 1.9	3.8 ± 1.0	3.3 ± 1.9	5.8 ± 0.9	6.4 ± 0.8	9.2 ± 1.5	2.8 ± 1.4	8.7 ± 1.4

Data in the table refer to the mean ± SEM percent of each call category (i.e. USV subtype) per 6 min under saline (SAL), 1 mg/kg morphine (MOR 1), 3 mg/kg morphine (MOR 3), and 1 mg/kg amphetamine (AMPH) when tested singly and in pairs. The number in brackets in a given drug condition corresponds to the number of subjects with non-missing values (i.e. rats that failed to emit any calls did not have calculable percent values and therefore did not contribute to the mean).

Table S5: Experiment 1.3: Number of calls of each category

USV subtype	SAL (24)	MOR 1 (24)	MOR 3 (24)
Complex	1.4 ± 0.5	0.8 ± 0.4	0.0 ± 0.0
Upward ramp	0.2 ± 0.1	0.0 ± 0.0	0.0 ± 0.0
Downward ramp	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Flat	1.3 ± 0.5	0.4 ± 0.2	0.1 ± 0.1
Short	3.0 ± 0.7	1.0 ± 0.6	0.1 ± 0.1
Split	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Step up	1.3 ± 0.6	0.4 ± 0.3	0.0 ± 0.0
Step down	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.1
Multi-step	0.7 ± 0.3	0.2 ± 0.1	0.0 ± 0.0
Trill	2.8 ± 1.0	1.4 ± 1.1	0.0 ± 0.0
Flat-trill combination	2.2 ± 0.8	0.8 ± 0.5	0.0 ± 0.0
Trill with jumps	0.3 ± 0.1	0.2 ± 0.2	0.0 ± 0.0
Inverted-U	0.3 ± 0.2	0.2 ± 0.1	0.0 ± 0.0
Composite	0.9 ± 0.4	0.6 ± 0.4	0.0 ± 0.0

Data in the table refer to the mean ± SEM calls / 3 min of each call category (i.e. USV subtype) emitted by rats (n = 24) under saline (SAL), 1 mg/kg morphine (MOR 1), and 3 mg/kg morphine (MOR 3).

Table S6: Experiment 1.3: Percentage of calls of each category

USV subtype	SAL (19)	MOR 1 (11)	MOR 3 (3)
Complex	11.1 ± 5.2	15.9 ± 9.0	0.0 ± 0.0
Upward ramp	0.8 ± 0.4	0.5 ± 0.5	0.0 ± 0.0
Downward ramp	0.5 ± 0.5	0.8 ± 0.8	0.0 ± 0.0
Flat	6.8 ± 2.3	13.9 ± 9.3	20.0 ± 20.0
Short	40.6 ± 9.3	25.0 ± 11.5	55.6 ± 29.4
Split	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Step up	5.2 ± 2.1	11.9 ± 8.9	0.0 ± 0.0
Step down	0.3 ± 0.2	1.2 ± 0.9	13.3 ± 13.3
Multi-step	2.6 ± 0.9	2.1 ± 1.1	0.0 ± 0.0
Trill	15.2 ± 4.4	7.9 ± 4.1	11.1 ± 11.1
Flat-trill combination	11.8 ± 2.9	13.9 ± 8.9	0.0 ± 0.0
Trill with jumps	1.0 ± 0.4	0.6 ± 0.6	0.0 ± 0.0
Inverted-U	1.2 ± 0.9	1.5 ± 0.9	0.0 ± 0.0
Composite	3.0 ± 1.2	4.7 ± 3.0	0.0 ± 0.0

Data in the table refer to the mean ± SEM percent of each call category (i.e. USV subtype) per 3 min under saline (SAL), 1 mg/kg morphine (MOR 1), 3 mg/kg morphine (MOR 3). The number in brackets in a given drug condition corresponds to the number of subjects with non-missing values (i.e. rats that failed to emit any calls did not have calculable percent values and therefore did not contribute to the mean).

Table S7: Experiment 2: Number of calls of each category for rats conditioned with 1 mg/kg morphine

	USV during conditioning		Conditioned USVs	
USV subtype	**SAL (12)**	**MOR 1 (12)**	**SAL (12)**	**MOR 1 (12)**
Complex	1.6 ± 0.8	2.7 ± 1.5	1.3 ± 1.1	0.2 ± 0.1
Upward ramp	0.7 ± 0.4	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.2
Downward ramp	0.1 ± 0.1	0.4 ± 0.2	0.0 ± 0.0	0.0 ± 0.0
Flat	12.6 ± 7.9	5.7 ± 2.0	4.9 ± 3.1	1.8 ± 0.6
Short	15.8 ± 8.6	8.5 ± 3.5	3.0 ± 1.5	1.6 ± 0.8
Split	0.3 ± 0.3	0.5 ± 0.5	0.0 ± 0.0	0.1 ± 0.1
Step up	6.9 ± 4.7	5.2 ± 2.6	2.6 ± 1.7	0.8 ± 0.5
Step down	0.4 ± 0.3	1.0 ± 0.4	0.6 ± 0.5	0.1 ± 0.1
Multi-step	0.8 ± 0.5	1.1 ± 0.5	0.8 ± 0.6	0.5 ± 0.2
Trill	14.8 ± 8.7	16.7 ± 10.1	7.5 ± 4.9	2.8 ± 1.4
Flat-trill combination	4.1 ± 1.9	3.5 ± 2.0	1.7 ± 0.8	0.8 ± 0.3
Trill with jumps	0.3 ± 0.2	0.9 ± 0.4	0.2 ± 0.1	0.1 ± 0.1
Inverted-U	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.1
Composite	0.1 ± 0.1	0.5 ± 0.2	0.2 ± 0.1	0.0 ± 0.0

Data in the table refer to the mean ± SEM calls each call category (i.e. USV subtype) emitted by rats (n = 12) during conditioning with saline (SAL) and morphine **1 mg/kg** (MOR 1) (first 2 columns) and when they were restricted to the saline and morphine paired floor textures (last two columns). The number in brackets in a given drug condition corresponds to the number of subjects.

Table S8: Experiment 2: Number of calls of each category for rats conditioned with 3 mg/kg morphine

	USV during conditioning		Conditioned USVs	
USV subtype	**SAL (12)**	**MOR 3 (12)**	**SAL (12)**	**MOR 3 (12)**
Complex	1.7 ± 1.2	0.3 ± 0.1	0.7 ± 0.4	0.4 ± 0.3
Upward ramp	0.6 ± 0.2	0.1 ± 0.1	0.0 ± 0.0	0.2 ± 0.2
Downward ramp	0.6 ± 0.3	0.2 ± 0.1	0.2 ± 0.1	0.0 ± 0.0
Flat	9.6 ± 6.5	2.3 ± 1.0	1.3 ± 0.7	2.9 ± 2.5
Short	13.6 ± 8.1	3.3 ± 1.8	2.7 ± 1.1	3.0 ± 2.0
Split	0.3 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.2
Step up	3.7 ± 2.2	0.7 ± 0.4	0.7 ± 0.4	1.1 ± 0.8
Step down	0.9 ± 0.7	0.2 ± 0.1	0.3 ± 0.2	0.3 ± 0.3
Multi-step	1.3 ± 0.7	0.3 ± 0.2	0.3 ± 0.3	0.4 ± 0.3
Trill	5.7 ± 3.2	2.0 ± 1.7	2.1 ± 1.0	0.8 ± 0.4
Flat-trill combination	4.5 ± 2.9	0.5 ± 0.3	0.6 ± 0.4	1.0 ± 0.6
Trill with jumps	0.6 ± 0.4	0.3 ± 0.3	0.2 ± 0.1	0.3 ± 0.3
Inverted-U	0.8 ± 0.7	0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.1
Composite	0.4 ± 0.2	0.2 ± 0.1	0.0 ± 0.0	0.2 ± 0.2

Data in the table refer to the mean ± SEM calls each call category (i.e. USV subtype) emitted by rats (n = 12) during conditioning with saline (SAL) and morphine **3 mg/kg** (MOR 3) (first 2 columns) and when they were restricted to the saline and morphine paired floor textures (last two columns).

Table S9: Experiment 2: Percentage of calls of each category for rats conditioned with 1 mg/kg morphine

	USV during conditioning		Conditioned USVs	
USV subtype	**SAL (10)**	**MOR 1 (10)**	**SAL (12)**	**MOR 1 (10)**
Complex	4.5 ± 2.0	3.7 ± 1.3	5.7 ± 4.2	2.8 ± 2.5
Upward ramp	2.1 ± 1.9	1.1 ± 0.7	2.1 ± 1.7	5.2 ± 2.7
Downward ramp	0.0 ± 0.0	2.8 ± 1.4	0.0 ± 0.0	0.0 ± 0.0
Flat	26.3 ± 7.6	23.5 ± 5.9	18.3 ± 6.3	32.0 ± 9.5
Short	32.3 ± 6.0	23.4 ± 6.2	32.1 ± 8.0	13.1 ± 8.3
Split	0.2 ± 0.2	0.7 ± 0.7	0.0 ± 0.0	0.3 ± 0.3
Step up	4.0 ± 1.9	10.8 ± 1.8	6.2 ± 2.8	5.5 ± 2.8
Step down	1.1 ± 1.0	2.8 ± 1.0	1.2 ± 0.9	1.7 ± 1.7
Multi-step	2.9 ± 2.0	2.4 ± 1.2	2.9 ± 1.4	12.7 ± 5.1
Trill	16.9 ± 5.9	16.1 ± 6.3	20.9 ± 7.6	19.1 ± 6.9
Flat-trill combination	7.4 ± 3.0	6.6 ± 1.8	9.1 ± 4.3	7.0 ± 2.8
Trill with jumps	0.1 ± 0.1	1.6 ± 0.7	0.8 ± 0.6	0.3 ± 0.3
Inverted-U	1.1 ± 0.7	1.2 ± 1.0	0.4 ± 0.3	0.3 ± 0.3
Composite	1.0 ± 1.0	3.4 ± 1.7	0.4 ± 0.3	0.0 ± 0.0

Data in the table refer to the mean ± SEM percent of each call category (i.e. USV subtype) during conditioning with saline (SAL) and morphine **1 mg/kg** (MOR 1) (first 2 columns) and when they were restricted to the saline and morphine paired floor textures (last two columns). The number in brackets in a given drug condition corresponds to the number of subjects with non-missing values (i.e. rats that failed to emit any calls did not have calculable percent values and therefore did not contribute to the mean).

Table S10: Experiment 2: Percentage of calls of each category for rats conditioned with 3 mg/kg morphine

	USV during conditioning		Conditioned USVs	
USV subtype	**SAL (11)**	**MOR 3 (10)**	**SAL (9)**	**MOR 3 (9)**
Complex	3.5 ± 2.6	1.1 ± 0.6	3.8 ± 1.6	5.9 ± 5.5
Upward ramp	5.2 ± 3.1	1.0 ± 1.0	0.0 ± 0.0	0.2 ± 0.2
Downward ramp	2.4 ± 1.4	3.9 ± 3.3	13.9 ± 11.1	0.0 ± 0.0
Flat	14.5 ± 3.3	21.9 ± 7.5	15.1 ± 7.5	27.3 ± 14.2
Short	45.5 ± 7.8	44.3 ± 10.5	39.3 ± 10.0	45.8 ± 14.2
Split	0.4 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	1.7 ± 1.5
Step up	7.4 ± 2.0	2.9 ± 1.7	5.4 ± 2.8	4.2 ± 2.4
Step down	3.4 ± 3.0	0.9 ± 0.7	1.2 ± 0.8	0.5 ± 0.5
Multi-step	2.5 ± 1.3	6.0 ± 4.9	1.4 ± 1.4	1.8 ± 1.5
Trill	7.4 ± 2.9	14.6 ± 10.1	12.7 ± 5.7	6.8 ± 3.5
Flat-trill combination	4.2 ± 2.3	2.2 ± 1.4	3.4 ± 2.0	5.1 ± 3.0
Trill with jumps	0.6 ± 0.4	0.7 ± 0.7	1.6 ± 1.1	0.4 ± 0.4
Inverted-U	1.7 ± 1.3	0.0 ± 0.0	2.2 ± 2.2	0.1 ± 0.1
Composite	1.3 ± 0.9	0.5 ± 0.4	0.0 ± 0.0	0.2 ± 0.2

Data in the table refer to the mean ± SEM percent of each call category (i.e. USV subtype) during conditioning with saline (SAL) and morphine **3 mg/kg** (MOR 3) (first 2 columns) and when they were restricted to the saline and morphine paired floor textures (last two columns). The number in brackets in a given drug condition corresponds to the number of subjects with non-missing values (i.e. rats that failed to emit any calls did not have calculable percent values and therefore did not contribute to the mean).

Table S11: Experiment 3: Number and percent of calls of each category

	Number of USVs during conditioning		Percentage of USVs during conditioning	
USV subtype	**SAL (16)**	**MOR (16)**	**SAL (16)**	**MOR (16)**
Complex	0.9 ± 0.4	1.1 ± 0.4	3.6 ± 1.2	8.1 ± 2.7
Upward ramp	0.4 ± 0.2	0.3 ± 0.2	1.7 ± 1.0	3.5 ± 2.7
Downward ramp	0.4 ± 0.4	0.2 ± 0.1	1.1 ± 1.1	6.8 ± 6.2
Flat	4.7 ± 0.8	4.1 ± 1.1	29.3 ± 5.2	34.8 ± 5.5
Short	6.8 ± 1.2	2.6 ± 0.6	41.7 ± 4.3	28.3 ± 5.9
Split	0.2 ± 0.1	0.0 ± 0.0	0.6 ± 0.3	0.0 ± 0.0
Step up	0.9 ± 0.3	0.4 ± 0.2	3.7 ± 1.2	1.6 ± 1.0
Step down	1.0 ± 0.5	0.5 ± 0.2	5.5 ± 3.1	3.5 ± 1.4
Multi-step	0.8 ± 0.6	0.4 ± 0.3	1.2 ± 0.9	1.3 ± 0.7
Trill	3.4 ± 1.8	2.0 ± 0.9	9.5 ± 3.9	7.9 ± 3.3
Flat-trill combination	1.0 ± 0.8	1.4 ± 1.0	1.6 ± 1.1	3.6 ± 2.0
Trill with jumps	0.1 ± 0.1	0.0 ± 0.0	0.3 ± 0.2	0.0 ± 0.0
Inverted-U	0.1 ± 0.1	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0
Composite	0.1 ± 0.1	0.2 ± 0.1	0.2 ± 0.2	0.6 ± 0.5

Data in the table refer to the mean ± SEM calls each call category (i.e. USV subtype) emitted by rats (n = 16) during conditioning with saline (SAL) and **1 mg/kg** morphine (MOR) (first 2 columns) and mean ± SEM *percent* of each call category during conditioning (last two columns). The number in brackets in a given drug condition corresponds to the number of subjects.

Intervening Section 3

Chapter 3 revealed a critical role of noradrenergic transmission in the AMPH-induced 50-kHz USV response to AMPH. Furthermore, it was determined that the rate enhancing-effect and subtype profile produced by AMPH are mediated through $\alpha 1$ vs. β receptor mechanisms, respectively. However, the possible contribution of dopaminergic transmission remained unclear. Therefore, Chapter 5 examined the USV response to saline or AMPH following pretreatment with antagonists targeting D1-like or D2-like DA receptors. The results, together with the findings of Chapter 3, suggested that both dopaminergic and noradrenergic mechanisms contribute to the USV response to systemic AMPH. However, it was unclear whether increasing extracellular levels of DA and NA would be *sufficient* to elicit 50-kHz calling. This question was addressed using selective DAT and NET reuptake inhibitors, tested alone and in combination.

CHAPTER 5: The role of dopaminergic transmission through D1-like and D2-like receptors in amphetamine-induced rat ultrasonic vocalizations

Jennifer M. Wright, May R.S. Dobosiewicz, Paul B.S. Clarke

Psychopharmacology (Berl) (2013), **225**(4): 853-68

ABSTRACT

Rationale Systemic amphetamine (AMPH) administration increases the rate of 50-kHz ultrasonic vocalizations (USVs) in adult rats, and preferentially enhances the 'trill' subtype; these effects of AMPH critically depend on noradrenergic transmission, but the possible contributions of dopamine are unclear. *Objective* To assess the role of dopamine in 50-kHz USVs emitted drug-free and following systemic AMPH administration. *Methods* Adult male Long-Evans rats pre-selected for high AMPH-induced calling rates were tested with AMPH (1 mg/kg, IP) and saline following pretreatment with the following dopamine receptor antagonists: SCH 23390 (0.005-0.02 mg/kg, SC), SCH 39166 (0.03-0.3 mg/kg, SC), haloperidol (0.1, 0.2 mg/kg, IP), sulpiride (20-80 mg/kg, SC), raclopride (0.1-0.5 mg/kg, SC), clozapine (4 mg/kg, SC), risperidone (0.5 mg/kg, SC), and pimozide (1 mg/kg, IP). The dopamine and noradrenaline reuptake inhibitors (GBR 12909 and nisoxetine, respectively) were also tested, alone and in combination. *Results* SCH 23390, SCH 39166, haloperidol and raclopride, dose-dependently inhibited vocalizations under AMPH, and suppressed the proportion of trill calls. Sulpiride, however, had no discernable effect on call rate or profile, even at a high dose that reduced locomotor activity. Single doses of clozapine, risperidone, and pimozide all markedly decreased calling under saline and AMPH. Finally, GBR 12909 and nisoxetine failed to promote 50-kHz USVs detectably or alter the subtype profile, when tested alone or in combination. *Conclusions* The rate of 50-kHz USVs and the call subtype profile following systemic AMPH administration depends on dopaminergic neurotransmission through D1-like and D2-like receptors. However, inhibiting dopamine and/or noradrenaline reuptake appears insufficient to induce calling.

Keywords: Ultrasonic vocalization, Amphetamine, Dopamine, Noradrenaline, Atypical antipsychotic, Dose-response, D1 receptor, D2 receptor, Hedonia, Affect

INTRODUCTION

Higher-frequency ultrasonic vocalizations (USVs) emitted by adult laboratory rats, generally termed '50-kHz calls' (for review, see Brudzynski 2009; Wohr and Schwarting 2010), are frequently associated with appetitive stimuli (Burgdorf et al. 2011; Knutson et al. 2002) and have been proposed to reflect positive affect (Brudzynski 2007; Burgdorf and Moskal 2009; Burgdorf et al. 2011). However, 50-kHz USVs are acoustically diverse, with many identified subtypes including flat (i.e. constant frequency) calls and at least 12 types of frequency-modulated (FM) calls (Wright et al. 2010). The relative prevalence of the different call subtypes, which we have termed the "call profile" (Wright et al. 2010), can be experimentally modified independently of the overall rate of 50-kHz call emission (Ciucci et al. 2007; Ciucci et al. 2009; Wright et al. 2012b).

Dopaminergic (DAergic) neurotransmission appears to play a key role in USV emission. Notably, acute systemic injection of the DA agonist apomorphine promoted 50-kHz calls (Williams and Undieh 2010), and intraccumbens administration of the D_2/D_3 agonist quinpirole modulated USV production in a dose-related triphasic fashion (Brudzynski et al. 2012). Conversely, DA receptor antagonists are reported to inhibit 50-kHz USVs elicited by several natural and artificial rewarding stimuli, namely systemic cocaine (Williams and Undieh 2010), intracerebral AMPH and glutamate (Thompson et al. 2006; Wintink and Brudzynski 2001), tickling (Burgdorf et al. 2007), electrical brain

stimulation (Burgdorf et al. 2007), and copulation-related contexts (Bialy et al. 2010; Ciucci et al. 2007; Ciucci et al. 2009).

The psychostimulant amphetamine (AMPH), which enhances both DAergic and noradrenergic transmission (McKittrick and Abercrombie 2007), exerts two principal effects on 50-kHz vocalizations: it increases the overall call rate (Ahrens et al. 2009; Simola et al. 2009; Wintink and Brudzynski 2001; Wright et al. 2010; Wright et al. 2012b), and in relative terms, it shifts the "call profile", thereby enhancing the trill subtype while suppressing flat calls (Wright et al. 2010; Wright et al. 2012b). These rate-enhancing and call profile-altering effects of AMPH are critically dependent on α_1 and β adrenergic receptor function, respectively (Wright et al. 2012b). To our knowledge, however, it has not been determined whether the effects of systemic AMPH administration on 50-kHz USV emission are also dependent on DAergic transmission.

The first main aim of the present study was therefore to test the hypothesis that DAergic neurotransmission is required for 50-kHz calls that are emitted when tested drug-free or following systemic AMPH administration. The second, related, aim was to determine whether either D1-like or D2-like DA receptors (Le Foll et al. 2009) play a role. These questions were addressed in Experiments 1-7, in which we tested the effects of acute pretreatment with several D1- or D2-like DA receptor antagonists in combination with systemic saline or AMPH challenge (see Table 1). During testing, it emerged that the atypical antipsychotic drug sulpiride (Rama Rao et al. 1981) did not inhibit AMPH-induced calling, in striking contrast to two classical D2 antagonists (i.e. haloperidol and raclopride). Therefore as a third aim, we assessed whether sulpiride's lack of effect reflected its atypical

antipsychotic profile, by testing two other atypical neuroleptic drugs (clozapine and risperidone) and one additional classical D2 antagonist (pimozide). We also recorded USVs and locomotor activity simultaneously (Experiment 7), in order to confirm that sulpiride was behaviorally active despite its failure to influence 50-kHz calling.

A final aim was to address whether enhancing DA or noradrenaline (NA) transmission is *sufficient* to induce 50-kHz USVs or affect the call profile (Experiments 8-10 – see Table 1). To this end, rats were acutely challenged with the selective DAT inhibitor GBR 12909 and the selective NET inhibitor nisoxetine, given alone and in combination.

METHODS

Subjects

Subjects were 114 male Long-Evans rats (Charles River Laboratories, St Constant, Quebec, Canada), weighing 376±50 g (mean±SD) at the start of the experiment. They were housed 2 or 3 per cage (25 x 48 x 20 cm^3) in a temperature- and humidity-controlled colony room (19–20˚C, 50–60%) at the McGill University Animal Research Center. Rats were maintained on a reverse 12:12 light/dark cycle, with lights off at 0700 h. All behavioral testing took place during the dark phase of the cycle. Food and water were available ad libitum, except during testing sessions. In all experiments, rats were initially drug- and experimentally-naïve, with the following exceptions: in Experiments 3 and 4, rats had received 4 prior systemic injections of AMPH (0.25, 0.5, 1, and 2 mg/kg, IP), and in Experiment 7, rats had received 4 prior administrations of morphine (1 mg/kg, SC). All procedures were approved

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

Overview of Experiments

Ten experiments were performed, as summarized in Table 1. Briefly, Experiments 1-7 tested the effects of antagonist pretreatment on the USV response (i.e. call rate and subtype profile) to systemic AMPH. Experiment 7 additionally examined locomotor activity during the USV recording. The acute USV responses to the DA and NA reuptake inhibitors (i.e. GBR 12909 and nisoxetine), given alone or in combination, were examined in Experiments 8-10.

Experimental protocol

AMPH screen A significant minority of rats emit few calls in response to systemic AMPH (Wright et al. 2010). Therefore, subjects in most experiments were initially screened for AMPH-induced calling. Exceptionally, in order to reduce pre-experiment drug exposure, subjects in Experiments 3, 4 and 7 were not screened since they had already received prior AMPH or morphine administration (see above). The AMPH screening method was as described previously (see Wright et al. 2012b for further details). Briefly, rats received three administrations of AMPH (1 mg/kg, IP) spaced two days apart; rats with the lowest rate of calling on the third AMPH test were excluded from subsequent testing. Only the third AMPH test session was analyzed because the first two sessions are not necessarily indicative of a rat's subsequent USV response to AMPH (unpublished observation). In total, 52 rats (out of 126 rats that underwent screening) were excluded on this basis.

Drug testing All experiments featured a fully parametric within-subject design, whereby each rat was tested once under each drug/dose condition (see Table 1 for details). Thus, in

Experiments 1-7, rats received all combinations of pretreatment and treatment drugs including all vehicle controls. After the pretreatment time interval had elapsed, each rat was injected with saline or AMPH (1 mg/kg, IP) and immediately placed in a test chamber and recorded for 20 min. Similarly, in Experiments 8-10, every rat was tested under the following conditions: vehicle, AMPH (1 mg/kg – positive control), and each dose of the drug(s) being tested. Here, recording sessions were of 20 min duration except for the GBR 12909 dose-response study (Experiment 8), where rats were tested for 40 min. Within each experiment, the order of testing was counterbalanced as far as possible given the number of subjects. Test sessions were always spaced 2 days apart in order to minimize possible carry-over effects of the drugs.

Drugs

All test drugs, doses, routes of administration, and pretreatment/treatment time intervals are shown in Table 1. Drugs were: d-amphetamine sulphate (Sigma-Aldrich, Poole, UK); haloperidol and S(-)-sulpiride (both from Sigma-Aldrich, St. Louis, MO); pimozide, R(+)-SCH-23390 HCl, SCH 39166 HBr (i.e. Ecopipam), raclopride, and risperidone (all from Tocris Bioscience, Ellisville, MO); clozapine, GBR 12909 2HCl, and (±)-nisoxetine HCl (all from the NIMH Chemical Synthesis and Drug Supply Program). Doses of the different compounds refer to the form indicated above. GBR 12909 was administered in a volume of 2 ml/kg; all other drugs were administered in a volume of 1 ml/kg. Sulpiride was dissolved in a few drops of glacial acetic acid and diluted with sterile saline. Clozapine, GBR 12909, haloperidol, pimozide, and risperidone were dissolved in a 0.1 M tartaric acid solution. All other drugs were dissolved in sterile saline. Drug vehicles were used for control injections.

The pH of GBR 12909 could not be raised beyond 4.5 (with NaOH) without precipitation. In case the lower pH affected call emission, each rat was tested twice with AMPH in Experiment 10, once with the standard drug solution and once with the same solution acidified with HCl to pH 4.5. Since there was no difference in call rate or profile between the two AMPH tests, data from these tests were pooled for the remainder of the analysis.

Behavioral recording

USV recordings were conducted as previously described (Wright et al. 2012b). With the exception of Experiment 7 (see below), recordings took place in four clear Plexiglas experimental chambers (ENV-007CT, Med Associates, St Albans, VT), each of which was enclosed in a melamine compartment lined with sound-attenuating acoustic foam (Primacoustic, Port Coquitlam, British Columbia). A condenser ultrasound microphone (CM16/CMPA, Avisoft Bioacoustics, Berlin, Germany) was securely inserted through a small (5-cm diameter) hole located centrally in the top panel of each experimental chamber. Consequently, the microphones were 15–30 cm from rats during testing. Microphone signals were fed into an UltraSoundGate 416H data acquisition device (Avisoft Bioacoustics) with a sampling rate of 250-kHz and 16-bit resolution.

For Experiment 7, USV recordings were made in rectangular, open-topped chambers (58 cm long x 29 cm wide x 53 cm high) to allow simultaneous recording of USVs and locomotor activity, as previously described (Wright et al. 2012a). Two ultrasound microphones were secured inside each chamber at opposite corners, approximately 10 cm from the top (i.e. 40 cm above the floor). Sound-attenuating acoustic foam enveloped the walls and extended 20 cm above the top of each chamber. A video tracking system

(EthoVision v 3.0, Noldus Information Technology, Leesburg, VA, USA) measured locomotor activity (expressed as the total horizontal distance moved) during the second half (i.e. min 11-20) of the session to allow AMPH to take effect.

All lights were off during behavioral testing, except for Experiment 7, where far-red (wavelength > 650 nm) illumination using a Kodak GBX-2 safelight filter (Vistek, Toronto, Ontario, Canada) provided darkroom lighting.

Analysis and classification of ultrasonic vocalizations

Acoustical analysis was performed using Avisoft SASLab Pro (version 5.1, Avisoft Bioacoustics), as previously described (Wright et al. 2012b). Calls were selected manually from spectrograms by an individual who was masked to the treatment condition. Each identified 50-kHz call was classified into 1 of 14 distinct categories: complex, upward ramp, downward ramp, flat, short, split, step-up, step-down, multi-step, trill, flat–trill combination, trill with jumps, or composite (see Wright et al (2010) for criteria for call identification and classification, several examples of each call type, as well as descriptive statistics relating to acoustic parameters). A few representative 50-kHz calls are shown in Fig. 1. This method of manual call selection has been validated by surgical devocalization, and classification is associated with high inter- and intra-rater reliability (Wright et al. 2010). 22-kHz calls were not analyzed since they were rarely observed in this study (specifically, one rat made 2 calls under sulpiride 40 mg/kg plus AMPH 1 mg/kg, and another rat made 20 calls under sulpiride 80 mg/kg plus AMPH 1 mg/kg).

Data Analysis and Statistics

Data were analyzed using commercial software (Systat v11, SPSS, Chicago, IL; GraphPad Prism 4, GraphPad Software, La Jolla, CA). For Experiments 1-7, USVs that occurred during minute 12, 14, and 16 of the 20 min session were counted and classified. These minutes were chosen since AMPH-induced calling becomes most pronounced within the 10-20 min time interval following AMPH administration (Wright et al. 2010). In Experiment 8, data throughout the entire 40 min session were analyzed. Finally, for Experiments 9 and 10, USV analysis was performed for minutes 3, 8, 13, and 18 of the 20-min session (i.e. we chose 4 min of time-sampling, and spread it evenly across the session). One rat was removed for the call *subtype* analysis in Experiment 2 (SCH 39166) because it only emitted one call at the highest dose, making it an extreme outlier when evaluating the percentage data. Repeated measures ANOVA was performed to determine the effect of the within-subjects factors "pretreatment" and "treatment", where appropriate. Pairwise comparisons were performed using paired t-tests or Wilcoxon tests; the choice of test depended on the distribution of the raw data. ANOVA p values were subject to the Huynh-Feldt correction, where appropriate. Multiple comparisons relating to the call rate data were subject to Holm-Bonferroni corrections, except where stated. However, for the call subtype analysis, pairwise comparisons were performed using uncorrected tests, in order to maintain statistical power. For all analyses, a two-tailed p-value < 5% (after any correction) was considered significant.

RESULTS

Note: Statistically significant results were found for certain of the less frequent call subtypes, but they were not consistently observed across doses or drugs of the same class, and are likely to be false positives; hence, these results are not reported here.

Experiments 1 and 2: Effects of the D1 antagonists SCH 23390 and SCH 39166

As expected, AMPH given alone (i.e. with vehicle pretreatment) greatly increased the rate of 50-kHz calling (Wilcoxon Z=2.80 and 3.06, both $p<0.01$; Fig. 2a and b). The call rate under AMPH was dose-dependently reduced by both SCH 23390 and SCH 39166, with significant effects at the two higher doses (SCH 23390: Wilcoxon Z=2.70 and 2.70, $p<0.05$; SCH 39166: Wilcoxon Z=2.90 and 3.06, $p<0.01$; Fig.2). Each antagonist, given alone, tended to suppress calling below the already-low baseline call rate, but a statistically significant inhibitory effect only occurred at the highest dose of SCH 39166 (Wilcoxon Z=2.80, $p<0.05$; Fig. 2b).

Higher doses of the D1-like antagonists also significantly affected the call profile. More specifically, the proportion of trill calls under AMPH was dose-dependently suppressed by both SCH 23390 (0.01 and 0.02 mg/kg vs. vehicle, Wilcoxon Z=2.29 and 2.19, $p<0.05$; Fig. 3a) and SCH 39166 (0.3 mg/kg vs. vehicle, Wilcoxon Z=2.52, $p<0.05$; Fig. 3c). In addition, SCH 39166 significantly enhanced the proportion of flat calls under AMPH at the highest dose tested (i.e. 0.3 mg/kg) (Wilcoxon Z=2.38, $p<0.05$; Fig. 3d). Although the proportion of flat calls appeared to be enhanced by SCH 23390, this failed to reach statistical significance (Fig. 3b). No other call subtype was significantly altered.

Experiments 3-5: Effects of the D_2 antagonists haloperidol, sulpiride, and raclopride

Call rate under AMPH: Haloperidol, at both doses tested (0.1 and 0.2 mg/kg) significantly inhibited calling following AMPH administration (respectively, Wilcoxon Z=2.31, p<0.05, and 3.06, p<0.01; Fig. 4a); sulpiride (20 and 40 mg/kg), in contrast, had no effect (Fig. 4b). Sulpiride was tested again at a higher dose (Experiment 5), this time in parallel with raclopride (Fig. 4c). Sulpiride again failed to affect the rate of calling after AMPH treatment, whereas raclopride behaved similarly to haloperidol, inhibiting 50-kHz calling at all doses tested (Wilcoxon Z=3.06, 2.98, and 3.06, p<0.01; Fig. 4c).

Call rate after antagonist alone: Haloperidol did not alter the call rate after saline challenge (Fig. 4a); here, however, control call rates were very low (i.e. <3 calls/min). Sulpiride, tested alone, significantly reduced calling at only one dose (40 mg/kg Wilcoxon Z=2.28, p<0.05; Fig. 4c), and this apparent effect was not replicated across experiments (i.e. Experiment 4 *vs.* 5; see Fig. 4b *vs.* 4c). In contrast, raclopride tested alone significantly inhibited the call rate at all doses tested (0.1, 0.2, and 0.5 mg/kg vs. vehicle, Wilcoxon Z=2.85, 3.06, and 3.06, p<0.01; Fig. 4c).

Call profile: Haloperidol and raclopride dose-dependently suppressed the proportion of trill calls following AMPH challenge (haloperidol 0.2 mg/kg *vs.* vehicle, Wilcoxon Z=2.51, p<0.05; raclopride 0.5 mg/kg vs. vehicle, Wilcoxon Z=2.1, p<0.05; Fig. 5a, c). This effect appeared less potent than the rate-inhibiting effect (Fig. 4a and 4c). Raclopride (0.2 mg/kg) also increased the proportion of flat calls under AMPH (mean±SEM percent of flat calls following pretreatment with vehicle *vs.* 0.2 mg/kg raclopride: 12.7±2.9 vs. 32.8±4.5; Wilcoxon Z=2.5, p<0.05). In contrast, sulpiride marginally *increased* the proportion of trill

calls at 40 mg/kg in Experiment 5 (Wilcoxon Z=2.19, $p<0.05$; Fig. 5c) but not in Experiment 4 (Fig. 5b).

Experiment 6: Effects of pimozide and the atypical antipsychotics clozapine and risperidone

Pimozide, clozapine, and risperidone were all tested at a single, high dose. All three antagonists markedly inhibited both USV after saline treatment and AMPH-induced USV production (see Fig. 6). Despite low rates of calling, call subtype analysis revealed that pimozide significantly reduced the proportion of trill calls under AMPH (mean±SEM percent of trills: vehicle *vs.* pimozide, 36.4±6.5 *vs.* 13.3±11.4, respectively; Wilcoxon Z=2.37, $p<0.05$).

Experiment 7: Effect of high-dose sulpiride on 50-kHz USVs and locomotor activity

Sulpiride (80 mg/kg) significantly decreased AMPH-induced locomotor activity (ANOVA pretreatment x treatment interaction: $F_{1,15}=14.85$, $p<0.01$; Fig. 7). Sulpiride also reduced locomotor activity when given alone ($t_{15}=3.39$, $p<0.01$; Fig. 7a). In contrast, sulpiride exerted no detectable effect on either the call rate (Fig. 7b) or profile (not shown).

Experiments 8-10: Effect of GBR 12909 and nisoxetine, alone and in combination

Unlike AMPH, neither GBR 12909 nor nisoxetine significantly promoted 50-kHz calling at any dose tested; all comparisons were statistically non-significant after Holm-Bonferroni correction (Experiments 8 and 9, respectively; Fig. 8a, b). In Experiment 8, GBR 12909 tended to increase the call rate at 10 mg/kg, especially in the first half of the 40-min session, i.e. time 20-40 min post-injection (Supplemental Fig. S1). Accordingly, this shorter post-injection interval was used when this drug was retested in Experiment 10. Here,

selected doses of GBR 12909 (i.e. 10 mg/kg) and nisoxetine (i.e. 12 mg/kg) were administered, not only alone but also in combination; there was still no significant enhancement (or suppression) of call rate (Fig. 8c). Notably, the 10 mg/kg dose of GBR 12909 which appeared to increase calling in Experiment 8 no longer showed such a trend (Fig. 8c).

The reuptake inhibitors, given alone or in combination, failed to mimic the effect of AMPH on the call profile. For example, in Experiment 10, AMPH significantly increased the relative prevalence of trill calls, but neither GBR 12909, nor nisoxetine, nor their combination showed this effect (mean±SEM percent trills: vehicle *vs.* AMPH, 22.9±5.2 *vs.* 46.3±6.7, respectively; Wilcoxon $Z=2.58$, $p<0.01$). Conversely, a significant reduction in the proportion of flat calls was observed following the co-administration of GBR 12909 and nisoxetine, yet AMPH unexpectedly did not reduce the proportion of flat calls in this particular experiment (mean±SEM percent flat calls: vehicle *vs.* GBR 12909+nisoxetine, 21.9±6.3 *vs.* 10.3±6.0, respectively; Wilcoxon $Z=2.67$, $p<0.01$).

DISCUSSION

The present study provides the first evidence that D1-like and D2-like receptor antagonists modulate the effects of systemic AMPH administration on the 50-kHz call rate and profile. Exceptionally, sulpiride, which is a D2-like antagonist with atypical antipsychotic features, consistently failed to affect USV emission. In addition, neither GBR 12909 (DAT inhibitor) nor nisoxetine (NET inhibitor), nor their combination, mimicked the effects of AMPH on USV production. Below, we argue that both D1-like and D2-like DA receptors play a critical

role in 50-kHz USV emission, and we suggest mechanisms contributing to sulpiride's lack of effect. We subsequently review antagonist-induced USVs suppression in the context of other behavioral and clinical effects of the same drugs. Finally, we discuss whether enhanced DA or NA transmission is sufficient to promote USV emission.

D1 dopaminergic receptor antagonism

The D1-like antagonists SCH 23390 and SCH 39166 dose-dependently inhibited the 50-kHz call rate and the percentage of trill calls following AMPH challenge; both antagonists also tended to reduce the call rate below control (i.e. drug-free) levels, although a significant reduction was only seen at the highest dose of SCH 39166. SCH 23390 and SCH 39166 both bind with high affinity to D1 and D5 receptors, with negligible affinity for D2-like receptors (i.e. D2, D3, and D4) (Tice et al. 1994). While SCH 23390 also has considerable affinity for serotonin receptors, namely $5HT_2$ and $5HT_{1C}$ (Bischoff et al. 1986; Nicklaus et al. 1988), SCH 39166 does not (Alburges et al. 1992; McQuade et al. 1991a; McQuade et al. 1991b; Wamsley et al. 1991). To our knowledge, these drugs do not have any other significant off-target effects. Thus, DA D1-like receptors appear critical to the USV-altering effects of systemic AMPH and may also regulate USV emission in the absence of this drug.

D2 dopaminergic receptor antagonism

All six D2-like antagonists, with the notable exception of sulpiride, markedly inhibited or abolished the stimulatory effect of AMPH on call rate. Additionally, haloperidol and raclopride dose-dependently decreased the proportion of trill calls under AMPH. The latter finding is in line with previous studies showing a reduction in the proportion of FM calls in response to sexual odours following systemic haloperidol pretreatment (Ciucci et al. 2007;

Ciucci et al. 2009). It appears likely that DA transmission through D2-like receptors is critical for both the call rate and profile following AMPH, since several possibilities exist as to why sulpiride is anomalous:

(1) Sulpiride may exert an additional (as yet unidentified) action which functionally counteracts D2 receptor blockade. Indeed, studies with muscarinic cholinergic and adenosine A2A receptor antagonists have provided such a precedent, in that these drugs can reverse the behavioral effects of DA receptor blockade (Collins et al. 2012; Morpurgo and Theobald 1964).

(2) The phenomenon of D2-like receptor heteromerization (Maggio et al. 2009) suggests another plausible mechanism by which sulpiride might exert functional effects that are distinct from those of other D2-like antagonists.

(3) It is unlikely that our doses of sulpiride were insufficient to antagonize USV emission, since comparable or even lower doses have proven effective in a number of DA-dependent behavioral assays, i.e. apomorphine hyperactivity and stereotypy (de Paulis et al. 1985), the AMPH cue (Nielsen and Andersen 1992; Nielsen and Jepsen 1985), conditioned place preference (CPP) induced by food or testosterone (Guyon et al. 1993; Schroeder and Packard 2000), and intravenous self-administration of nicotine or cocaine (Sorge and Clarke 2009). Importantly, a high dose of sulpiride that failed to affect the call rate, did at the same time reduce AMPH-induced hyperactivity (present study - Experiment 7); the latter effect is consistent with previous findings (Ljungberg and Ungerstedt 1985; Moore and Kenyon 1994; Sharp et al. 1986; White et al. 1992).

(4) Sulpiride, in contrast to many D2-like antagonists, possesses considerably lower affinity at D4 compared to D2 and D3 receptors (Rondou et al. 2010; Seeman et al. 1997;

Seeman and Van Tol 1994). However, it is unlikely that D4 receptors are critical to USV emission since raclopride (Experiment 5) markedly reduced USVs despite also having very low affinity at D4 receptors (Seeman and Van Tol 1994).

(5) The 'atypical' antipsychotic properties of sulpiride do not appear related to its lack of effect on USV emission, since the atypical drugs clozapine and risperidone clearly inhibited calling.

(6) Since D2-like antagonists tend to be pharmacologically non-selective (Jafari et al. 2012), it is conceivable that all the D2-like antagonists tested, except for sulpiride, fortuitously suppressed calling through some shared non- DAergic mechanism. However, this possibility seems remote since the compounds were drawn from multiple, structurally heterogeneous chemical classes (Jafari et al. 2012), and we are unaware of any such shared receptor candidate. Notably, α1 adrenergic receptor blockade abolishes AMPH-induced calling (Wright et al. 2012b) but some DA antagonists (e.g. raclopride) lack significant affinity for this receptor (Hall et al. 1986; Ishiwata et al. 2001; Ogren et al. 1986).

Behavioral mechanisms

The USV-related effects produced by the DA-like antagonists in the present study are summarized in Table 2, together with several other behavioral effects of the same drugs reported in the literature. Antagonist doses that inhibited saline- or AMPH-induced USVs frequently overlapped with those affecting other behavioral measures. However, as discussed below, no particular behavioral measure matched our USV findings completely.

USVs vs. motor function. Several DA antagonists (i.e. haloperidol, clozapine, risperidone, pimozide) inhibited USV emission at doses expected to markedly suppress drug-free or

AMPH-associated locomotion (Table 2). In general, however, there was no consistent relationship between motor impairment and USV emission. In particular, raclopride inhibited drug-free USV production even at low doses which tend not to inhibit locomotion, and conversely, sulpiride inhibited drug-free and AMPH-induced locomotion without detectably affecting USV production (Table 2).

AMPH cue. The discriminative stimulus effects of AMPH are of particular interest since they serve to model the drug's subjective effects in humans (Brauer et al. 1997). The USV-stimulatory and cue effects of AMPH appear similarly affected by our D1 and D2 antagonists, but only the latter is attenuated by sulpiride (see Table 2 for references).

USVs vs. reward/aversion. Since 50-kHz USVs have been proposed as a measure of drug reward, it is potentially informative to compare our results with published work using the conventional reward measure of CPP, while acknowledging that the latter reflects conditioned rather than unconditioned drug effects. Both D1 antagonists appeared to inhibit 50-kHz calling under saline treatment, allowing for the low rate of drug-free calling. However, it is unclear whether D1 receptor blockade reliably produces a conditioned place aversion (CPA) in rats (Table 2), since D1 antagonist effects are either mixed (SCH 23390) or unreported (SCH 39166). In contrast, D2-like antagonists consistently fail to produce a CPP or CPA in adult rats (Tzschentke 1998). The lack of D2 antagonist-induced CPP or CPA does not appear to reflect a learning or memory deficit, since D2 receptor blockers do not inhibit the acquisition of all types of CPP or CPA (Tzschentke 1998). Thus, D2 receptor antagonists appear neutral in the CPP/CPA test, yet all our D2 receptor antagonists (with the exception of sulpiride) tended to inhibit calling under saline treatment.

The acquisition of AMPH CPP is inhibited by D1 and D2 receptor antagonists, according to most reports (Table 2). However, our USV findings reveal two striking differences: (1) sulpiride did not inhibit AMPH-induced calling (present study), whereas it inhibited AMPH CPP (Hiroi and White 1991), and (2) clozapine abolished AMPH-induced calling, yet failed to inhibit AMPH CPP (Hoffman and Donovan 1995a). Importantly, these studies employed comparable doses of antagonist and AMPH.

USVs vs. affect. Although classic antipsychotics (e.g. haloperidol) do not produce a CPA in rats (see above), they often produce dysphoria in human subjects (Emerich and Sanberg 1991; Voruganti and Awad 2004). Atypical antipsychotics, in contrast, appear far less commonly associated with dysphoria, as evidenced by sulpiride, clozapine, and risperidone (Mehta et al. 1999; Potvin et al. 2003; Voruganti et al. 2000). Although the latter two drugs produced profound alterations in USV emission in the present study, circulating levels of these three DA antagonists probably far exceeded the clinical range.

USVs vs. AMPH euphoria. The dose of AMPH employed in the present study (i.e. 1 mg/kg) appears comparable to euphorigenic doses in human studies (Grilly and Loveland 2001). FM 50-kHz ultrasonic vocalizations (USVs) have been proposed to reflect hedonia (Burgdorf and Moskal 2009), and the trill subtype in particular appears most closely associated with rewarding doses of AMPH and cocaine (Wright et al. 2010; Wright et al. 2012b). Although trill calls following AMPH were preferentially inhibited by both D1-like and some D2-like (i.e. haloperidol, raclopride, and pimozide) antagonists in the present study, it is important to note that animal and human studies do not strongly support a role for DA in hedonia, but rather in incentive salience or 'wanting' (Brauer and de Wit 1997;

Leyton et al. 2005; Leyton et al. 2007; Smith et al. 2011). Therefore, in view of the present findings, we speculate that emission of FM 50-kHz calls, and trills in particular, may relate to incentive salience rather than hedonia. Flat calls, in contrast to trill calls, were significantly increased in relative terms by certain doses of SCH 39166 and raclopride, possibly as a consequence of trill call suppression. Flat calls have been proposed to have a social-coordinating function unrelated to positive affect (Wöhr et al. 2008).

Dopamine and noradrenaline reuptake inhibitors

AMPH and cocaine, which increase both DA and NA transmission (McKittrick and Abercrombie 2007), enhance USV production and modulate the call profile (Wright et al. 2010; Wright et al. 2012b). The results of the present study together with previous findings (Wright et al. 2012b) suggested that both DA and NA transmission are *necessary* for the observed effects of AMPH on USV emission. The question of *sufficiency* was addressed by subsequently examining whether the selective DAT and NET inhibitors GBR 12909 (Andersen 1989) and nisoxetine (Wong et al. 1982; Wong and Bymaster 1976), respectively, could mimic the USV effects of AMPH or cocaine. Neither GBR 12909 nor nisoxetine, alone or in combination, mimicked the effect of AMPH on the call rate or profile in the present study. At doses tested here, GBR 12909 would be expected to elevate extracellular DA, and co-administration of a NET blocker would likely potentiate this increase (Carboni et al. 2006). To our knowledge, there are no studies directly examining extracellular NA following nisoxetine administration in rats. Instead, the doses of nisoxetine were chosen based on their ability to generalize to the cues produced by the non-selective β-adrenergic agonist isoproterenol (Crissman and O'Donnell 2002) and the

NET blocker reboxetine (Millan and Dekeyne 2007); the latter drug produces a marked increase in extracellular levels of NA (Dekeyne et al. 2001).

GBR 12909 and nisoxetine, unlike AMPH, appear to exert their behavioral effects solely through transmitter reuptake inhibition. We have previously found that the DA/NA reuptake blocker cocaine moderately stimulated 50-kHz calling, while mimicking AMPH's ability to promote trill calls preferentially (Wright et al. 2012b). It is unclear why GBR 12909 and nisoxetine failed to exert either of these effects; here, cocaine's ability to inhibit the 5-HT reuptake transporter (Wall et al. 1995) or enhance exocytotic DA release (Ramsson et al. 2011) may be relevant.

Limitations

Adult rats exhibit large variability in their USV response to systemic AMPH (Taracha et al. 2012; Wright et al. 2010). In order to examine drug effects on AMPH-induced calling, we identified low responders using an initial AMPH screen in most experiments. A substantial number of subjects were then excluded, resulting in a selected population that may differ in other behavioral or neurochemical respects (Burgdorf et al. 2008b). Notably, the failure of sulpiride to modify the call rate or profile was independent of whether rats were screened or not (compare Experiment 5 with Experiments 4 and 7). The present method of selecting adult rats based on their acute response to AMPH helps to address the issue of low baseline call rates and high individual differences. Other approaches include selective breeding (Burgdorf et al. 2008b), and possibly through prior social manipulations (Vivian and Miczek 1991).

Due to the labour-intensive nature of this type of USV analysis, only a small fraction of the entire session (i.e. 3 or 4 min) was time-sampled for most experiments. It is possible that USV effects outside our chosen time intervals were missed. This method of time-sampling therefore limits interpretation of the present findings.

Finally, certain drugs, namely sulpiride, GBR 12909, and nisoxetine, exerted no discernable effects on USV emission. In the case of sulpiride, we performed an additional experiment (Experiment 7) where USVs and locomotion were assessed simultaneously. However, the negative findings with GBR 12909 and nisoxetine (or their combination) were not followed up with additional behavioral testing. While GBR 12909 would be expected to stimulate locomotor activity at all the doses tested (Hooks et al. 1994; Powell et al. 2001), nisoxetine does not appear to affect this measure in adult rats (Davids et al. 2002; Powell et al. 2001). The lack of positive controls in Experiments 8-10 is a limiting factor when interpreting these results.

Conclusion

USVs are a potentially rich source of information about the rat's subjective state. The present study furthers our understanding of the neurochemical substrates regulating USV production in adult rats. DA transmission appears critical for the 50-kHz USV response to systemic AMPH, since antagonism of either D1-like or D2-like receptors (with the notable exception of sulpiride) reversed the effects of AMPH on the call rate and profile. DA transmission also appears to modulate drug-free call emission. It appears that although both DA and NA are required, inhibition of DA and NA reuptake per se is not sufficient to elicit an AMPH-like USV response.

Table 1: Summary of Experiments

Experiment	Pretreatment	Doses (mg/kg)	Route	Time before saline/AMPH (min)	n
1	SCH23390	0.005, 0.01, 0.02	SC	20	10
2	SCH39166	0.03, 0.1, 0.3	SC	30	12
3	haloperidol	0.1, 0.2	IP	60	12
4	sulpiride	20, 40	SC	60	12
5	raclopride	0.1, 0.2, 0.5	SC	30	12
	sulpiride	40, 80	SC	30	
6	clozapine	4	SC	30	12
	risperidone	0.5	SC	30	
	pimozide	1	IP	30	
7	sulpiride	80	SC	30	16
Experiment	**Drug**	**Doses (mg/kg)**	**Route**	**Time before testing (min)**	**n**
8	GBR 12909	5, 10, 20	IP	20	8
9	nisoxetine	4, 8, 16	IP	15	8
10	GBR 12909	10	IP	20	12
	nisoxetine	12	IP	15	

Table 2: Effects of antagonists on USVs and other behavioral measures

Pretreatment		USV results (present study)			Other behavioral effects					
DRUG	**DOSE**	**Drug-free USV rate[a]**	**USV rate under AMPH**	**Trills under AMPH**	**Spontaneous LMA**	**Catalepsy**	**AMPH[b]-induced LMA**	**CPA/CPP**	**AMPH[c] CPP**	**AMPH[d] cue**
SCH 23390	**0.005**	—	—	—	—[10,22,30,43,44]	—[11,35,40]	?	—[1,26]	—[19]	—[9,37,38]
	0.01	—	↓	↓	—[10,22,43], ↑[31], ↓[30,44]	—[11,35,40]	↓[40]	—[1,26], CPA[49,50]	—[19]	—[9,37-39], ↓[4]
	0.02	—	↓	↓	—[10,48], ↓[30,44]	—[11,35,40]	↓[40]	—[1,26], CPA[49,50]	—[19], ↓[2]	↓[4,13,37,38,51]
SCH 39166	**0.03**	—	—	—	—[8]	—[17], yes[42]	?	?	↓[2]	?
	0.1	—	↓	—	—[8]	—[17], yes[42]	?	?	↓[2]	↓[58]
	0.3	↓	↓	↓	—[8], ↓[12]	—[17], yes[42]	?	?	↓[2]	↓[58]
Haloperidol	**0.1**	—	↓	—	—[45]	—[11,27], yes[23,35]	↓[5,21,23,41]	—[21,53]	↓[21]	↓[13,38,39]
	0.2	—	↓	↓	↓[45]	—[11,27], yes[23,35,45]	↓[5,21,23,33,41]	—[53]	↓[21,33,53]	↓[6,13,38,39]
(-)Sulpiride	**20**	—	—	—	—[14,36]	—[25,54]	↓[28,34,41,59]	—[50]	—[19]	—[37,38]
	40	— or ↓	—	— or ↑	—[14,36]	—[25,54]	↓[28,34,41,59]	—[50]	↓[19]	—[38], ↓[37]
	80	—	—	—	↓[10, present study]	—[25,54]	↓[present study, 28,34,41,47,59]	?	↓[19]	↓[37,38]
Raclopride	**0.1**	↓	↓	—	—[16,18,44,48], ↓[32]	—[18,23,40,56,57]	—[40], ↓[21,23]	—[16,21]	?	—[15,37], ↓[55]
	0.2	↓	↓	—	—[18,44], ↓[32,48]	—[18,23,57], yes[40]	↓[21,23,40]	—[21]	↓[16]	—[15,37], ↓[39]
	0.5	↓	↓	↓	↓[16,18,32,44,48]	—[18], yes[23,40,57]	↓[21,23,40]	—[21]	↓[16,21]	↓[15,37,39]
Clozapine	**4**	↓	↓	—	↓[5,45]	—[23,27,45]	↓[7,21,23]	—[21]	—[21]	↓[6,37,38]
Risperidone	**0.5**	↓	↓	—	—[5]	—[7], yes[23]	↓[5,21,23]	—[21]	↓[21]	↓[6]
Pimozide	**1**	↓	↓	—	↓[3,24,46,52]	—[29], yes[11]	↓[41,46]	?	?	↓[20]

— : no significant change; ? : currently no published data (to our knowledge); ↑ or ↓: significant increase or decrease, respectively. [a]Particularly for SCH 23390, haloperidol, and sulpiride, inhibitory effects might have been masked by the low rate of drug-free calling, [b] 0.5-3.5 mg/kg AMPH, [c] 1-2 mg/kg AMPH, [d] 0.3-1 mg/kg AMPH

1. Acquas et al. 1989; **2.** Acquas and Di Chiara 1994; **3.** Agmo and Soria 1999; **4.** Arnt 1988; **5.** Arnt 1995; **6.** Arnt 1996; **7.** Arnt and Skarsfeldt 1998; **8.** Batsche et al. 1994; **9**. Callahan et al. 1991; **10**. Cervo and Samanin 1996; **11**. Christensen et al. 1984; **12**. Collins et al. 2010; **13**. Exner et al. 1989; **14**. Ferrari and Giuliani 1995; **15**. Furmidge et al. 1991; **16**. Garcia Horsman and Paredes 2004; **17**. Hietala et al. 1992; **18**. Hillegaart and Ahlenius 1987; **19**. Hiroi and White 1991; **20**. Ho and Huang 1975; **21**. Hoffman and Donovan 1995a; **22**. Hoffman and Beninger 1985; **23**. Hoffman and Donovan 1995b; **24**. Horvitz and Ettenberg 1991; **25**. Imperato and Di Chiara G. 1985; **26**. Leone and Di Chiara G. 1987; **27**. Liao et al. 1999; **28**. Ljungberg and Ungerstedt 1985; **29**. McMillen et al. 1980; **30**. Menzaghi et al. 1997; **31**. Meyer et al. 1993; **32**. Millan et al. 2004; **33**. Mithani et al. 1986; **34**. Moore and Kenyon 1994; **35**. Morelli and Di 1985; **36**. Morgenstern et al. 1983; **37**. Nielsen and Andersen 1992; **38**. Nielsen and Jepsen 1985; **39**. Nielsen et al. 1989; **40**. Ouagazzal et al. 1993; **41**. Poncelet et al. 1987; **42**. Prinssen et al. 1993; **43**. Sacaan et al. 1996; **44**. Salmi et al. 1998; **45**. Sanchez et al. 1991; **46**. Schaefer and Michael 1984; **47**. Sharp et al. 1986; **48**. Shen et al. 2010; **49**. Shippenberg and Herz 1987; **50**. Shippenberg and Herz 1988; **51**. Smith et al. 1989; **52**. Spivak and Amit 1986; **53**. Spyraki et al. 1982; **54**. Tagliamonte et al. 1975; **55**. Varty and Higgins 1997; **56**. Wadenberg et al. 2000a; **57**. Wadenberg et al. 2000b; **58**. West et al. 1995; **59**. White et al. 1992

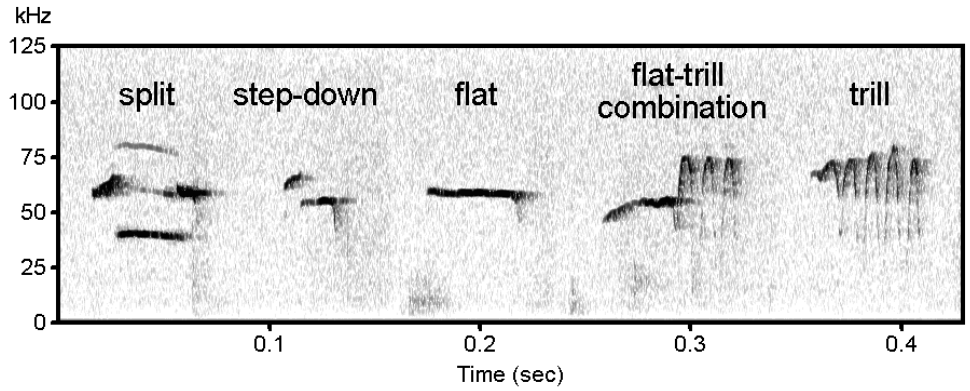

Fig. 1 Spectrogram containing individual 50-kHz calls representative of the following subtypes (left to right): split, step-down, flat, flat-trill combination, and trill. See Wright et al. (2010) for additional examples of all fourteen 50-kHz call subtypes

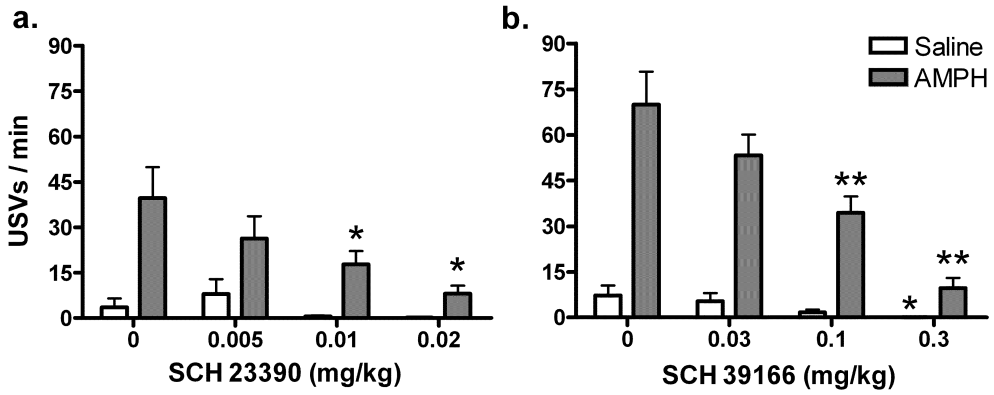

Fig. 2 Experiments 1 and 2: The D_1 antagonists SCH 23390 (**a**) and SCH 39166 (**b**) dose-dependently inhibited the call rate under AMPH. The y-axes represent mean+SEM calls/min. Each rat was tested under all pretreatment/treatment conditions (SCH 23390 group n=10; SCH 39166 group n=12). SCH 39166 alone also decreased the call rate at the highest dose tested (i.e. 0.3 mg/kg). *p<0.05, **p<0.01 *vs.* corresponding vehicle pretreatment condition

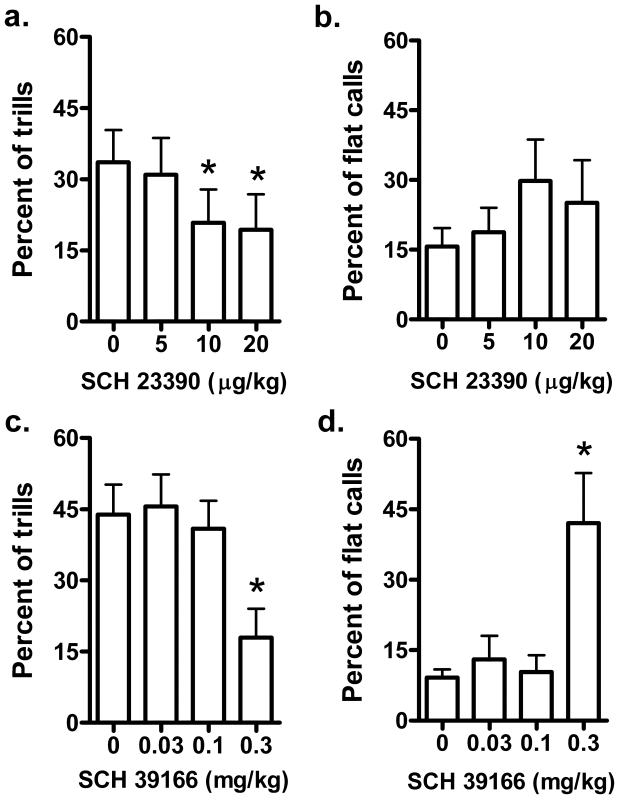

Fig. 3 Experiments 1 and 2: Pretreatment with the D_1 antagonists SCH 23390 (**a**) and SCH 39166 (**c**) before AMPH dose-dependently reduced the percent of trill calls. SCH 39166 also increased the percent of flat calls at the highest dose tested (0.3 mg/kg) (**d**). The apparent increase in the percent of flat calls with SCH 23390 was statistically non-significant (**b**). Each rat was tested under all pretreatment/treatment conditions (SCH 23390 group n=10; SCH 39166 group n=12). *p<0.05 *vs.* vehicle control

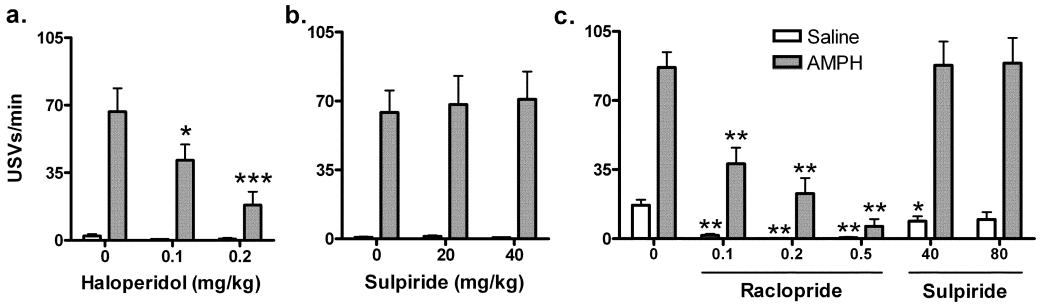

Fig. 4 Experiments 3-5: Haloperidol (**a**) and raclopride (**c**) dose-dependently inhibited USV emission under AMPH (grey bars) at all doses tested, while sulpiride (**b, c**) was ineffective. Raclopride (**c**) also reduced the call rate following saline treatment (open bars). Sulpiride only modestly reduced the drug-free call rate at 40 mg/kg in Experiment 5 (**c**). Each rat was tested under all pretreatment/treatment conditions (n=12 rats per experiment). *p<0.05, **p<0.01 *vs.* corresponding vehicle pretreatment

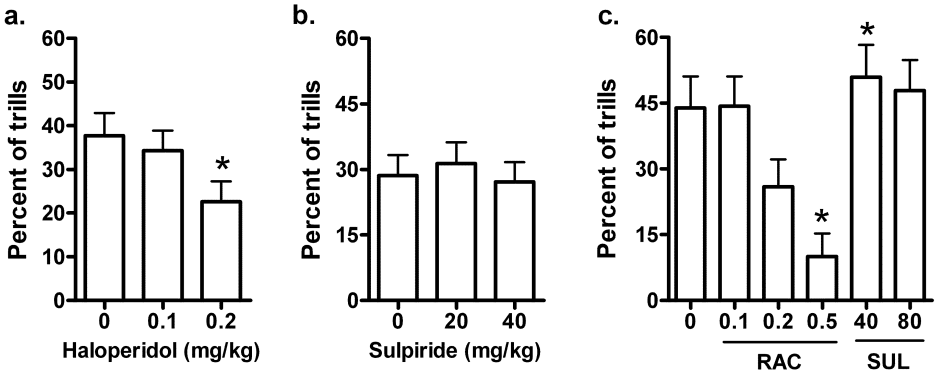

Fig. 5 Experiment 3 and 5: Haloperidol (**a**) and raclopride (RAC) (**c**) suppressed trills (as a proportion of all 50-kHz calls) following AMPH administration at the highest doses tested. Sulpiride (SUL), in contrast, was largely ineffective (**b, c**), except for an increase in the proportion of trills at 40 mg/kg in Experiment 5 (**c**). Each rat was tested under all pretreatment/treatment conditions (n=12 rats per experiment). *p<0.05 vs. vehicle control

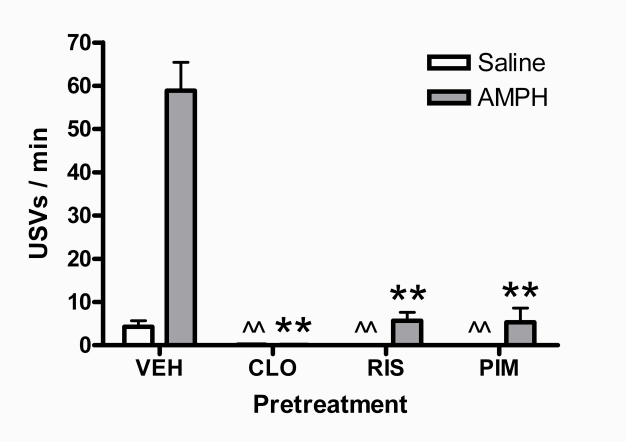

Fig. 6 Experiment 6: Single doses of clozapine (4 mg/kg, SC; CLO), risperidone (0.5 mg/kg, SC; RIS), and pimozide (1 mg/kg, IP; PIM), all markedly reduced the 50-kHz call rate under saline (open bars) and AMPH 1 mg/kg IP (grey bars). Each rat was tested under all pretreatment/treatment conditions (n=12 rats). ^^p<0.01 *vs.* vehicle/saline control, **p<0.01 *vs.* vehicle/AMPH control

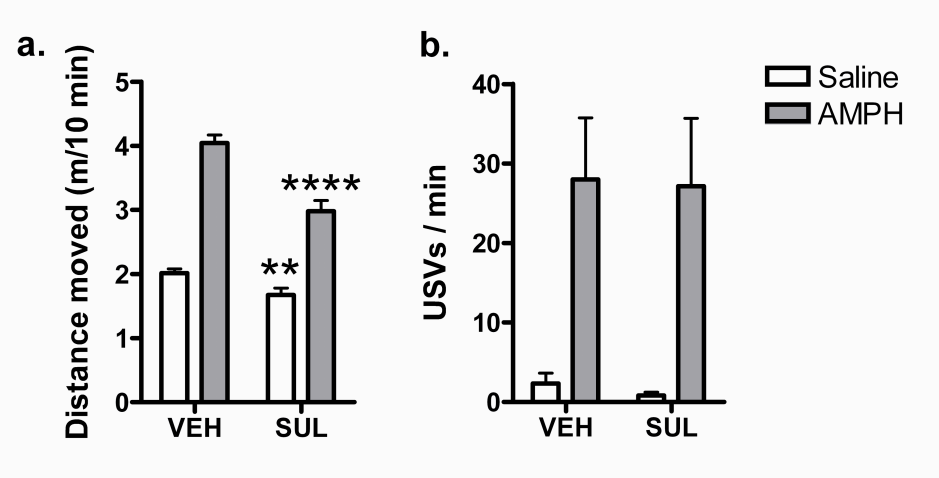

Fig. 7 Experiment 7: Sulpiride (SUL; 80 mg/kg, SC) significantly inhibited AMPH-induced locomotor activity (panel a) (ANOVA pretreatment x treatment interaction: $F_{1,15}$=14.85, p<0.01) but produced no detectable effect on the rate of USV emission (panel b). The y axes represent mean+SEM total horizontal distance (metres) travelled (panel a) or the 50-kHz call rate (panel b), following administration of saline (open bars) or AMPH (grey bars). **p<0.01, p<0.0001 *vs.* corresponding vehicle (VEH) control

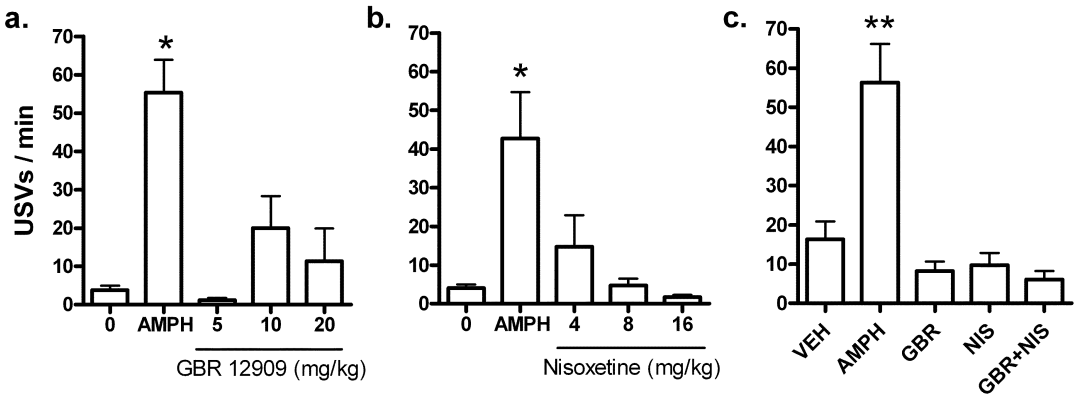

Fig. 8 Experiments 8-10: GBR 12909 (**a**) and nisoxetine (**b**) failed to significantly promote 50-kHz calling at any dose tested. Panel c shows that single doses of GBR 12909 (GBR, 10 mg/kg IP) and nisoxetine (NIS, 12 mg/kg IP), given either alone or even in combination (GBR+NIS), still failed to modify the call rate detectably (**c**). *p<0.05, **p<0.01 *vs.* vehicle control (VEH)

Supplementary Material for Chapter 5

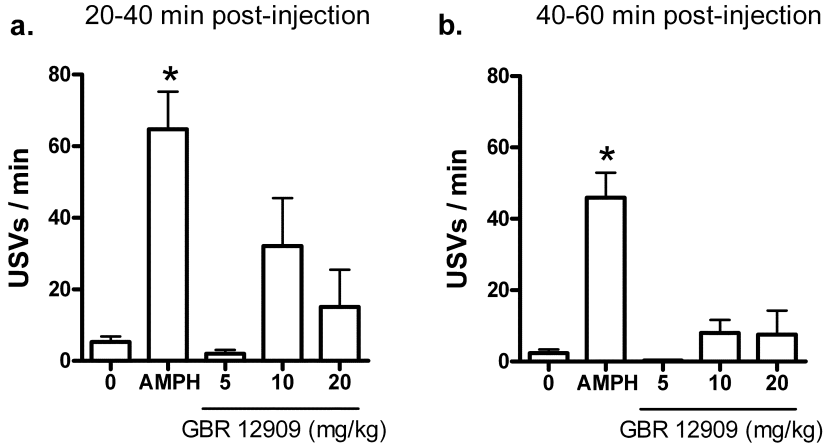

Figure S1 Experiment 8: GBR 12909 failed to significantly promote 50-kHz calling at any dose 20-40 min post-injection (**a**) and 40-60 min post-injection (**b**). *p<0.05 *vs.* vehicle control (Wilcoxon with Holm-Bonferroni correction)

CHAPTER 6: General Discussion

Jennifer M. Wright

Summary

This thesis revealed that the 50-kHz ultrasonic vocalizations (USVs) emitted by adult rats are considerably more heterogeneous than described previously, with as many as fourteen call *subtypes* (Chapter 2). Notably, the relative proportion of individual call subtypes emitted depended on drug or social context. The psychostimulant AMPH increased the rate of calling in the 50 kHz range and promoted trill calls in particular. Both adrenergic (Chapter 3) and dopaminergic (Chapter 5) mechanisms appear to be involved in these effects of AMPH. Cocaine mimicked the trill-enhancing effect of AMPH, but was comparatively less efficient at increasing the overall call rate (Chapter 3). Finally, rewarding and non-sedative doses of morphine failed to promote trill calls (Chapter 4), unlike AMPH and cocaine, suggesting that not all rewarding drugs share this effect on USV emission.

Overall, the results of this thesis suggest that the emission of acoustically distinct 50-kHz calls is not random, but rather deliberate, and that individual call subtypes do not necessarily share the same neurochemical basis or behavioural significance.

Future directions

Understanding the potential meaning of rat USVs

Future studies are needed to elucidate the potential communicative value of the 50-kHz call subtypes described in this thesis. Several approaches would be useful in this regard; for example, an in-depth analysis of the calls produced in appetitive contexts other than those examined here (e.g. tickling, food reward, and sexual encounters) would help to further characterize the relationship between trill calls and positive affect. Conversely, a thorough

investigation of the 50-kHz USVs emitted in aversive situations could also be quite revealing (see below). Finally, as discussed in the next section, ultrasonic playback combined with a variety of behavioural measures could provide a better understanding of the potential information encoded in different 50-kHz USV subtypes.

Ultrasonic Playback

Adult rat USVs, whether emitted in naturalistic contexts or played through a loudspeaker, have been shown to modify behaviour in other rats (see Introduction). The nature of the behavioural change has helped to elucidate the communicative value of 50-kHz *vs.* 22-kHz calls. As an approach towards gaining a better understanding of rat USVs, ultrasonic playback is particularly useful for two reasons. First, any behavioural change can be attributed to the nature of the sound alone independently of any visual, olfactory, or other cues that rats might normally transmit. Second, ultrasonic playback allows the experimenter to control which types of calls the rats are subjected to. The latter is especially advantageous when trying to determine the effects of individual call subtypes on behaviour (see below).

The usefulness of ultrasonic playback is exemplified in two published studies. In the first, rats performed an operant task (nosepoke) in order to receive playback of different types of 50-kHz calls (Burgdorf et al. 2008a). Rats self-administered the playback of frequency-modulated (FM) calls but did not increase responding for flat calls and avoided playback of 22-kHz calls (Burgdorf et al. 2008a). These findings suggest that rats can distinguish between different USV subtypes played through a loudspeaker, and that the reinforcing properties of call playback are subtype-specific. In the second study, playback

of a 50-kHz call sequence (comprising both FM and flat calls in a ratio of 10:3) induced behavioural activation and approach towards the loudspeaker, whereas playback of 22-kHz calls resulted in behavioural inhibition (as measured by a reduction in locomotor activity)(Wohr and Schwarting 2007). This study also examined the effects of artificial sine wave tones having the same duration, frequency, and amplitude as the natural 50-kHz calls, but lacking the frequency and amplitude modulation. As such, these artificial tones closely resembled natural flat calls. Playback of these tones produced behavioural changes analogous to those elicited by the natural 50-kHz calls, albeit less efficiently (Wohr and Schwarting 2007). Taken together, the above two studies suggest that FM calls have a greater appetitive value than flat calls.

However, as described in Chapter 2, FM calls can be sub-classified into at least a dozen categories. Hence, it would be informative, and naturally extend the work in this thesis, to examine how playback of *each subtype* defined in Chapter 2 affects behaviour. The appetitive value of each subtype could be assessed by measuring approach, conditioned place preference, and self-administration of playback. These studies would help validate (and perhaps refine) the categorization scheme described in this thesis, and help elucidate the communicative value of different call subtypes.

50-kHz USVs in non-appetitive contexts

The emission of 50-kHz USVs by adult rats has been reported to occur in both appetitive and aversive contexts (see Introduction). Of the fourteen subtypes of 50-kHz calls described in this thesis, only one subtype (namely the trill call) was repeatedly associated with appetitive stimuli. It is not clear what call subtypes are emitted in aversive contexts.

Hence, it would be informative to perform a detailed subtype analysis of the 50-kHz calls emitted during aggressive encounters (e.g. resident-intruder paradigm), drug-withdrawal, and pain (see Introduction). These experiments would shed light on whether other non-trill 50-kHz USVs subtypes predominate under such conditions, and whether aversive situations cause the emergence of call categories not yet described. Accordingly, these studies would be an important step towards gaining a better understanding of the information encoded in different subtypes of 50-kHz USVs.

Neurochemical mediators of USV production and recognition

Throughout this thesis, drugs were administered systemically (through IP, IV, or SC injection) in order to target certain neurotransmitter systems and receptors rather than specific brain regions *per se*. To this end, it was determined in Chapters 3 and 5 that DA and NA play a critical role in AMPH-induced USV emission. However, administration of a DAT and/or NET blocker failed to mimic the USV altering effects of AMPH or cocaine (Chapter 5), suggesting that enhancing extracellular DA and NA alone is not the mechanism by which these psychostimulants affect 50-kHz USV production. Here, consideration of phasic dopamine release may be important (see Introduction section "Psychostimulants: mechanism of action").

In order to examine the neuroanatomical substrates involved in USV emission, researchers have used various standard techniques such as intracerebral drug injections, electrical brain stimulation, and brain lesions (e.g. Burgdorf et al. 2007; Ciucci et al. 2009; Thompson et al. 2006). While these studies have elucidated some key brain areas involved in USV emission (see Introduction section "CNS mechanisms of 50-kHz USV production and

recognition"), two powerful neuroscience techniques now exist that could yield important additional insight about USV production in rats:

The first is optogenetics: a technique combining genetic strategies and laser illumination that enables the instantaneous activation (or silencing) of particular neurons within a circuit (Fiala et al. 2010). It has the advantage over electrical brain stimulation in that it can be cell-type-specific (Zhang et al. 2007). Combining optogenetics with USV recording could address whether activating particular neuronal pathways is sufficient to induce 50-kHz USV production.

The second is fast-scan cyclic voltammetry (FSCV): an electrochemical technique that can monitor extracellular monoamine levels with high temporal (subsecond) resolution (Clark et al. 2010). FSCV can be performed in freely moving rats and would be able to elucidate the precise temporal relationship between catecholamine release and USV production (Clark et al. 2010). It is important to note however, that DA and NA produce indistinguishable cyclic voltammograms and thus cannot be differentiated by electrochemistry alone (Robinson et al. 2003; Robinson and Wightman 2007). This is because these neurotransmitters are very similar in chemical structure and the same hydroxyl groups are oxidized on each compound (Robinson et al. 2003). Therefore, in order to identify the signal as either DA or NA, it is first necessary to consider the anatomical location of the recording site (Robinson et al. 2003; Robinson and Wightman 2007). Second, it is highly desirable to use pharmacological manipulations (e.g. administration of a receptor antagonist or transport blocker) to help verify which compound is producing the voltammetric signal (Robinson et al. 2003).

The source of vocalizations during pair-testing

We found that pair-testing not only enhances the overall rate of calling (on a per rat basis), but also affects the call profile such that trills and flat-trill combination calls were preferentially increased (Chapter 2). One significant limitation when interpreting the USV data obtained by rats tested in pairs is the inability to discern which subject is vocalizing. For example, when two rats are tested in a pair, it is not clear whether they call at similar rates or if one rat predominates. In the latter case, it would be valuable to examine what other differences might exist between the rats that could explain the variation in response (e.g. social dominance). Moreover, knowing which USV *subtypes* were emitted by each rat during behavioural interactions (e.g. sexual or resident-intruder encounter) could help further our understanding of the communicative value of the different call categories described in Chapter 2.

To address the above issue, some researchers have performed surgical devocalization of one member of the rat dyad. Thus, vocalizations recorded during sexual and aggressive encounters have been attributed to the male *vs.* female and resident *vs.* intruder, respectively (Takahashi et al. 1983; Thomas et al. 1983; Thomas and Barfield 1985; White et al. 1990b). However, a major limitation of devocalization is that it was shown to disrupt normal behaviour and interaction with conspecifics (Thomas et al. 1981). Moreover, playback of 50-kHz USVs elicited call production in recipient rats, suggesting that some calls are emitted *in response* to those made by conspecifics (Wohr and Schwarting 2007). Therefore, devocalization is not an ideal method for attributing calls to each member of a rat pair.

One possible method for discerning which rat in a pair is vocalizing is by determining differences in amplitude and/or time of arrival of the signal at two spatially distributed microphones. This approach models two of the most important binaural auditory system mechanisms for sound localization in humans and other mammals (Grothe et al. 2010). The difference in arrival time of the sound at the two microphones can be analyzed using the statistical technique of cross-correlation (Valin et al. 2003). However, these binaural cues are not sufficient to disambiguate sound source locations in the vertical plane as well as in-front *vs.* back. Therefore, the auditory system uses additional mechanisms; for example, sound gets filtered as it interacts with the head and the pinnae, which provide additional cues for where the sound originated (Grothe et al. 2010). Head rotation and tilt also help in this regard (Wightman and Kistler 1999). To compensate for the latter complexities of the auditory system, as many as *eight* microphones were necessary to locate an audible sound source (using differences in arrival time and amplitude) (Valin et al. 2003). Sound reverberation is another issue that the auditory system must contend with. In a reverberant environment, reflected sounds reach the listener from all directions, interfering with the direct sound (i.e. the portion of sound that travels directly from the source to the listener). A mechanism called "the precedence effect" enables the direct sound, which arrives first, to dominate human perception and allow for accurate localization of the sound source (Litovsky et al. 1999). However, reverberation represents a considerable limitation for sound localization using a microphone array (Valin et al. 2003). The latter might be particularly problematic in the case of rat USVs, since higher frequency sounds scatter more easily (Brudzynski and Fletcher 2010). Therefore,

although it may be possible to ascertain which rat is vocalizing using multiple microphones, it is a non-trivial challenge.

Perhaps a more promising method for determining which rat in a pair is emitting USVs would be to monitor some physiological process involved in vocal production. A recent study examined the activity of the laryngeal muscles during USV emission in freely moving rats using intramuscular electromyography (EMG) (Riede 2013). It was determined that two intrinsic laryngeal muscles, i.e. thyroarytenoid and cricothyroid, are engaged during call emission, and that different call subtypes lead to characteristic EMG activity in those muscles (Riede 2013). A potential limitation of this approach is that implantation the EMG electrodes could cause tension or damage to the muscles or laryngeal framework, affecting the rat's ability to produce USVs (Riede 2011). However, no significant effects on USV acoustic parameters were noted in the study by Riede (2013).

Need for an automated system for call identification and classification

For all experiments in this thesis, USV identification was performed by visually scanning the spectrogram and manually delineating the beginning and end of each call. Following identification, calls were then classified based on the criteria outlined in Chapter 2.

The advantage of manually selecting the calls from the spectrogram is its high accuracy compared to currently available automated approaches such as those based on intensity thresholds. However, under certain conditions, rats vocalize at very high rates (e.g. ~50-75 calls per minute), rendering the method of manual call selection especially time-consuming. For example, following the administration of AMPH, a single rat typically produces over 1000 calls during a 20-min session. Accordingly, the number of calls to

identify reaches the tens of thousands, in a typical experiment with 8-12 subjects and multiple test days. In order to analyze the data within a reasonable amount of time, we resorted to time-sampling from our recordings for most experiments. Consequently, a significant amount of the acquired data (up to 80-85% of recorded calls) remains unanalyzed. An intrinsic disadvantage of this method is the possibility that the analyzed portions are not truly representative or that some effects were missed.

While the process of call classification takes less time than manual call selection in a practiced observer, it also presents some drawbacks. Since there exists a high degree of variability in acoustic structure *within* each call category, call classification is not always unambiguous. Although clearly unclassifiable calls were identified as such, and we were able to obtain high inter- and intra-rater reliability scores (Chapter 2), there is nevertheless some degree of inherent subjectivity in the classification process.

The ability to automate USV analysis would obviate the issues noted above, namely the time-consuming process of call identification and any possible bias during call classification. Here, the artificial neural network approach holds promise (Reby et al. 1997), as it has already been successfully employed to classify mice species based on their calls (Tian and Shang 2006).

Categorization of 50-kHz call subtypes: considerations

Fourteen call categories were described in this thesis. This categorization scheme was devised following visual inspection of thousands of 50-kHz USVs. Importantly, all of these calls were emitted by adult male rats, tested alone or with a cage-mate, drug-free and under various doses of AMPH (Chapter 2). Subsequent experiments (i.e. Chapters 3-5), in

which many additional pharmacological manipulations were employed, did not see the emergence of new call types. However, it remains to be determined whether the classification scheme described here is generalizable to other social or behavioural contexts, to female rats, and to other rat strains.

As mentioned in the previous section, there exists considerable acoustic variability within each call category. It is therefore possible that the criteria for classification are too broad, and that important effects may be observed in more subtle acoustic parameters. Alternatively, it could be argued that since similar characteristics between categories are evident in some cases (e.g. trill components are a feature of 3 or 4 subtypes), it would be worthwhile to determine criteria for collapsing call subtypes into fewer, more manageable categories. Indeed, in Chapter 5, statistically significant results were obtained for certain of the less frequent call subtypes, but they were not consistently observed across doses or drugs of the same class. As these results were likely false positives, they were essentially disregarded. Fewer call categories might preserve these rarer calls, and reduce the likelihood of false positives during statistical analysis. However, few call categories were actually positively correlated within a given behavioural context (Chapter 2), and in general, which call categories happen to be correlated may well depend on the particular experimental condition. Hence, the rationale to pool certain call subtypes would be rather subjective, and could render it more difficult to establish whether individual call subtypes encode unique information.

Concluding Remarks

The 50-kHz ultrasonic vocalizations emitted by adult rats have been regarded as a behavioural index of positive affect. However, in-depth examination of these calls has revealed a high degree of variability in acoustic structure; indeed, adult rat USVs can be classified into at least 14 categories according to characteristic patterns of frequency modulation. The finding that certain call subtypes are emitted preferentially under certain social or pharmacological conditions strongly suggests that different call subtypes may communicate unique information. However, in order to capitalize on the potential wealth of information conveyed by these calls, we first need a better understanding of *why* rats preferentially emit certain call subtypes in particular contexts, the neuroanatomical substrates that mediate their emission, and how subtype playback affects behaviour. Importantly, such studies may lead to the development of novel animal models in several fields (e.g. affective neuroscience, drug addiction, pain) in which the ability to gauge subjective states would be invaluable.

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APPENDIX: Copies of published articles

Psychopharmacology (2010) 211:1–13
DOI 10.1007/s00213-010-1859-y

ORIGINAL INVESTIGATION

Identification of multiple call categories within the rich repertoire of adult rat 50-kHz ultrasonic vocalizations: effects of amphetamine and social context

Jennifer M. Wright · Jim C. Gourdon · Paul B. S. Clarke

Received: 6 February 2010 / Accepted: 1 April 2010 / Published online: 6 May 2010

Abstract
Rationale 50-kHz ultrasonic vocalizations (USVs) emitted by adult rats are heterogeneous; they occur over a wide frequency range, show varying degrees of frequency modulation, and appear to differ in their behavioral significance. However, they have not been extensively categorized.
Objectives The main objective of this study was to identify subtypes of 50-kHz USVs emitted by adult rats and to determine how amphetamine (AMPH) or social testing condition affects their relative and absolute production rate and acoustic characteristics. A second objective was to determine the extent of individual differences in call rate, call subtype profile, and acoustic parameters (i.e., duration, bandwidth, and mean peak frequency).
Methods Adult male Long–Evans rats were administered systemic amphetamine (0.25–2 mg/kg, IP) and tested individually or with a cage mate for 20 min. Call categories were defined based on visual inspection of over 20,000 USV spectrograms. Surgical devocalization was performed on a subset of AMPH-tested rats in order to confirm the authenticity of call subtypes.
Results Fourteen categories of 50-kHz USVs were recognized. Call subtypes were differentially affected by social context, AMPH dose, and time within session. In contrast, the acoustic characteristics of call subtypes were notably stable. Marked and stable inter-individual differences occurred with respect to overall 50-kHz call rate, acoustic parameters, and call profile.
Conclusions The present findings, obtained under saline and amphetamine test conditions, provide the first detailed classification of adult rat 50-kHz USVs. Consideration of 50-kHz USV subtypes may advance our understanding of inter-rat communication and affective state.

Keywords Ultrasonic vocalizations · Amphetamine · Reward · Frequency-modulated · Trill · Dose–response · Individual differences

Electronic supplementary material The online version of this article (doi:10.1007/s00213-010-1859-y) contains supplementary material, which is available to authorized users.

J. M. Wright · P. B. S. Clarke (✉)
Department of Pharmacology and Therapeutics,
McGill University,
3655 Promenade Sir William Osler,
Montreal, QC H3G 1Y6, Canada
e-mail: paul.clarke@mcgill.ca

J. C. Gourdon
Comparative Medicine and Animal Resources Center,
McGill University,
3655 Promenade Sir William Osler,
Montreal, QC H3G 1Y6, Canada

P. B. S. Clarke
Center for Studies in Behavioral Neurobiology,
Concordia University,
Montreal, QC, Canada

Introduction

Ultrasonic vocalizations (USVs) have been observed in a number of rodent species (Sales 1972). In adult laboratory rats, two main types of USVs have been described: 22-kHz and 50-kHz calls (see Brudzynski 2009 for review). The 22-kHz call type has been termed a distress or "alarm" vocalization (Litvin et al. 2007), as it can be elicited by the presentation of a predator, painful stimuli, startling noises, and intermale aggression (Blanchard et al. 1991; Calvino et al. 1996; Han et al. 2005; Kaltwasser 1991; Thomas et al. 1983). In contrast, calls of the 50-kHz category have been detected in naturalistic appetitive contexts, such as during play, mating behavior,

exploratory activity, or in anticipation of food reward (Burgdorf et al. 2000; Knutson et al. 1998; Sales 1972). 50-kHz calls have also been elicited by several non-natural appetitive stimuli, particularly rewarding electrical brain stimulation and amphetamine (AMPH) administration (Ahrens et al. 2009; Burgdorf et al. 2000, 2001a, 2007; Simola et al. 2009; Thompson et al. 2006; Wintink and Brudzynski 2001). Of note, the 50-kHz class of calls encompasses a wide frequency range (30–90 kHz) (Kaltwasser 1990; Sales and Pye 1974), and these calls vary considerably in spectrographic structure (see below).

Until recently, rodent USVs were typically detected by means of frequency-division or heterodyne recording devices. These approaches allowed counting of calls but provided little or no information about acoustic parameters (Parsons 2000). In contrast, the use of high-frequency sampling of untransformed microphone signals has shown that adult rat 50-kHz USVs are heterogeneous, comprising "flat" (i.e., constant frequency) and "frequency-modulated" (FM) calls (Ahrens et al. 2009; Burgdorf et al. 2007, 2008a; Burgdorf and Panksepp 2006; Ciucci et al. 2009; Simola et al. 2009; Wohr et al. 2008). Somewhat more detailed classification schemes have been described, each comprising three or four subtypes of 50-kHz calls (Kaltwasser 1990; Vivian and Miczek 1993; White et al. 1990), but individual spectrograms seem to indicate a much richer diversity (Burgdorf et al. 2008a; Ciucci et al. 2009; Schwarting et al. 2007; Wohr et al. 2008). Recently, five USV subtypes were defined in adult mice (Panksepp et al. 2007) and as many as ten USV subtypes were documented in mouse pups (Scattoni et al. 2008), suggesting that a detailed classification of adult laboratory rat USVs would also be warranted.

Adult rat USVs can play a communicative role, as evidenced by the effects of devocalization and USV playback on rat behavior (Brudzynski and Chiu 1995; Burgdorf et al. 2008a; Thomas et al. 1981; Wohr and Schwarting 2007). Interestingly, FM and flat calls appear to differ in their neurochemical basis and behavioral significance (Ahrens et al. 2009; Burgdorf et al. 2007, 2008a; Burgdorf and Panksepp 2006). For example, FM calls have been suggested to signal a dopamine-dependent reward state (Burgdorf et al. 2008a) and, on preliminary evidence, are increased by the prototypical DA agonist AMPH more than flat calls (Simola et al. 2009). In contrast, flat calls may serve a social-coordinating function (Wohr et al. 2008). Rat 50-kHz USVs have frequently been detected even in the absence of conspecifics (e.g. Burgdorf et al. 2000; Schwarting et al. 2007), but whether rats make the same kinds of 50-kHz USVs when tested singly vs. paired with a conspecific has not, to our knowledge, been investigated.

Stable and pronounced inter-individual differences with respect to USV production rates have been noted in several studies (Burgdorf et al. 2001b; Mallo et al. 2007; Schwarting et al. 2007; Wohr et al. 2008, 2009; Wohr and Schwarting 2009). However, it has not been reported whether individual rats differ in terms of the specific call subtypes that they preferentially emit, that is, whether each rat possesses a characteristic "call profile". In addition, there have been no reports of stable differences in acoustic parameters of calls.

The present study therefore addressed the following hypotheses: (1) the rich heterogeneity of 50-kHz USVs is captured within a moderate number of discrete call categories, (2) call categories are differentially modulated by AMPH treatment and the presence of conspecifics, and (3) individual differences exist, not only in terms of the overall number of 50-kHz calls emitted, as previously shown, but also in relation to call subtypes and acoustic parameters. A final experiment was performed in order to confirm the authenticity of individual 50-kHz USV subtypes by means of a surgical devocalization procedure (Roberts 1975; White and Barfield 1990).

Methods

Subjects

In Experiment 1, subjects were 24 experimentally-naïve male Long-Evans rats (Charles River Laboratories, St. Constant, Quebec, Canada), weighing 319–380 g at the beginning of the experiment. For Experiment 2, subjects were 36 experimentally naïve male Long–Evans rats (Charles River Laboratories, St. Constant, Quebec, Canada), weighing 267–330 g at the start of the experiment. Subjects were housed three (Experiment 1) or two (Experiment 2) per cage (25×48×20 cm) in a temperature- and humidity-controlled colony room (19–20°C, 50–60%) at the McGill University Animal Research Center. The rats were maintained on a reverse 12:12 light/dark cycle, with lights off at 0700 h. All behavioral testing took place during the dark phase of the cycle. Food and water were available ad libitum, except during testing. Before the start of the experiment, animals were handled for approximately 3 min daily for 2 days. All procedures were approved by the McGill Animal Care Committee in accordance with the guidelines of the Canadian Council on Animal Care.

Acoustic data acquisition, analysis, and classification of ultrasonic vocalizations

Testing took place in clear Plexiglas experimental chambers (ENV-007CT, Med Associates, St. Albans, VT), each of which was enclosed in a melamine compartment lined with sound-attenuating acoustic foam (Primacoustic, Port Coquitlam, British Columbia). Electret microphones with a frequency response range of 15 to 125 kHz (FG-23329-C05, Knowles Acoustic, Itasca, IL) were securely placed through small holes

located centrally in the top panels of the experimental chambers. Consequently, the microphones were 15–30 cm from the rats during testing. Microphone signals were fed into a preamplifier (QuadMic, RME, Germany) and an anti-aliasing filter (Krohn-Hite 3323, Brockton, MA) before the analog signal was digitized through an A/D card (PCI-6251, National Instruments, Austin, TX) with a sampling rate of 200-kHz and a 16-bit resolution.

Acoustical analysis of the recordings was performed using Avisoft SASLab Pro (Version 4.2, Avisoft Bioacoustics, Berlin, Germany). Spectrograms were generated with a fast Fourier transform (FFT)-length of 512 points and an overlap of 75% (FlatTop window, 100% frame size). Correspondingly, spectrograms had a frequency resolution of 390 Hz and a time resolution of 0.64 ms. Calls were selected manually with section labels and classified based on pre-defined frequency pattern criteria (see below); call classification was performed masked to the treatment condition. Three acoustic properties of each call were determined by the automatic parameter measurements feature of the software: duration, bandwidth (i.e., difference between the maximum and minimum peak frequency), and mean peak frequency (i.e., time-averaged peak energy frequency within each call). In order to improve accuracy of parameter measurements by the software, a threshold between −40 and −50 dB was set (setting: "Reject if peak amplitude <"). If background noise still interfered with proper measurement of acoustic parameters, those calls were discarded from the parameter analysis.

Each ultrasonic "call" had to meet three spectrographic criteria: (1) temporal continuity (i.e., maximal interruption of 20 ms), (2) fundamental frequency between 20 and 95 kHz, and (3) sound intensity and structure that was clearly distinct from background noise when seen under optimal viewing settings. From a visual inspection of over 20,000 USV spectrograms, 15 call categories were recognized (see Fig. 1):

(1) *Complex*: contain two or more directional changes in frequency of at least 3 kHz each
(2) *Upward ramp*: monotonically increasing in frequency, with a mean slope not less than 0.2 kHz/ms
(3) *Downward ramp*: monotonically decreasing in frequency, with a mean negative slope not less than 0.2 kHz/ms
(4) *Flat*: near-constant frequency greater than 30 kHz with a mean slope between −0.2 and 0.2 kHz/ms
(5) *Short*: duration less than 12 ms
(6) *Split*: middle component "jumps" to a lower frequency and contains a harmonic
(7) *Step up*: instantaneous frequency change to a higher frequency
(8) *Step down*: instantaneous frequency change to a lower frequency
(9) *Multi-step*: two or more instantaneous frequency changes
(10) *Trill*: rapid frequency oscillations with a period of approximately 15 ms (either sinusoidal or appearing as repeated "inverted-Us").
(11) *Flat/trill combination*: a trill that is flanked on one or both sides by a monotonic portion that is no less than 10 ms
(12) *Trill with jumps*: a trill that contains one or more higher-frequency components
(13) *Inverted U*: a monotonic increase followed by a monotonic frequency decrease, each of at least 5 kHz
(14) *Composite*: calls (other than flat/trill combinations) that comprise two or more categories
(15) *22-kHz calls*: near-constant frequency calls between 20 and 25 kHz

Finally, a few (1%) other calls were classified as "miscellaneous" because they did not fit any of the above call categories. A proportion of calls (7%) was spectrographically unclear and categorized as "unclassifiable".

Inter-rater reliability in call category identification was assessed using a subset of 500 calls, which was independently rated by the main experimenter (J.M.W.) and a trained student. Reliability was high (Cohen's kappa=0.95), such that 96% of calls received the same classification from both individuals. Intra-rater reliability, assessed by a repeat scoring of 500 calls by the main experimenter one week apart, was also high (Cohen's kappa=0.96), such that 97% of calls were assigned the same classification between scorings.

Drugs

D-Amphetamine sulfate (Sigma Aldrich, Oakville, ON) was dissolved in sterile 0.9% saline and administered by IP injection in a volume of 1 ml/kg; doses are expressed as salt.

Experimental protocol

Experiment 1: Systemic AMPH dose–response in singly- and pair-tested rats

The experiment comprised an initial habituation day followed immediately by six test days, spaced 2 days apart in order to minimize possible carry-over effects of the drug. Rats were housed three per cage: one rat from each home cage (n=8 rats) was randomly assigned to be tested singly throughout the experiment, and the other two rats from the same home cage were always tested together (n=8 pairs). On the first day (Habituation), rats were placed in the test chambers for 10 min. Over the six test days, each rat or rat

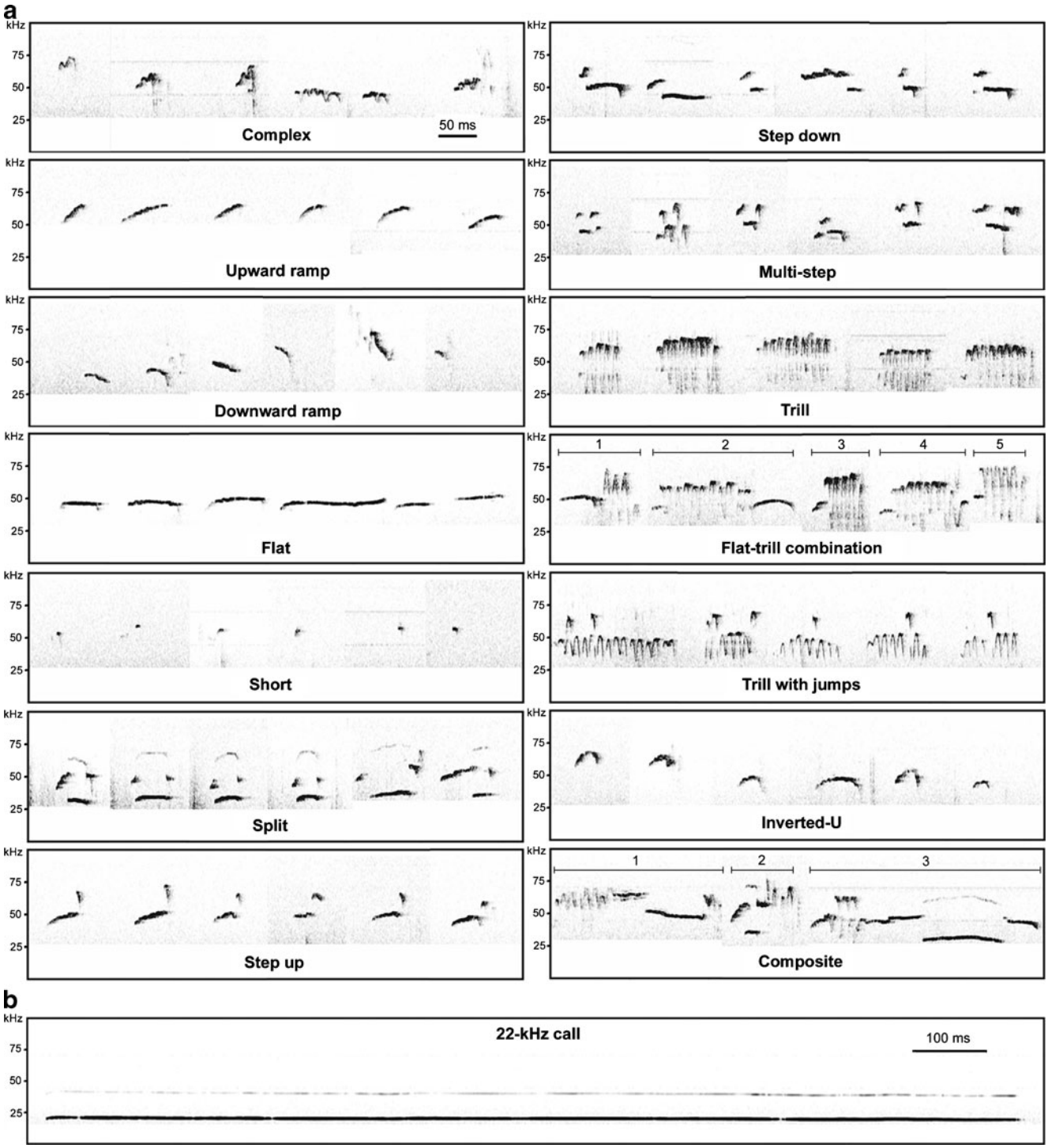

Fig. 1 Representative calls for each of the 14 categories of 50-kHz USVs (**a**) and a 22-kHz USV (**b**). Several exemplar calls are shown for each 50-kHz call category; these examples are not necessarily consecutive nor made by the same rat. Individual "flat–trill combination" and "composite" calls are differentiated by the accompanying line brackets. The time scale for all 50-kHz calls is indicated in the top left panel

pair received two administrations of saline and all four doses of amphetamine (0.25, 0.5, 1, and 2 mg/kg), counterbalanced as fully as possible. Immediately following drug administration, the animals were placed in the test chambers and recorded for 20 min.

Experiment 2: Effects of surgical devocalization

This experiment was conducted in two parts (using 16 and 20 subjects, respectively). Rats were initially tested with AMPH in order to screen out low-rate callers as follows. Every 2 days

over 5 days, each rat (n=36) received an IP injection of AMPH (1 mg/kg) and was immediately placed in the test chamber alone and recorded for 20 min. The 14 rats with the lowest number of USVs were excluded from the study. The remaining 22 rats were randomly allocated to two groups of comparable pre-surgery call rates (mean±SEM 73±7 vs. 77±6 calls/min). One group received devocalization surgery; the other was sham-operated (see "Surgery"). Following a 6-day recovery period, rats received one additional test session under AMPH. One sham-operated rat was excluded from analysis since data collection failed in the post-surgery session due to a technical problem.

Surgery

Under general anesthesia, achieved by 2–4% isoflurane in conjunction with pure oxygen, a 2-cm incision was made on the ventral surface of the neck. Local anesthesia was provided by infiltration of lidocaine. The sternohyoideus muscle was separated to expose the trachea and locate the recurrent laryngeal nerves. Once located, approximately 3 mm of the nerve on both sides (bilateral section) was removed (devocalized), or left intact (sham). The incision was closed with subcutaneous suture and 1–2 staples. Analgesic carprofen (5 mg/kg SC) was administered to rats at surgery and again every 24 h for the following 3 days. One devocalized rat died the day following surgery. The rats were allowed to recover for 6 days before testing.

Data analysis and statistics

Data were analyzed using commercial software (Systat v11, SPSS Inc., Chicago, Illinois). The number of USVs acquired for the pair-tested rats was divided by two in order to express the results on a per-rat basis. Only USVs that occurred during minutes 3, 8, 13, and 18 (Experiment 1) or during minutes 13 and 18 (Experiment 2) of each 20-min recording were selected. When specifically examining the effect of AMPH on USVs in Experiment 1, only the latter two sampled time intervals were used in order to allow time for AMPH to take effect. In this study, 22-kHz calls accounted for less than 1% of USVs and were not analyzed further.

The statistical analysis of acoustic parameters (duration, bandwidth, and mean peak frequency) was performed by repeated-measures analysis of variance (ANOVA). The fact that not all subjects emitted every call subtype under every condition posed a challenge, since this type of ANOVA discards subjects with *any* missing values. As a first step, we excluded from analysis four call categories as well as four singly-tested rats, which had a large number of missing values. Subsequently, the missing values for each remaining rat were replaced by the mean of its other test conditions. In total, 5% of the raw data values were interpolated.

Data from the two saline test sessions were analyzed as follows. For the correlation of the total number of calls between the two saline tests, Spearman's correlation coefficient was calculated, and unprotected paired t tests were performed for each call category. For the remainder of the analysis, the number of calls was averaged across the first and second saline test. ANOVA was performed to test the effects of "group" (i.e., between subjects; singly- *vs.* pair-tested), call "category" (within subjects; 14 call subtypes), "time" (within subjects; minutes 3, 8, 13, and 18), "dose" (within subjects; five dose levels), and "rat" (between subjects; eight singly-tested and eight pair-tested rats), where appropriate, and was performed on each call subtype either in absolute terms, or as a proportion of all calls. Only call categories with a proportion greater than 2% in at least one of the groups were included in this analysis. Two-sample independent t tests or paired t tests were used where appropriate. All ANOVA p values were subject to the Huynh–Feldt correction where applicable. For all tests, a two-tailed p value less than 5% was considered significant.

Results

Experiment 1: Systemic AMPH dose–response in singly- and pair-tested rats

Classification of rat ultrasonic vocalization subtypes

Over 20,000 calls were examined in this experiment, from which 14 categories of 50-kHz calls were recognized; spectrograms of typical calls of each category are shown in Fig. 1. Three call subtypes were particularly prevalent, together comprising approximately 50% of the observed calls pooled across all experimental conditions: the trill (29% of calls), flat/trill combination (16%), and flat (14%). The least common 50-kHz subtype was the split call, accounting for only 0.5% of calls. Of the 93% of calls that were considered "classifiable" (see "Methods"), only 1% did not fall into one of the 14 categories. These infrequent miscellaneous calls varied widely in appearance.

Ultrasonic vocalizations during saline test sessions

All 50-kHz call categories occurred during saline test sessions. The total number of calls made by each rat during the first and second saline test were significantly correlated (Pearson r=0.63, df 14, p<0.01; Fig. 2a). The mean number of each call subtype did not differ significantly between the two saline tests in either the singly- or pair-tested rats. For all further analysis, the number of USVs was averaged across the two saline sessions.

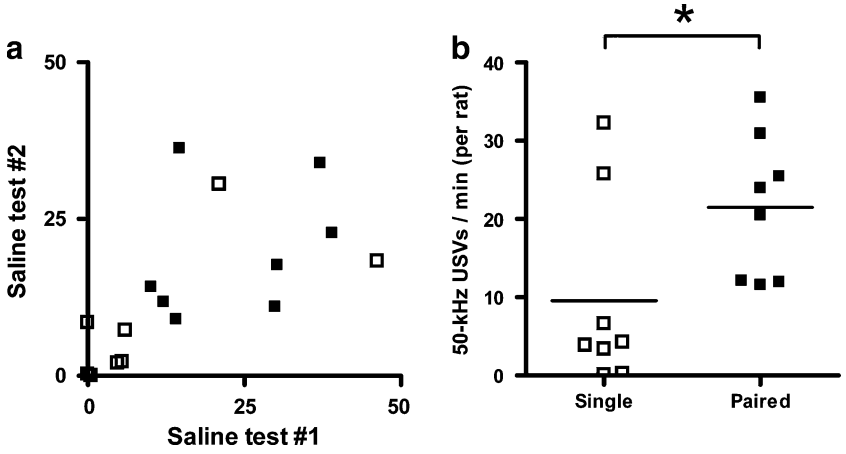

Fig. 2 Rate of 50-kHz calling in the two saline test sessions. Each rat or rat pair is represented by *open* or *closed squares*, respectively. The rate of 50-kHz calling was averaged across four time intervals (minutes 3, 8, 13, and 18 post-injection) and expressed as calls/min per rat. The 50-kHz call rate was significantly correlated between the first and second saline test sessions (Spearman $r=0.81$, df 16, $p<0.01$) (**a**). Pair-tested rats emitted significantly more 50-kHz USVs (per rat) than singly-tested rats during saline test sessions (**b**). *$p<0.05$, *t* test

During saline tests, pair-tested rats emitted approximately twice as many calls as singly-tested rats, on a per-rat basis (main effect of group: $F(1,14)=4.84$, $p<0.05$; Fig. 2b). The call profile differed markedly between singly- and pair-tested rats (Fig. 3). In particular, pair-tested rats emitted a significantly higher proportion of trills ($t=2.86$, df 10, $p<0.05$) and flat–trill combinations ($t=2.24$, df 14, $p<0.05$) than singly-tested rats. The proportion of flat calls appeared greater in the singly-tested rats, but this difference was not significant, perhaps because this measure was highly variable across singly-tested rats.

Effect of AMPH on ultrasonic vocalizations

AMPH dose-dependently increased the total number of 50-kHz USVs per minute ($F(4,56)=13.30$, $p<0.0001$; Fig. 4). Of note, all doses of AMPH tested, including 0.25 mg/kg, caused a significant increase in calls relative to the saline

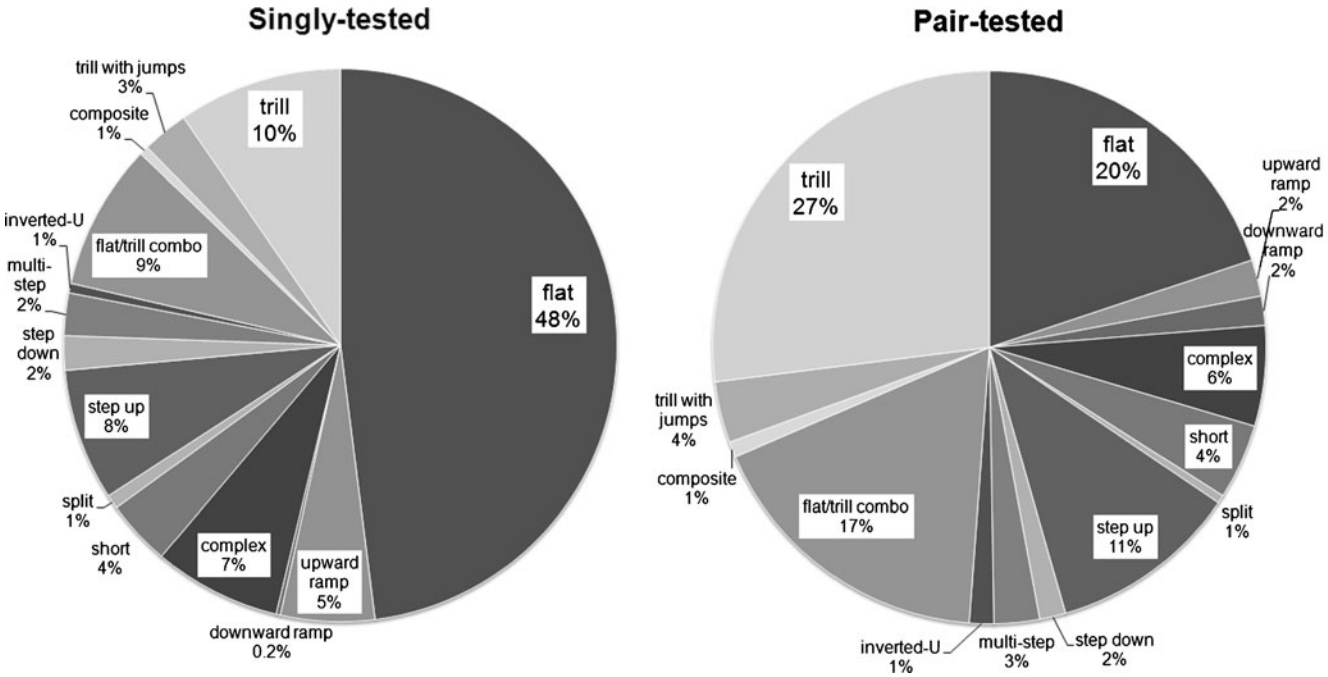

Fig. 3 Call profiles differed between singly-tested and pair-tested rats during saline test sessions. Each sector represents the group mean number of calls in a given category, expressed as a percentage of all classifiable calls ($n=8$ rats or rat pairs). Pair-tested rats emitted a significantly higher proportion of trills and flat–trill combinations than singly-tested rats (see text), whereas singly-tested rats tended to emit a higher percentage of flat calls

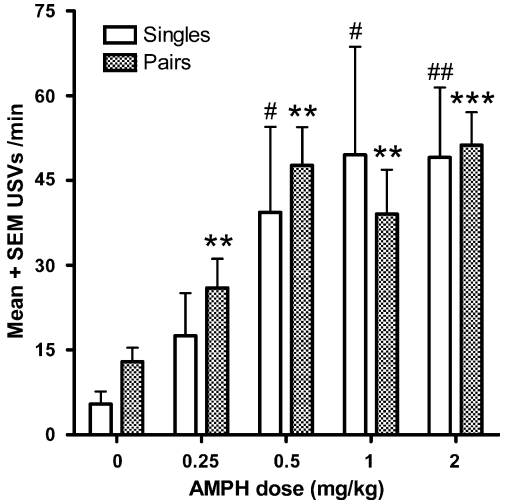

Fig. 4 AMPH dose-dependently increased the number of USVs emitted by singly- and pair-tested rats. The rate of 50-kHz calling was averaged across two time intervals (minutes 13 and 18 post-injection) and expressed as calls/min per rat. #$p<0.05$, ##$p<0.01$, **$p<0.01$, ***$p<0.001$ vs. corresponding saline condition (paired t tests; n= 8 rats or 8 rat pairs, respectively)

condition (paired t tests with Bonferroni correction, n=16, $p<0.01$–0.0001). The two groups evinced comparable absolute increases in call rate (per rat) in response to AMPH. In order to assess whether repeated AMPH administration induced behavioral sensitization, we examined whether the overall rate of calling increased across test days; however, no significant change was observed.

AMPH administration did not result in the emergence of new call types, but it clearly altered the call profile (Fig. 5; see Table 1 for statistical analyses). The relative proportion of trill calls dose-dependently increased with AMPH in both singly- and pair-tested rats. The other call types that were preferentially affected by AMPH were the flats and shorts. Similar to the saline condition, pair-tested rats emitted a significantly higher proportion of trills and trills with jumps than singly-tested rats, irrespective of the AMPH dose.

Effect of time within session

The overall call rate decreased over the test session ($F(3,42)$= 3.320, $p<0.05$), independently of AMPH dose or social condition (Fig. S1a). However, AMPH-induced calling (i.e., calls emitted under AMPH minus those under saline) was most pronounced during the last two sampled time intervals of the 20-min test session (i.e., minutes 13 and 18, Fig. S1b). The call profile also changed over time (category×time interaction, $F(39,507)=2.63$, $p<0.05$) irrespective of whether rats were tested singly or in pairs; the only significant time-dependent change was in the proportion of flat calls ($F(3,42)=5.31$, $p=0.02$), which became less frequent relative to other calls as the session progressed (i.e., minutes 3 vs. 8, 13, or 18: paired t tests, $p<0.05$).

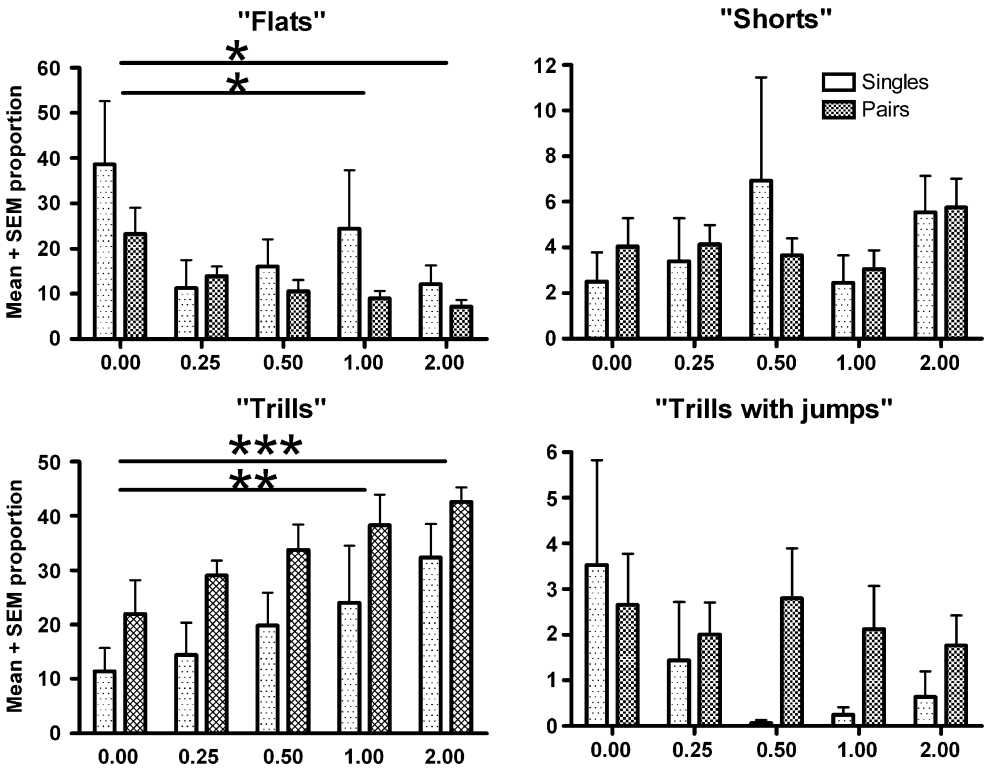

Fig. 5 Amphetamine dose-dependently increased the proportion of trill calls and suppressed flat calls. The proportion (mean±SEM) of each call category for singly- and pair-tested rats is plotted against increasing AMPH dose (only subtypes that exhibited significant changes under AMPH are shown; see supplemental Fig. S4 for *all* call categories). Note also the overall higher proportion of trills and trills with jumps in the pair-tested rats compared to the singly-tested rats. Amphetamine-induced changes with respect to flat, short, and trill calls were not significantly group-dependent (Table 1); pooling the two groups (i.e., single and pair-tested), significant differences from the saline condition are indicated by asterisks (*$p<0.05$, **$p<0.01$, ***$p<0.001$, paired t tests,

Table 1 ANOVA results for the call profile data (i.e. proportion of calls in each category)

Call type	S/P F (1,12)	AMPH F (4,48)	S/P×AMPH F (4,48)
Complex	1.75	0.93	0.37
Upward ramp	1.44	0.21	0.10
Downward ramp	0.00	0.69	1.17
Flat	1.34	**4.05***	0.29
Short	1.07	**3.15***	0.74
Split	n/a	n/a	n/a
Step up	0.12	1.34	**2.94***
Step down	1.17	1.33	1.34
Multi-step	1.94	1.56	**4.18****
Trill	**6.26***	**4.63****	0.21
Flat/trill combo	0.32	0.77	0.92
Trill with jumps	**4.91***	0.96	0.40
Inverted U	3.77	1.25	0.95
Composite	0.99	1.31	1.08

Two-way analyses of variance (ANOVAs) were performed separately for each call subtype, with between-subjects factor S/P (singly- vs. pair-tested), and within-subjects factor AMPH (i.e., dose of amphetamine). n/a, split calls were excluded from this analysis (see "Methods"). n=8 (rats or rat pairs). Significant F values are shown in bold *p< 0.05, **p< 0.01. Corresponding data are shown in Fig. 5

Correlation between call subtypes

To test whether rats that preferentially emitted a particular call subtype would also preferentially emit (or avoid emitting) any other call subtypes, we performed an exploratory correlational analysis (Table S1). Among the more frequent call subtypes, there was a significant positive correlation between step-ups and flats and between step-ups and flat–trill combinations. In contrast, a significant negative correlation occurred between trills and flats and between trills and step-ups.

Acoustic parameters (duration, bandwidth, and mean peak frequency)

Acoustic data and related statistical analyses are presented in Tables 2 and S2. Acoustic parameters were largely unaffected by social testing condition and AMPH dose (Table S2). The only exception was a slight (approximately 2 kHz) but significant decrease in bandwidth at the lowest dose of AMPH relative to saline (not shown). There was no difference, in any acoustic parameter examined, between singly-tested and pair-tested rats. Not surprisingly, acoustic parameters differed markedly between 50-kHz call subtypes (main effects of call category: p< 0.0001). All 50-kHz USV subtypes had a mean peak frequency between 48 and

Table 2 Call parameters (duration, bandwidth, and mean peak frequency) of each call category

Category	Duration (ms)				Bandwidth (kHz)				Mean peak frequency (kHz)			
	n	Mean	SEM	5th and 95th percentile	n	Mean	SEM	5th and 95th percentile	n	Mean	SEM	5th and 95th percentile
Complex	1,017	28.0	0.32	15.3, 47.3	972	9.1	0.2	3.5, 25.0	996	54.6	0.2	41.1, 64.9
Upward ramp	395	30.3	0.57	15.3, 52.3	378	7.9	0.3	3.5, 19.5	383	52.1	0.3	42.5, 61.8
Downward ramp	88	23.9	1.41	10.2, 55.1	77	8.7	0.8	3.1, 25.5	83	50.4	1.0	37.2, 65.1
Flat	2,595	34.8	0.44	13.4, 70.2	2,401	3.5	0.0	0.4, 7.5	2,460	48.8	0.1	40.2, 59.3
Short	657	9.6	0.07	7.0, 12.1	575	1.7	0.1	0.4, 4.2	624	57.3	0.3	42.2, 67.9
Split	51	105.5	19.26	37.9, 388.4	51	26.5	1.2	14.5, 39.8	51	40.3	0.9	31.2, 51.4
Step up	1,649	34.7	0.30	16.6, 55.6	1592	19.0	0.1	12.1, 27.8	1,617	52.9	0.1	44.2, 62.9
Step down	222	36.6	1.30	16.0, 71.2	219	16.3	0.5	7.8, 31.9	222	52.0	0.4	41.6, 62.2
Multi-step	439	39.1	0.66	19.2, 66.9	434	19.2	0.3	11.9, 33.1	437	53.4	0.3	41.5, 64.5
Trill	4,773	45.7	0.31	20.4, 83.8	4528	15.0	0.2	3.5, 40.2	4,644	59.9	0.1	48.2, 68.7
Flat–trill	2,923	60.6	0.51	30.0, 114.5	2874	22.2	0.2	12.8, 39.1	2,902	55.1	0.1	45.7, 64.0
Trill with jumps	383	67.5	1.62	31.3, 129.5	378	23.9	0.5	13.3, 43.7	382	54.4	0.3	42.6, 64.4
Inverted U	106	27.1	1.04	15.2, 45.9	99	9.7	0.8	5.1, 29.3	103	51.7	0.7	42.1, 64.6
Composite	191	87.2	3.60	36.4, 192.4	191	25.5	0.8	12.5, 46.1	191	55.3	0.5	42.7, 64.9
22-kHz	117	795.2	49.54	218, 1,910	117	9.4	0.7	1.5, 22.3	117	21.9	0.2	18.7, 25.1

Calls are from all testing conditions in Experiment 1. If background noise interfered with accurate measurement of acoustic parameters by the software, those calls were excluded from analysis (see "Methods"). In some cases, determination of only a subset of parameters was possible for a given call

60 kHz, except split calls, whose mean peak frequency was considerably lower (mean=40.3 kHz, see Table 2). Split calls were also longer than other 50-kHz USV subtypes, with a mean duration of 105 ms vs. 10–90 ms for other call subtypes (Table 2).

Individual differences in rate of calling, acoustic parameters, and call profile

Pronounced individual differences occurred in all three respects. First, individual rats differed consistently in terms of rate of calling. This was the case both within sessions and across AMPH doses (Cronbach's alpha=0.979 and 0.907, respectively; Fig. S2a, b). Analogous differences occurred between rat pairs (Cronbach's alpha=0.862 and 0.844, respectively; Fig. S2c, d). Second, individual differences in *acoustic parameters* (duration, bandwidth, and mean peak frequency) were found for almost every 50-kHz USV subtype; this is illustrated for trills and flats in Fig. 6. Finally, individual rats differed in their *call profiles* (rat× category interaction, $F(91,377)=1.68$, $p<0.05$; Fig. S3), and analogous differences were found between dyads of pair-tested rats ($F(91,416)=2.08$, $p<0.01$).

Experiment 2: Devocalization

Rats that were ultimately selected for surgery emitted 43–113 USVs/min during the pre-surgery AMPH screen. A comparison of the pre- and post-surgery AMPH tests showed that devocalization surgery suppressed USVs (group×time interaction: $F(1,18)=25.87$, $p<0.001$); the devocalized group emitted significantly fewer calls (paired $t=6.25$, df 3, $p<0.0001$) following surgery, whereas the rate of USVs emitted by sham-operated rats did not change significantly. Every call subtype was emitted by each group prior to surgery. USVs were completely abolished in 6 out of 10 devocalized rats, and the remaining rats emitted very few calls (mean±SEM 4±3 USVs/min) when tested under AMPH. Almost every 50-kHz call subtype was significantly reduced following devocalization surgery (Mann–Whitney *U* test, $p<0.05$–0.001). The only exceptions were the highly infrequent split and downward ramp calls; here, statistical power was limited. Sham surgery did not appear to alter the spectrographic appearance of 50-kHz calls or the call profile (data not shown); the low number of calls emitted by devocalized rats precluded analysis of these parameters. Finally, devocalized rats tended to gain less weight after

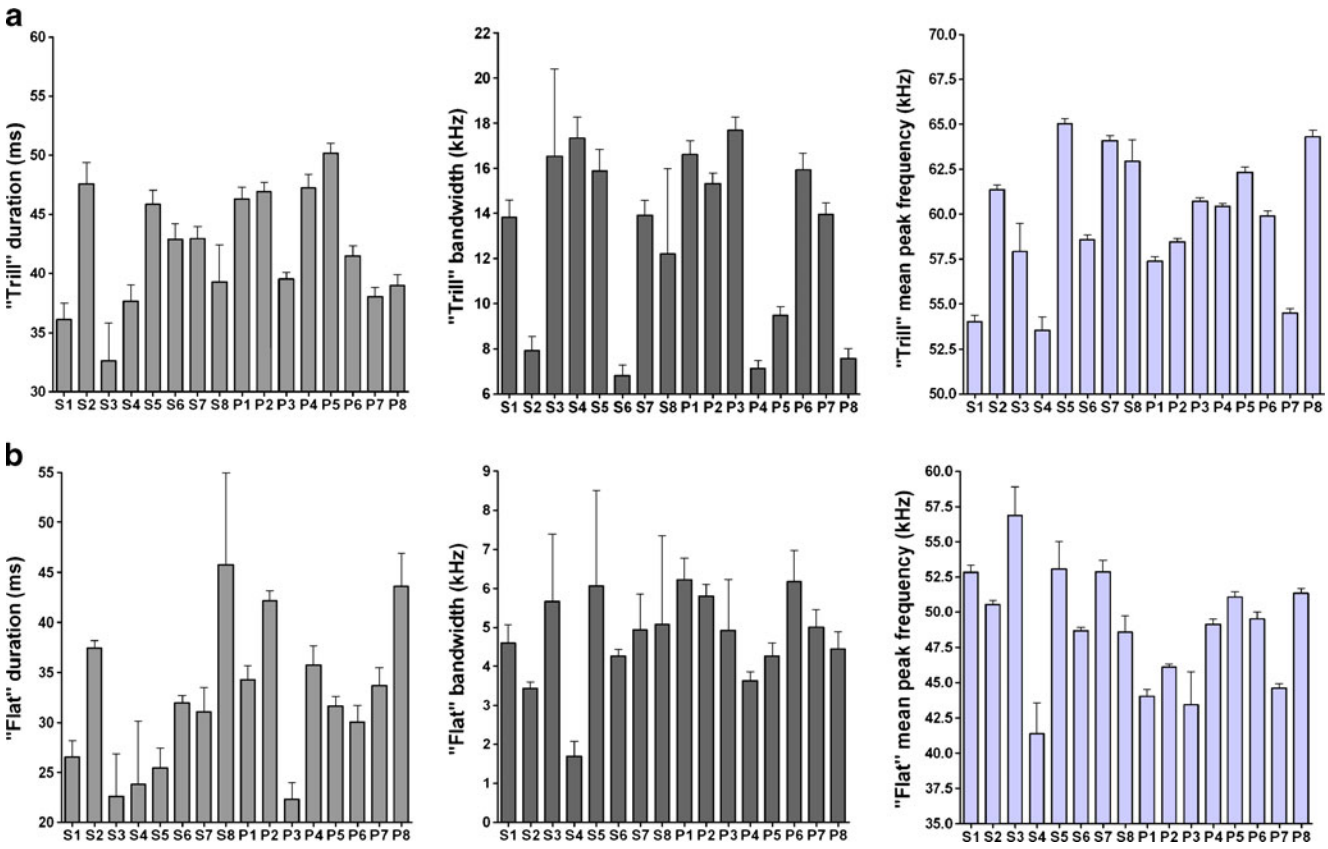

Fig. 6 Individual differences in acoustic parameters of 50-kHz calls. Differences in duration, bandwidth, and mean peak frequency were observed for every call subtype, and are exemplified here for trills (**a**) and flat calls (**b**). The *y*-axis variables are expressed as mean+SEM. The *x*-axis shows each singly-tested rat (S1–S8) and each pair-tested rat (P1–P8). One-way ANOVAs; $n=9$–760 calls; $p<0.0001$

surgery than the sham-operated rats (mean±SEM, respectively: 30.3±17.2 vs. 76.8±3.5 g; t=2.65, df 3, p=0.07).

Discussion

The present study provides the first detailed classification of adult rat 50-kHz USVs under saline and amphetamine test conditions. The authenticity of these calls was confirmed through surgical devocalization. Our analysis yielded several novel findings. Systemic amphetamine administration increased 50-kHz calling dose-dependently. More interestingly, call profiles (i.e., relative frequency of each call category) were differentially affected by social context, drug, and time within session. In particular: (1) pair-tested rats produced a higher proportion of trill calls than did singly-tested rats under both drug and saline conditions, (2) amphetamine altered the call profile such that trills became more prominent while flat calls became less so, and (3) flat calls became proportionately less frequent late in the session. In contrast, the acoustic characteristics of individual call subtypes (i.e., duration, bandwidth and mean peak frequency) were notably stable. Stable inter-individual differences were also found, not only with respect to overall 50 kHz call rate, as previously reported, but also in terms of acoustic parameters and call profile.

All the 14 USV subtypes described here are likely to be authentic rat vocalizations, since they virtually all disappeared after surgical transection of the recurrent laryngeal nerve. The origin of adult rat 50-kHz ultrasounds has been the subject of some debate, with suggestions that some calls might represent a byproduct of locomotion (Blumberg 1992) or serve a thermoregulatory role (Blumberg and Moltz 1987). The locomotor artifact hypothesis, however, has been countered by several lines of evidence (Knutson et al. 2002; Simola et al. 2009). For example, doses of caffeine that would be expected to increase locomotor activity are reported not to increase the rate of 50-kHz calling in adult rats (Simola et al. 2009). A thermoregulatory role of 22-kHz USVs has been proposed in adult rats, in the context of sexual behavior (Blumberg and Moltz 1987). As in the case of copulation, the higher doses of amphetamine used in the present study likely produced mild (1–2°C) hyperthermia in the periphery (Lin et al. 1980; Ulus et al. 1975) and therefore quite possibly in the brain as well. However, it seems unlikely that the increased rate of 50-kHz calling seen after amphetamine administration would reflect an attempt at thermoregulation, insomuch as these calls are much briefer and less intense than 22-kHz USVs.

Previous 50-kHz classification schemes have comprised only three or four subtypes (Kaltwasser 1990; Vivian and Miczek 1993; White et al. 1990). We now describe a considerably larger repertoire of calls. We cannot of course exclude the possible occurrence of additional call subtypes in other behavioral contexts, stages of development, or indeed by other rat strains. Importantly, our 14 call categories have nearly all been depicted previously (see Table 3), and they appear to capture all published spectrographic examples of rat 50-kHz USVs. Interestingly, eight of the ten USV subtypes that were recently described in mice appear to have acoustic counterparts in adult rats (compare Fig. 1 with Fig. 2 of Scattoni et al. 2008). This high degree of similarity possibly reflects physical constraints on call production; it remains to be determined whether these seemingly homologous call subtypes signal the same information in rats as in mice.

Automated call categorization has been achieved for a limited number of mouse USV subtypes (Holy and Guo 2005); a similar procedure for categorizing our 14 call subtypes would be highly desirable, for several reasons. First, manual categorization is highly labor-intensive. Second, although categorization was usually unambiguous (as reflected in high inter- and intra-rater reliabilities), mathematical modeling of 50-kHz calls could potentially eliminate any observer bias. Third, a modeling procedure might reveal further heterogeneity within our existing classification scheme.

In the present study, paired cage mates called significantly more, on a per-rat basis, than rats tested alone under drug-free conditions. This result is in line with previous findings by Brudzynski and Pniak (2002) using unfamiliar conspecifics, and may be unsurprising given that USVs appear to have a communicative role (see "Introduction"). It should however be noted that our testing protocol did not distinguish between vocalizations emitted by individual pair-tested rats, and consequently, it is not clear whether each pair-tested rat was similarly affected by AMPH or cage-mate presence.

In our study, AMPH dose-dependently increased the rate of 50-kHz calling, consistent with previous investigations that employed single systemic AMPH doses (Ahrens et al. 2009; Simola et al. 2009; Wintink and Brudzynski 2001). Of note, even the lowest dose of AMPH tested (0.25 mg/kg), a dose considered to be a "low" behaviorally effective dose in rats (Grilly and Loveland 2001), caused a highly significant increase in USVs, indicating that USVs are a relatively sensitive behavioral measure.

While systemic administration of AMPH has been widely reported to increase 50-kHz calling, effects on 50-kHz *call subtypes* have received less attention. In particular, Ahrens et al. (2009) showed that trill, but not flat, calls increased with repeated AMPH administration. In another study, a single, relatively high dose of AMPH (2 mg/kg) increased the FM-flat ratio (Simola et al. 2009). By examining the effect of AMPH on each of the 14 subtypes of 50-kHz USVs, we found that AMPH dose-dependently increased the proportion of trill calls and decreased the

Table 3 The 50-kHz call categories defined in the present study (i.e., call subtype) in relation to published spectrographic evidence from adult rats

Call subtype	Sex	Rat strain	Age/weight	Condition
Complex	Male[a,b,c]	Sprague-Dawley,[a] Wistar[b,c]	192–226 g,[b] 220–260 g,[a] 280–310 g[c]	Intra-NAc carbachol,[a] heterospecific play,[b] cage exploration[c]
Upward ramp	Male[a,d,e]	Sprague–Dawley,[a] Wistar,[d] Long–Evans[e]	220–260 g,[a] 250–450 g,[d] 300–400 g[e]	Intra-NAc carbachol,[a] intracerebral glutamate,[d] aggression[e]
Downward ramp	Male[e]	Long–Evans[e]	300–400 g[e]	Aggression[e]
Flat[f,g]	Male[h,i,a,d,b,j,e,c,k]	Long-Evans,[h,i,j,e,l] Sprague–Dawley,[a] Wistar[d,b,c,k]	380–540 g,[a], 9–15 months,[i] 220–260 g,[a] 250–450 g,[d] 192–226 g,[b] 6–8 months,[j] 300–400 g,[e] 280–310 g[c,k]	IV AMPH,[h] sexual behavior,[i,l] intra-NAc carbachol,[a] intracerebral glutamate,[d] IP caffeine or AMPH,[j] heterospecific play,[b] aggression,[e] cage exploration[c,k]
Short				
Split	Male[i,a]	Long–Evans,[i] Sprague–Dawley[a]	9–15 months,[i] 220–260 g,[a]	Sexual behavior,[i] intra-NAc carbachol,[a]
Step up	Male[a]	Sprague–Dawley[a]	220–260 g[a]	Intra-NAc carbachol[a]
Step down	Male[a,c]	Sprague–Dawley,[a] Wistar[c]	220–260 g,[a] 280–310 g[c]	Intra-NAc carbachol,[a] cage exploration[c]
Multi-step	Male[a,m,k]	Black Rat,[m] Sprague–Dawley,[a] Wistar[k]	220–260 g,[a] 280–310 g[k]	Aggression,[m] intra-NAc carbachol,[a] cage exploration[k]
Trill	Male[b,j,k]	Black Rat,[m] Long–Evans,[j] Wistar[b,k]	192–226 g,[b] 6–8 months,[j] 280–310 g[k]	IP caffeine or AMPH,[j] cage exploration,[k] heterospecific play,[b] sexual behavior[m]
Flat–trill combination	Male[b]	Long–Evans,[l] Wistar[b]	192–226 g,[b]	Sexual behavior,[l] heterospecific play[b]
Trill with jumps	Male[h,n,i]	Long–Evans[h,n,i]	380–540 g,[h] 6 months,[n] 9–15 months[i]	IV AMPH,[h] sexual behavior[n,i]
Inverted U	Male[a]	Sprague–Dawley[a]	220–260 g[a]	Intra-NAc carbachol[a]
Composite[f,g]	Male[c,k]	Wistar[c,k]	280–310 g[c,k]	Cage exploration[c,k]

[a] Fendt et al. (2006); [b] Schwarting et al. (2007); [c] Wohr and Schwarting (2007); [d] Fu and Brudzynski (1994); [e] Vivian and Miczek (1993); [f] Burgdorf et al. (2007); [g] Burgdorf et al. (2008a, b); [h] Ahrens et al. (2009); [i] Ciucci et al. (2009); [j] Simola et al. (2009); [k] Wohr et al. (2008); [l] White et al. (1990); [m] Kaltwasser (1990); [n] Ciucci et al. (2007)

proportion of flat calls. Interestingly, the proportion of the other 12 subtypes of 50-kHz USVs remained stable across AMPH doses, although most 50-kHz subtypes significantly increased in absolute number.

Analysis of rodent 50-kHz USVs has tended to emphasize the calling rate rather than the spectrographic characteristics of calls; however, acoustic call parameters are also susceptible to experimental manipulation, in some cases independently of call rate (Ciucci et al. 2007; Hodgson et al. 2008; Simola et al. 2009; Vivian and Miczek 1993). It has been reported that neither AMPH administration (Simola et al. 2009; Thompson et al. 2006) nor social testing conditions (Brudzynski and Pniak 2002) alter the acoustic parameters of 50-kHz calls; however, in these studies, little (or no) distinction was made between call subtypes. We now show a similar lack of effect across multiple 50-kHz call categories, the only exception being a modest effect of AMPH on bandwidth.

Inter-rat differences in 50-kHz call rates have been widely reported in response to various stimuli (Burgdorf et al. 2001b, 2005, 2008b; Mallo et al. 2007; Schwarting et al. 2007; Wohr et al. 2008, 2009; Wohr and Schwarting 2009). In the present study, such differences were maintained not only within session but also across different AMPH doses. To further elucidate the relationship between 50-kHz calls and AMPH's rewarding effects, it would be of interest to determine whether individual differences in AMPH-induced calling rate predicts intravenous self-administration or the magnitude of conditioned place preference.

To our knowledge, this study is the first to report individual differences in call profiles (i.e., the proportion of calls in each category). Thus, in addition to differences in absolute call rate, some rats, for example, favored complex calls over trills (or vice versa). Furthermore, individual differences in acoustic parameters, previously reported for 22-kHz calls (van der Poel and Miczek 1991), were evident with respect to 50-kHz calls. Specifically, we found inter-rat differences in the duration, bandwidth, and mean peak frequency of each 50-kHz call subtype. Consequently, we speculate that the combination of call profile and acoustic parameters may be sufficient to allow rats to recognize individual conspecifics, even in the absence of odor cues.

Recent evidence suggests that 50-kHz call subtypes may differ in their behavioral significance; for example, FM calls, but not flat, have been associated with appetitive stimuli (e.g. Ahrens et al. 2009; Burgdorf et al. 2007, 2008a; Burgdorf and Panksepp 2006; Wohr et al. 2008). In the present study, we used two stimuli: AMPH and the opportunity to interact with another similar-aged rat, both of which have been shown to be rewarding (Calcagnetti and Schechter 1992; Spyraki et al. 1982). Only trill calls were proportionally increased by both these conditions (flat–trill combinations increased only when comparing pair- to singly-tested rats). Therefore, we propose that among the 14 call subtypes, it is specifically trill calls that are reward-associated. Flat calls, in contrast, have been observed in social conditions that are not necessarily appetitive (Burgdorf et al. 2008a; Stevenson et al. 2009; Wohr et al. 2008). In the present study, flat calls tended to be more prevalent in singly-tested rats than pair-tested rats, consistent with the proposal that their purpose is to re-establish social contact (Wohr et al. 2008). We also found a negative correlation between flat calls and trills, further suggesting that they are functionally distinct.

Conclusion

Our new classification scheme represents a significant extension of previous work by others, but it is unlikely to be complete. For example, only future investigation will show whether it generalizes to adult male rats in other behavioral or pharmacological test conditions, female rats, other rat strains, and other stages of adult development. It is natural to speculate that the various individual call types each communicate unique information. Hence, in the short term, it will be important to try to elucidate the behavioral significance of 50-kHz call subtypes, for example by identifying experimental conditions that preferentially elicit them, and by studying the effects of call subtype playback on rodent behavior. If the meaning of 50-kHz call subtypes can be deciphered, this may offer new avenues for future behavioral and pharmacological studies.

Acknowledgments This study was supported by a Natural Science and Engineering Research Council of Canada discovery grant (155055, to P.B.S.C), a Canadian Institutes of Health Research of Canada operating grant (MOP-10516, to P.B.S.C), and a Master's Research Scholarship from the Fonds Québécois de Recherche sur la Nature et les Technologies (to J.M.W). We would like to thank Diala Chehayeb for valuable discussions and Laura Desrochers for help with the reliability testing. The authors have no financial relationship with the organizations that sponsored this research. Declaration: all experiments comply with the current laws of Canada.

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Neuropsychopharmacology (2012) 37, 808–821

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α- and β-Adrenergic Receptors Differentially Modulate the Emission of Spontaneous and Amphetamine-Induced 50-kHz Ultrasonic Vocalizations in Adult Rats

Jennifer M Wright[1], May RS Dobosiewicz[1] and Paul BS Clarke*[,1,2]

[1]Department of Pharmacology and Therapeutics, McGill University, Montreal, QC, Canada; [2]Center for Studies in Behavioral Neurobiology, Concordia University, Montreal, QC, Canada

Amphetamine (AMPH) increases adult rat 50-kHz ultrasonic vocalizations, preferentially promoting frequency-modulated (FM) calls that have been proposed to reflect positive affect. The main objective of this study was to investigate a possible noradrenergic contribution to AMPH-induced calling. Adult male Long-Evans rats were tested with AMPH (1 mg/kg intraperitoneal) or saline combined with various systemic pretreatments: clonidine (α2 adrenergic agonist), prazosin (α1 antagonist), atipamezole (α2 antagonist), propranolol, betaxolol, and/or ICI 118,551 (β1/β2, β1, and β2 antagonists, respectively), nadolol (β1/β2 antagonist, peripheral only), or NAD-299 ($5HT_{1A}$ antagonist). In addition, effects of cirazoline (α1 adrenergic agonist) and cocaine (0.25–1.5 mg/kg intravenous) were studied alone. AMPH-induced calling was suppressed by low-dose clonidine and prazosin. Cirazoline and atipamezole did not significantly affect calling rate. Propranolol, without affecting the call rate, dose dependently promoted 'flat' calls under AMPH while suppressing 'trills,' thus reversing the effects of AMPH on the 'call subtype profile.' This effect of propranolol seemed to be mediated by simultaneous inhibition of CNS β1 and β2 rather than by $5HT_{1A}$ receptors. Finally, cocaine elicited fewer calls than did AMPH, but produced the same shift in the call subtype profile. Taken together, these results reveal differential drug effects on flat *vs* trill *vs* other FM 50-kHz calls. These findings highlight the value of detailed call subtype analyses, and show that 50-kHz calls are associated with adrenergic α1- and β-receptor mechanisms. These preclinical findings suggest that noradrenergic contributions to psychostimulant subjective effects may warrant further investigation.

Neuropsychopharmacology (2012) **37,** 808–821; doi:10.1038/npp.2011.258; published online 26 October 2011

Keywords: ultrasonic vocalizations; rat; amphetamine; cocaine; noradrenaline; trills

INTRODUCTION

Adult laboratory rats emit two main types of ultrasonic vocalizations (USVs), commonly termed '22-kHz calls' and '50-kHz calls.' Evidence suggests that USVs may have a communicative role (Brudzynski, 2005; Burgdorf *et al*, 2008a; Wohr and Schwarting, 2009). Vocalizations of the 22-kHz type serve as alarm or distress calls (Covington and Miczek, 2003; Litvin *et al*, 2007), whereas 50-kHz calls are frequently elicited by appetitive stimuli (Burgdorf *et al*, 2010; Knutson *et al*, 2002).

The 50-kHz class of adult rat USVs encompasses a wide frequency range (30–90 kHz) (Kaltwasser, 1990; Sales and Pye, 1974; Wright *et al*, 2010) and comprises two main subclasses: flat (ie, constant frequency) and frequency-modulated (FM) calls. These two subclasses seem to differ in their behavioral significance and neurochemical basis (Ahrens *et al*, 2009; Barker *et al*, 2010; Burgdorf *et al*, 2007, 2008a; Burgdorf and Panksepp, 2006; Ciucci *et al*, 2009; Meyer *et al*, 2011; Simola *et al*, 2009; Wohr *et al*, 2008, 2009). FM 50-kHz USVs are diverse, with at least 13 acoustic subtypes, and the prevalent 'trill' call subtype, in particular, consistently occurs in appetitive situations (Burgdorf *et al*, 2008a). On this basis, it has been proposed that FM calls (and especially trill calls) reflect an emotional state homologous to positive affect in humans (Burgdorf and Moskal, 2009; Burgdorf *et al*, 2010).

The prototypical euphoriant D-amphetamine (AMPH) (Foltin and Fischman, 1991) increases the rate of 50-kHz call production in adult rats, both after systemic and central administration (Ahrens *et al*, 2009; Burgdorf *et al*, 2001; Simola *et al*, 2009; Thompson *et al*, 2006; Wintink and Brudzynski, 2001; Wright *et al*, 2010). In addition, AMPH has been shown to modify the 50-kHz call 'profile' (ie, the relative proportion of different call subtypes), preferentially

*Correspondence: Dr PBS Clarke, McIntyre Medical Building Room No. 1320, 3655 Promenade Sir William Osler, Montreal, QC H3G 1Y6, Canada, Tel: +1 514 398 3616, Fax: +1 514 398 6690, E-mail: paul.clarke@mcgill.ca
Received 6 June 2011; revised 13 September 2011; accepted 19 September 2011

increasing trills and decreasing flat calls (Wright *et al*, 2010). Cocaine administration is also reported to promote 50-kHz calling (Barker *et al*, 2010; Browning *et al*, 2011; Ma *et al*, 2010; Maier *et al*, 2010; Mu *et al*, 2009; Williams and Undieh, 2010), and a recent report shows a preferential increase in FM 50-kHz calls in response to intraperitoneal (IP) cocaine (Meyer *et al*, 2011). However, whether intravenous (IV) cocaine mimics the AMPH-induced shift in the call profile has not been reported.

Dopaminergic transmission seems to have a key role in the production of 50-kHz USVs. In particular, dopaminergic manipulations are reported to affect calls elicited by AMPH (Thompson *et al*, 2006), sex-relevant odors (Ciucci *et al*, 2009), tickling (Burgdorf *et al*, 2007), and intracerebral glutamate (Wintink and Brudzynski, 2001). However, the observation that dopamine (DA)-depleting lesions inhibited FM but not flat 50-kHz calls (Burgdorf *et al*, 2007; Ciucci *et al*, 2009) indicates that not all 50-kHz calls are necessarily DA dependent.

AMPH and cocaine promote noradrenergic, as well as dopaminergic neurotransmission (McKittrick and Abercrombie, 2007; Segal and Kuczenski, 1997). However, a possible noradrenergic role in the production of adult rat 50-kHz USVs has not, to our knowledge, been investigated, except in the context of social stress (Tornatzky and Miczek, 1994). This issue is of interest for several reasons. First, recent evidence supports a noradrenergic contribution to conventional reward-related behaviors, notably conditioned place preference (CPP) and reinstatement of IV self-administration (for review, see Weinshenker and Schroeder (2007); also see the 'Discussion' section). Second, noradrenaline (NA) also seems to contribute to the discriminative stimulus effects of AMPH in several species (Snoddy and Tessel, 1983, 1985); these cues potentially model subjective drug effects in humans (Stolerman, 1992). Third, early studies indicated that AMPH euphoria in human subjects is critically dependent on catecholaminergic transmission (Jonsson *et al*, 1969, 1971), and in some studies, AMPH euphoria seemed to be DA independent, suggesting a possible role for NA (Brauer and de Wit, 1997; Rothman *et al*, 2001; Sofuoglu *et al*, 2009).

The main aim of this study was to test the hypothesis that NA (or adrenaline) contributes to the emission of spontaneous and AMPH-induced 50-kHz USVs, potentially in a call subtype-selective manner. To this end, we first examined whether 50-kHz USV emission under AMPH was altered by acute pretreatment with the $\alpha 2$ agonist clonidine, administered at doses that decrease NA release (Schoffelmeer and Mulder, 1984). We then tested the $\alpha 1$ adrenergic antagonist prazosin, the $\alpha 1$ agonist cirazoline, the $\alpha 2$ antagonist atipamezole, and the $\beta 1/\beta 2$ blocker propranolol. Propranolol produced a dose-dependent shift in the call profile under AMPH, and we subsequently investigated the pharmacological mechanism underlying this effect: (1) To test for peripheral mediation, we administered nadolol, a non-selective hydrophilic β-blocker, which does not readily cross the blood–brain barrier (Schiff and Saxey, 1984); (2) We evaluated the contribution of $\beta 1$ *vs* $\beta 2$ receptor blockade using selective antagonists (betaxolol and ICI 118,551); and (3) As propranolol is a weak $5HT_{1A}$ receptor antagonist, we tested a selective antagonist of this receptor (NAD-299) (Ross *et al*, 1999). In a final experiment, we tested whether the call subtype-dependent effects produced by IP administration of AMPH would generalize to the IV route and also to cocaine.

MATERIALS AND METHODS

Subjects

Subjects were 77 male Long-Evans rats (Charles River Laboratories, St Constant, Quebec, Canada), weighing 307–425 g (ie, aged approximately 9–11 weeks) at the start of the experiment. They were housed 2 per cage ($25 \times 48 \times 20\,cm^3$) in a temperature- and humidity-controlled colony room (19–20°C, 50–60%) at the McGill University Animal Research Center. Rats were maintained on a reverse 12 : 12 light/dark cycle, with lights off at 0700 hours. All behavioral testing took place during the dark phase of the cycle. Food and water were available *ad libitum*, except during testing sessions. All procedures were approved by the McGill Animal Care Committee in accordance with the guidelines of the Canadian Council on Animal Care. In all experiments, rats were initially drug-naive and experimentally naive; Experiments 3 and 6 were each divided into two parts, with part b beginning within a week after the end of part a.

Overview of Experiments

Almost all experiments investigated the effects of various drug pretreatments on the USV response (ie, call rate and acoustic profile) to systemic (IP) AMPH. Exceptionally, Experiment 3a examined the acute USV response to cirazoline and atipamezole alone, and Experiment 7 comprised a dose-response study of IV cocaine given alone. Details of individual experiments are summarized in Table 1.

Protocol for Individual Experiments

AMPH screen. A significant minority of rats emit few USVs in response to systemic AMPH (Wright *et al*, 2010). To identify and exclude such subjects, rats were initially screened for AMPH-induced calling in three 20-min test sessions spaced 2 days apart. Immediately before each session, rats were administered AMPH (1 mg/kg, IP) and then placed in a test chamber. On the intervening days, rats remained in their home cages. Only the third AMPH test session was analyzed because the first two sessions are not necessarily indicative of a rat's subsequent USV response to AMPH (unpublished observation). USVs that were emitted 10–20 min after injection were counted; rats with the lowest rate of calling (ie, 20–43% of rats depending on the experiment) were excluded from subsequent testing. In total, 47 out of 124 rats were excluded on this basis.

Drug testing. Drug testing was initiated 2–5 days after the final AMPH screening session, with the exception of Experiment 7 (ie, 11 days) in which rats needed to recover from surgery before drug testing began. All experiments used a fully parametric within-subject design in which each rat was tested once under each drug condition (see Table 1 for details). Thus, in Experiments 1, 2, 3b, and 4–6, rats received all combinations of pretreatment and treatment

Table 1 Summary of Experiments

Expt	Pretreatment[a]					Treatment[b]				
	Drug		Doses (mg/kg)	Route	Time before treatment (min)	Drug		Doses (mg/kg)	Route	*n*
1	α2 agonist	Clonidine	0, 0.01, 0.02, 0.1	IP	20		AMPH	0, 1	IP	10
2	α1 antagonist	Prazosin	0, 0.3, 1	IP	30		AMPH	0, 1	IP	12
3a	—	—	—	—	—	α1 agonist	Cirazoline	0.5, 1	IP	12
	—	—	—	—	—	α2 antagonist	Atipamezole	0.3, 1	IP	
	—	—	—	—	—		AMPH	0, 1	IP	
3b	α2 antagonist	Atipamezole	0, 1	IP	20		AMPH	0, 1	IP	9[c]
4	β1/β2 antagonist	Propranolol	0, 1, 3, 10	IP	20		AMPH	0, 1	IP	8
5	β1/β2 antagonist	Propranolol	10	IP	20		AMPH	0, 1	IP	11
	β1 antagonist	Betaxolol	1	IP	20		AMPH	0, 1	IP	
	β2 antagonist	ICI 118,551	0.2	IP	20		AMPH	0, 1	IP	
	β1/β2 antagonist (peripheral)	Nadolol	5	IP	20		AMPH	0, 1	IP	
6a	β1/β2 antagonist	Propranolol	10	IP	20		AMPH	1	IP	12
	β1+β2 antagonist	Betaxolol + ICI 118,551	2.5 (BET), 1 (ICI)	IP	20		AMPH	1	IP	
	$5HT_{1A}$ antagonist	NAD-299	0.2	SC	20		AMPH	1	IP	
6b	β1 antagonist	Betaxolol	2.5	IP	20		AMPH	1	IP	
	β2 antagonist	ICI 118,551	1	IP	20		AMPH	1	IP	
7		—	—	—	—		Cocaine	0, 0.25, 0.75, 1.5	IV	12
		—	—	—	—		AMPH	0.5	IV	

[a]For Experiments 5 and 6, each rat was also tested under vehicle pretreatment combined with saline and AMPH treatment.
[b]In all experiments, treatments were administered immediately before placing the rat in the experimental chambers for recording, with the exception of Experiment 3a, in which cirazoline and atipamezole treatments were administered 20 min before. All test session durations were 20 min, except for Experiment 7 (ie, 10 min).
[c]The rats in Experiment 3b were the same as those used in Experiment 3a.

drugs including vehicle controls. Similarly, in Experiments 3a and 7, rats received a test with each of the following: vehicle, AMPH, and each dose of the drug(s) being tested. Within each experiment, the order of testing was counterbalanced as far as possible. Test sessions were of 20 min duration except in Experiment 7; here, subjects were administered IV cocaine or AMPH and were tested only 0–10 min after injection, ie, during the period of drug onset. Test sessions were spaced 2 days apart to minimize possible carry-over effects of the drugs.

For Experiment 7 (IV cocaine and AMPH), rats first underwent IV catheterization surgery (see below). After recovery, the experiment comprised an initial habituation day, whereby rats were placed in the test chambers for 10 min, then removed and immediately injected with 0.1 ml heparin-Baytril-saline solution to maintain catheter patency. On the five test days that followed, each rat received a 10-s infusion of drug directly after they were placed in the test chamber. Immediately after drug infusion, the tubing was disconnected and the session started.

IV Catheterization Surgery

General anesthesia was provided by ketamine HCl (80 mg/kg IP) and xylazine HCl (16 mg/kg IP). A 5-mm incision was made on the right ventral surface of the neck. A chronic indwelling silastic catheter (0.5 mm I.D. and 0.9 mm O.D., Fisher Scientific, Montreal, Quebec, Canada) was inserted in the right jugular vein and secured using silk sutures. The catheter was passed subcutaneously to a 2-cm incision on the head, where it was connected to a modified plastic cannula (Plastics One, Roanoke, VA), which was then anchored to the top of the skull with stainless steel mounting screws (Plastics One) and dental cement (Stoelting, Wooddale, IL). The cannula was blocked using a plastic stopper made from Tygon tubing (Fisher Scientific), and shielded with an aluminum cap when not in use. The analgesic carprofen (5 mg/kg SC) was administered during surgery to alleviate post-surgical pain. In all, 4 rats out of 19 died from anesthetic overdose during surgery. To verify catheter patency, each rat received an infusion of Na methohexital ('Brevital,' 1 mg in 0.1 ml, 2-s infusion, IV) once in their home cage, 3–5 days after surgery; three rats failed to show the expected sedative response and were therefore excluded from the experiment. The remaining rats were allowed 7–9 days of recovery before experimental testing began. Immediately after the habituation session and after each test session, the catheters were flushed with 0.1 ml of a sterile 0.9% saline solution containing 0.2 mg/ml heparin (Sigma-Aldrich, Oakville, Ontario, Canada) and 17 mg/ml Baytril (ICN Biomedicals, Cleveland, OH).

Acquisition and Classification of USVs

Testing took place in clear Plexiglas experimental chambers (ENV-007CT, Med Associates, St Albans, VT), each of

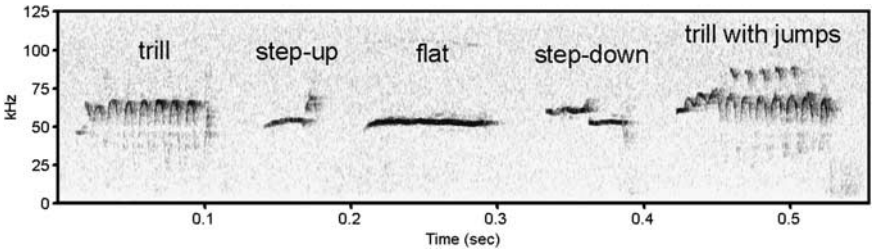

Figure 1 Spectrographic display of individual 50-kHz calls, which are representative of the following subtypes (left to right): trill, step-up, flat, step-down, and trill with jumps. See Wright *et al* (2010) for additional examples of all fourteen 50-kHz call subtypes so far recognized.

which was enclosed in a melamine compartment lined with sound-attenuating acoustic foam (Primacoustic, Port Coquitlam, British Columbia). Condenser ultrasound microphones (CM16/CMPA, Avisoft Bioacoustics, Berlin, Germany) were securely inserted through small (5-cm diameter) holes located centrally in the top panels of the experimental chambers. Consequently, the microphones were 15–30 cm from rats during testing. Microphone signals were fed into an UltraSoundGate 416H data acquisition device (Avisoft Bioacoustics) with a sampling rate of 250-kHz and a 16-bit resolution.

Acoustical analysis was performed using Avisoft SASLab Pro (version 4.2, Avisoft Bioacoustics). Spectrograms were generated with a fast Fourier transform length of 512 points and an overlap of 75% (FlatTop window, 100% frame size). Correspondingly, spectrograms had a frequency resolution of 490 Hz and a time resolution of 0.5 ms. Calls were selected manually from spectrograms by an individual who was masked to the treatment condition. Each identified 50-kHz call was classified into 1 of 14 distinct categories: complex, upward ramp, downward ramp, flat, short, split, step-up, step-down, multi-step, trill, flat–trill combination, trill with jumps, or composite (see Wright *et al* (2010) for criteria for call identification and classification, several examples of each call type, as well as descriptive statistics relating to acoustic parameters). This method of manual call selection has been validated by surgical devocalization, and classification is associated with high inter- and intra-rater reliability (Wright *et al*, 2010). Some representative 50-kHz USVs are shown in Figure 1. 22-KHz calls were rarely observed in this study and were not analyzed further.

Drugs

All test drugs, doses, and routes of administration are shown in Table 1. Drugs were: D-AMPH sulfate (Sigma-Aldrich, Poole, UK); cocaine HCl (Medisca, St-Laurent, Quebec, Canada); clonidine HCl, prazosin HCl, (±)-propranolol HCl, and nadolol (all from Sigma-Aldrich); NAD-299 HCl (ie, Robalzotan), betaxolol HCl, ICI 118,551 HCl, cirazoline HCl, and atipamezole HCl (all from Tocris Bioscience, Ellisville, MO). The doses of prazosin, propranolol, clonidine, and nadolol are expressed as the free base; all other drug doses are expressed as the salt. Drugs were dissolved in sterile 0.9% saline and administered in a volume of 1 ml/kg body weight with the following exceptions: (1) prazosin was dissolved in distilled water, (2) the combination of betaxolol and ICI 118,551 in Experiment 6 was administered in a volume of 4 ml/kg (divided into 2 separate injections), and (3) nadolol (Experiment 5), as well as betaxolol and ICI 118,551 in Experiment 5 were administered in a volume of 2 ml/kg. Control injections were of saline (Experiments 1 and 3–7) or water (for prazosin, Experiment 2) and administered in the same volume as the corresponding drug.

Data Analysis and Statistics

Data were analyzed using commercial software (Systat v11, SPSS, Chicago, IL; GraphPad Prism 4, GraphPad Software, La Jolla, CA). For the IV cocaine dose-response study (Experiment 7), only the USVs emitted during the first 30-s of each minute were analyzed. For Experiment 3a (effects of cirazoline and atipamezole), minutes 3, 8, 13, and 18 were analyzed. For all other experiments, analysis of USVs was restricted to minutes 12, 14, and 16 of the 20-min session to allow time for AMPH to take effect. In the analysis, 'call rate' was defined as the total number of 50-kHz calls (ie, calls of all categories) emitted per minute. ANOVA or Friedman's test was performed, where appropriate, to test the effects of the within-subject factors 'pretreatment' and 'treatment' (see Table 1), for both the call rate and for each call subtype expressed as a proportion of all calls. In addition, for Experiments 4, 6a, and 7, a *post hoc* analysis was performed on non-trill FM calls (ie, all call subtypes except trills, flats, and shorts). All ANOVA p-values were subject to the Huynh–Feldt correction. Multiple comparison tests were performed using Tukey's, Dunnett's, paired t-tests, or Wilcoxon's tests, depending on the type of comparisons to be made and the distribution of the data. For call rate, the latter two tests were subjected to the Holm–Bonferroni (H-B) correction. However, for call subtype analysis, pairwise comparisons were performed using unprotected tests to maintain statistical power. For all tests, a two-tailed p-value $<5\%$ was considered significant.

RESULTS

Experiments 1 and 2: Effects of Clonidine and Prazosin

As expected, AMPH administered alone (ie, with vehicle pretreatment) significantly increased the overall rate of calling (ie, sum of all 50-kHz call categories emitted per minute) (Figure 2a and b). This effect was significantly reduced by the lowest dose of the α2 adrenergic agonist clonidine (ie, 10 μg/kg) and abolished by the two higher doses (20 or 100 μg/kg; Figure 2a). Clonidine also seemed to decrease calls when administered alone (ie, under saline

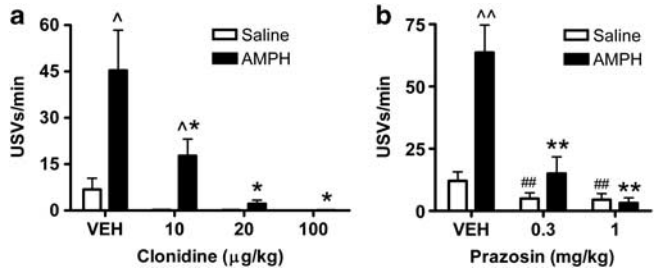

Figure 2 Experiments 1 and 2: Clonidine and prazosin dose dependently decreased the 50-kHz call rate (ie, calls of all categories). The *y* axis represents mean + SEM calls/min. Each rat was tested under all conditions (clonidine group $n = 12$, prazosin group $n = 12$). AMPH administration only significantly increased the call rate when rats were pretreated with vehicle (a, b) or with the lowest dose of clonidine (panel a). Under AMPH treatment, clonidine (panel a) and prazosin pretreatment (panel b) dose dependently reduced the call rate. Prazosin alone (ie, with saline treatment) also decreased the call rate at both doses tested. Clonidine (ie, saline treatment) appeared to decrease calls when administered alone (Friedman test, $p < 0.01$), but the trend did not reach statistical significance for any individual dose ($p > 0.05$). All pairwise comparisons were made by Wilcoxon's tests with Holm–Bonferroni (H-B) correction, $n = 12$ (per experiment). $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$ *vs* the corresponding saline treatment (ie, same pretreatment), $^{\#\#}p < 0.01$ *vs* VEH + saline condition, $^{*}p < 0.05$, $^{**}p < 0.01$ *vs* corresponding VEH pretreatment condition.

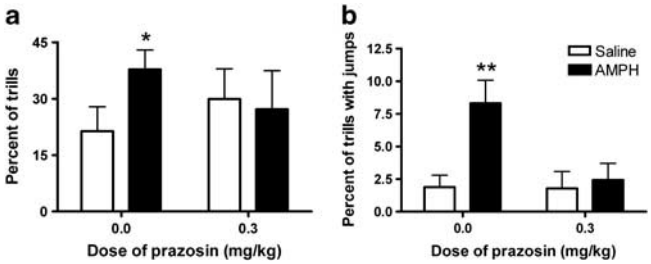

Figure 3 Experiment 2: Prazosin inhibited or blocked the AMPH-induced increase in the percentage of trills (a) and trills with jumps (b). The *y* axes represent mean + SEM percentage. Each rat was tested under all conditions ($n = 12$). Both two-way ANOVA interactions were significant (see main text). $^{*}p < 0.05$, $^{**}p < 0.01$ *vs* corresponding vehicle/saline condition (paired *t*-tests).

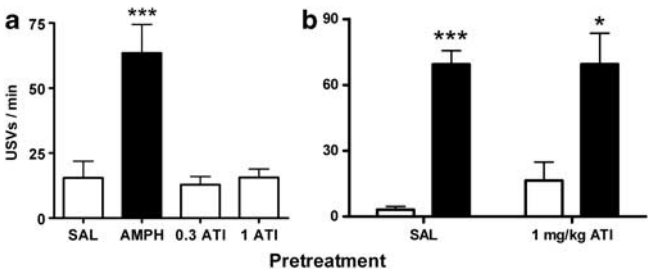

Figure 4 Experiment 3: (a) AMPH increased the call rate, whereas atipamezole (0.3 and 1 mg/kg ATI; $n = 10$) administered alone had no significant effect. The *y* axis represents the mean + SEM call rate per minute. $^{***}p < 0.001$ *vs* saline condition (paired *t*-test) (b) AMPH increased the call rate in rats pretreated with saline or atipamezole. Atipamezole alone did not significantly increase the call rate. The *y* axis represents the mean + SEM call rate per minute. Filled bars correspond to AMPH treatment and open bars correspond to saline treatment. Each rat was tested under all conditions ($n = 9$). $^{*}p < 0.05$, $^{***}p < 0.001$ compared with the same pretreatment with saline challenge (paired *t*-tests).

treatment; Friedman test, $Q_3 = 12.97$, $p < 0.01$; Figure 2a), but no individual dose of clonidine exerted a significant effect (Wilcoxon's tests with H-B correction, $Z \leqslant 2.37$, NS). The $\alpha 1$ antagonist prazosin alone significantly inhibited calling (Friedman test, $Q_2 = 18.48$, $p < 0.001$; Figure 2b), and even the lower dose (0.3 mg/kg) of this drug virtually abolished AMPH-induced calling (Wilcoxon's test with H-B correction, $Z = 2.59$, $p < 0.01$).

Clonidine and prazosin also modified the call profile. As many rats failed to make any calls at higher doses of these drugs, analysis was restricted to the following low-dose conditions: (1) AMPH alone *vs* clonidine (10 μg/kg) + AMPH and (2) all four combinations of vehicle or prazosin (0.3 mg/kg) with saline or AMPH. In the presence of AMPH, clonidine increased the proportion of multi-step calls, while decreasing the proportion of flat–trill combination calls (paired *t*-tests, $t_8 = 2.74$ and $t_8 = 2.83$, respectively, both $p < 0.05$; see Supplementary Figure S1). Prazosin (0.3 mg/kg) blocked the AMPH-induced increase in the proportion of 'trills' and 'trills with jumps' (Figure 3; ANOVA pretreatment × treatment interactions: trills: $F_{1,7} = 6.74$, $p = 0.036$; trills with jumps: $F_{1,7} = 23.31$, $p < 0.01$).

Experiment 3: Effects of Atipamezole and Cirazoline

3a: Effect of atipamezole and cirazoline alone. Neither dose of the $\alpha 2$ antagonist atipamezole (0.3 and 1 mg/kg), administered alone, significantly altered the call rate (Figure 4a) or altered the call profile (data not shown). The $\alpha 1$ agonist cirazoline at both doses tested (0.5 and 1 mg/kg) produced observable changes in the behavior in all rats, such that they disengaged from their cage mate and reared in one corner of the cage. In the first three test days, two rats died shortly after receiving either 0.5 or 1 mg/kg cirazoline, possibly due to pulmonary edema (Micheletti *et al*, 1987). Consequently, saline injection was substituted for cirazoline for the remainder of the experiment. Among rats that received cirazoline ($n = 5$), there was an apparent but non-significant decrease in the call rate (mean ± SEM values for saline and 1 mg/kg cirazoline were 10.7 ± 5.4 and 1.0 ± 1.0 calls/min, respectively).

3b: Effect of atipamezole in combination with AMPH. Atipamezole (1 mg/kg) alone tended to increase the call rate in this experiment, but not significantly (Wilcoxon's test, $Z = 1.89$, $p = 0.0584$; Figure 4b). Atipamezole did not affect the call rate under AMPH (Wilcoxon's test, $Z = 0.41$, NS; Figure 4b), and only had moderate effects on the AMPH call profile. In particular, the percentage of short calls, step-ups, and step-downs was increased by atipamezole (paired *t*-tests, $t_8 = 2.43$–3.08, each $p < 0.05$). Mean ± SEM values in the presence *vs* absence of atipamezole were 17.4 ± 3.1% *vs* 10.4 ± 1.8% (short calls), 11.5 ± 2.3% *vs* 6.5 ± 2.3% (step-ups), and 4.1 ± 0.9% *vs* 2.1 ± 0.7% (step-downs), respectively.

Experiment 4: Effect of Propranolol

Propranolol failed to change the call rate significantly (Figure 5). Although propranolol seemed to depress calling

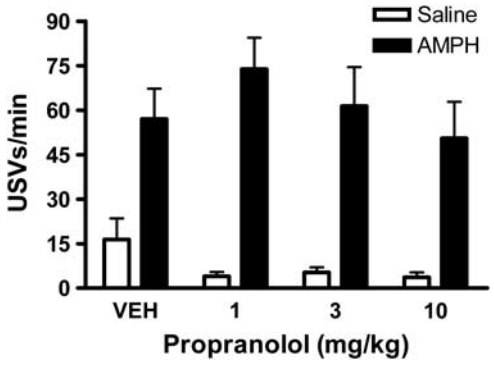

Figure 5 Experiment 4: AMPH-induced 50-kHz call rate was not altered by propranolol. The *y* axis shows mean + SEM calls/min ($n = 8$). Each rat was tested under all conditions. AMPH increased the call rate at all doses of propranolol (Wilcoxon's tests: $p < 0.05$). No dose of propranolol significantly altered the call rate under AMPH (paired *t*-tests, $p > 0.05$) or when administered alone (Wilcoxon's tests, $p > 0.05$). All other pairwise comparisons were subjected to H-B corrections.

Table 2 Effect of Propranolol on Percentage of Flat Calls and Trills under AMPH in Experiment 4

Propranolol dose (mg/kg)	Flat calls	Trills
1	−4.51**	1.86
3	−4.22**	2.7*
10	−9.1***	2.55*

Values in the table are the paired *t*-statistics of the propranolol pretreatment conditions *vs* the saline pretreatment control all under AMPH treatment, df = 7, *$p < 0.05$, **$p < 0.01$,***$p < 0.001$.

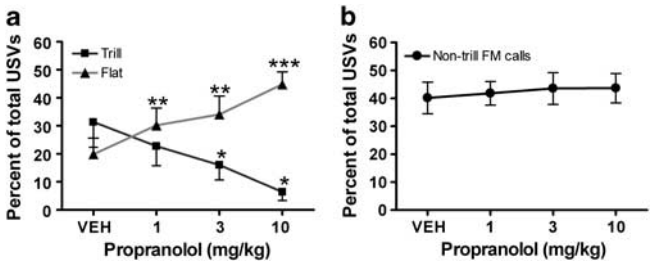

Figure 6 Experiment 4: Propranolol promoted flat calls and inhibited trill calls under AMPH. Line graphs showing (a) the dose-dependent increase in flat calls and concomitant decrease in trills, and (b) no significant difference in non-trill frequency-modulated calls, expressed as mean ± SEM percentage of total calls emitted (ie, calls of all 50-kHz categories). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with vehicle (VEH) pretreatment (paired *t*-tests, $n = 8$).

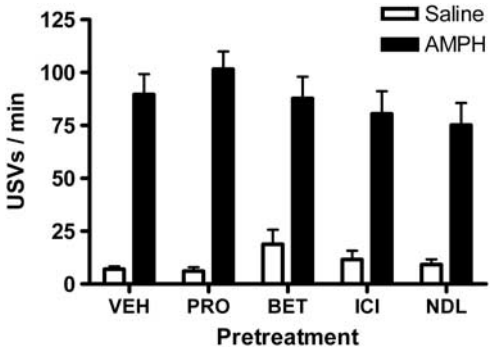

Figure 7 Experiment 5: AMPH-induced 50-kHz calling was not altered by propranolol (PRO; 10 mg/kg, IP), betaxolol (BET; 1 mg/kg, IP), ICI 118,551 (ICI; 0.2 mg/kg, IP), or nadolol (NDL; 5 mg/kg, IP). AMPH robustly increased the call rate under all pretreatment conditions (Wilcoxon's tests: $p < 0.05$–0.003). No pretreatment affected the call rate when administered alone (Wilcoxon's tests: $p > 0.05$) or when combined with AMPH (Wilcoxon's tests: $p > 0.05$). The *y* axis represents mean + SEM calls/min. Each rat was tested under all conditions ($n = 11$). All pairwise comparisons were subjected to H-B corrections.

when administered alone, no dose differed significantly from saline in this respect, even before correction for multiple comparisons (Wilcoxon's tests, $Z \leqslant 1.96$, NS for each dose). Propranolol also failed to affect 'AMPH-induced' calls (ie, AMPH minus saline difference score; ANOVA $F_{3,21} = 1.86$, NS; uncorrected paired *t*-tests, $t_7 = 0.4$–1.16, NS). In contrast, propranolol had a striking effect on the types of calls emitted (Supplementary Figure S2). In particular, under AMPH, propranolol dose dependently promoted flat calls while nearly abolishing trill calls (ANOVA: flat calls $F_{3,21} = 23.9$, $p < 0.0001$; trills $F_{3,21} = 5.66$, $p < 0.05$; see Table 2 for *t*-statistics comparing each propranolol dose with saline; Figure 6a). In contrast, all other non-trill FM calls collectively remained constant across propranolol doses (ANOVA $F_{3,21} = 0.18$, NS; Figure 6b). The absolute number of trills, flats, and non-trill FM calls are provided in Supplementary Table S1.

Experiment 5: Effects of Betaxolol, ICI 118,551, and Nadolol

In this experiment, the effects of the selective $\beta 1$ adrenergic antagonist betaxolol, the selective $\beta 2$ adrenergic antagonist ICI 118,551, and the hydrophilic $\beta 1/\beta 2$ blocker nadolol were examined. As with propranolol, none of these agents significantly affected the rate of calling after saline or AMPH treatment (Wilcoxon's tests; saline treatment: $Z = 0.62$–0.89, NS; AMPH treatment: $Z = 0.53$–1.95, NS; Figure 7). Analysis of individual call subtypes was restricted to AMPH treatment conditions, as saline test session yielded few calls (Figure 8). Propranolol again caused a highly significant shift in the call profile under AMPH (paired *t*-tests: proportion of (1) trills, $t_{10} = 6.54$, $p < 0.001$; (2) flat calls, $t_{10} = 4.45$, $p < 0.01$) (Figure 8a and b). Here, propranolol also had effects on other call subtypes: propranolol increased the proportion of flat–trill combinations (paired *t*-test, $t_{10} = 2.4$, $p < 0.05$) and split calls (paired *t*-test, $t_{10} = 2.47$, $p < 0.05$) (Figure 8c and d). However, betaxolol, ICI 118,551, and nadolol were all without effect on call profile (Figure 8). The absolute number of trills, flats, flat–trill combinations, and split calls are provided in Supplementary Table S2.

Given the possible sensitizing effects of AMPH on USVs (Ahrens *et al*, 2009), we assessed order effects by examining the call rate under AMPH as a function of the number of times the rat was exposed to AMPH. The call rate did not change significantly over multiple AMPH exposures in this experiment (Supplementary Figure S3).

Experiment 6: Effects of NAD-299 and Higher Doses of Betaxolol and ICI 118,551

The findings of Experiment 5 indicated that the observed effects of propranolol might require simultaneous $\beta1/\beta2$ receptor blockade, or might result from this drug's ability to antagonize $5HT_{1A}$ receptors; alternatively, our doses of betaxolol and ICI 118,551, chosen to ensure $\beta1$ *vs* $\beta2$ selectivity *in vivo* (see 'Notes' in Supplementary Material), might have been insufficient. Therefore, Experiment 6 examined the effects of (1) higher doses of betaxolol and ICI 118,551 alone or in combination and (2) the selective $5HT_{1A}$ antagonist NAD-299.

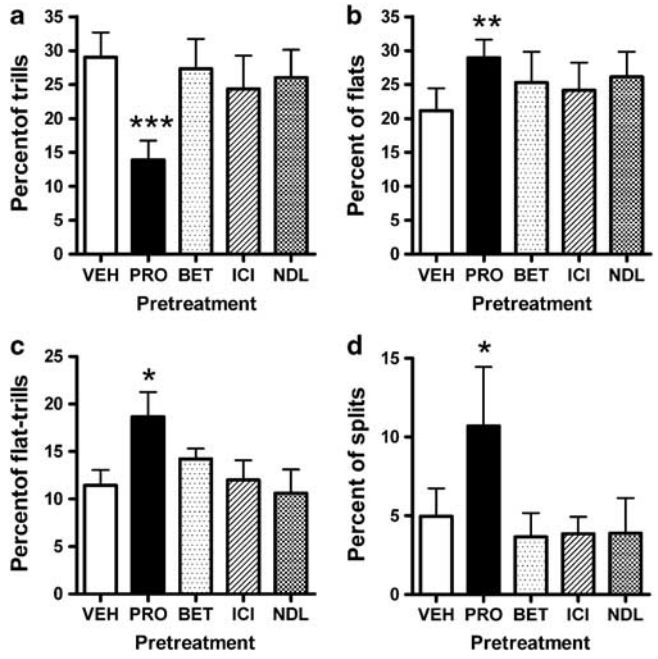

Figure 8 Experiment 5: Propranolol decreased the percentage of trills (a) and increased the percentage of flats, flat–trill combinations, and splits (b–d) under AMPH. The *y* axis represents mean + SEM percentage of total calls (ie, all 50-kHz categories). Each rat was tested under all conditions ($n = 11$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs* vehicle pretreatment condition (paired *t*-tests).

6a: Effects of betaxolol and ICI 118,551 in combination and NAD-299. AMPH treatment again produced a highly significant increase in call rate, and this effect was unaltered by pretreatment with either propranolol, the combination of betaxolol and ICI 118,551, or NAD-299 (Tukey's test: AMPH treatment conditions *vs* saline, $q = 9.07–11.22$, each $p < 0.001$; AMPH treatment alone *vs* drug pretreatment + AMPH, $q = 0.35–1.80$, NS; Supplementary Figure S4). As before, propranolol normalized the trill/flat profile shift induced by AMPH (Figure 9a and b; see Table 3 for statistical details), and in addition, it caused a significant decrease in the proportion of short calls (Figure 9c). The betaxolol/ICI 118,551 combination mimicked these effects of propranolol, whereas NAD-299 was without significant effect (Figure 9a–c). However, propranolol also caused an increase in the proportion of split calls, an effect not observed with the betaxolol/ICI 118,551 combination or with NAD-299 (Figure 9d). There was no significant change in the proportion of non-trill FM calls after any pretreatment in this experiment (Figure 9e).

6b: Effect of betaxolol and ICI 118,551 alone at higher doses. Here, betaxolol or ICI 118,551 was tested individually at the same doses as used in Experiment 6a (2.5 and 1 mg/kg, respectively) in combination with AMPH (1 mg/kg, IP). Neither antagonist affected USV rate or profile (Supplementary Figures S5 and S6).

Experiment 7: Effect of IV Cocaine and AMPH on 50-kHz USVs

The dose of AMPH used in this experiment (0.5 mg/kg IV) was chosen based on a preliminary dose-response study (0.1, 0.5, 1, and 2 mg/kg, IV; Supplementary Figure S7).

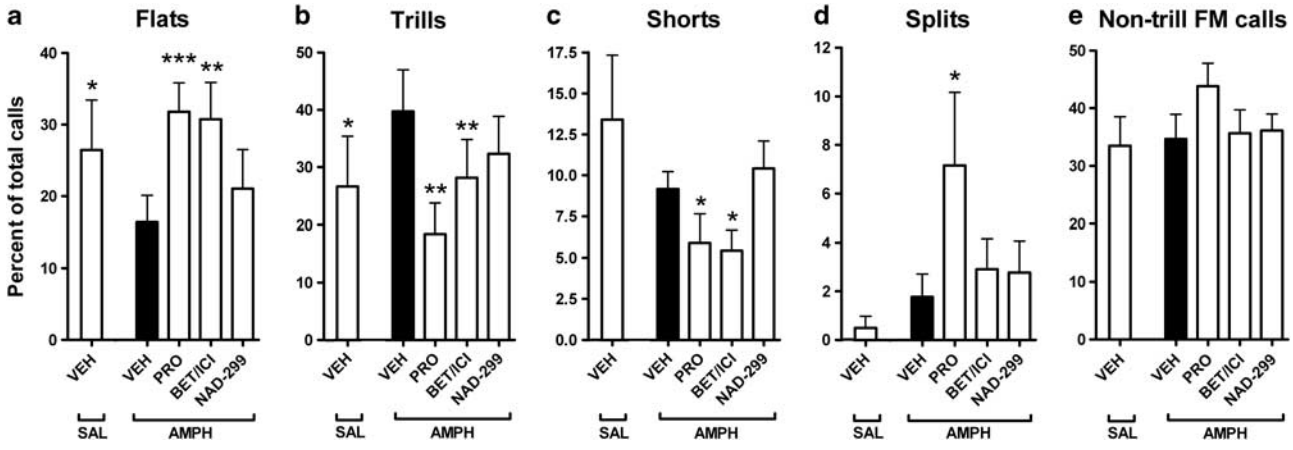

Figure 9 Experiment 6a: Propranolol (PRO; 10 mg/kg, IP) and the combination of betaxolol and ICI 118,551 (BET/ICI; 2.5 and 1 mg/kg IP, respectively) increased the percentage of flat calls under AMPH (a) while decreasing the percentage of trills (b) and shorts (c). In this experiment, propranolol also significantly increased the percentage of split calls (d), an effect not observed with the betaxolol/ICI 118,551 combination. There was no significant effect of any pretreatment on non-trill frequency-modulated calls (e). All pairwise comparisons between the PRO *vs* BET/ICI conditions were non-significant (paired *t*-tests, $p = 0.07–0.81$). NAD-299 failed to affect the percentage of any calls emitted. The *y* axis shows mean + SEM percent of total calls (ie, all subtypes) ($n = 12$). Pretreatments are listed immediately below the *x* axes, and saline or AMPH treatment conditions are indicated underneath each graph. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the VEH/AMPH condition.

Table 3 Call Profile Shifts in Experiment 6a

Pretreatment	Treatment	AMPH treatment alone				
		Flats	**Trills**	**Shorts**	**Splits**	**Non-trill FM calls**
Vehicle	Saline	2.48*	2.25*	0.95	1.17	0.2
Propranolol	AMPH	4.85***	4.06**	2.33*	2.41*	2.12
Betaxolol/ICI 118,551	AMPH	4.31**	3.23**	3.05*	1.36	0.29
NAD-299	AMPH	1.31	1.78	0.64	1.11	0.44

Values in the table refer to the paired *t*-statistics comparing the percentage of each call subtype under AMPH treatment alone with percentage under the pretreatment/treatments listed in the first two columns. df = 11, *$p<0.05$, **$p<0.01$,***$p<0.001$.

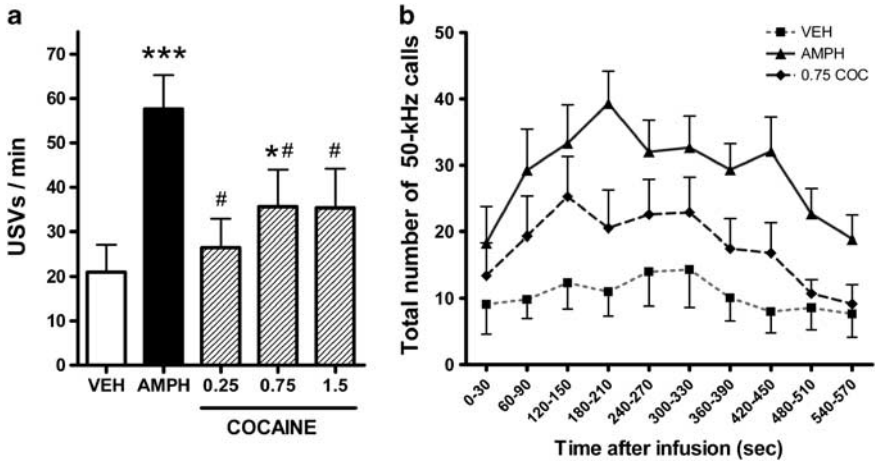

Figure 10 Experiment 7: Cocaine (0.25, 0.75, 1.5 mg/kg, IV) dose dependently increased the number of USVs emitted by rats, but significantly less so than amphetamine (0.5 mg/kg IV; AMPH). (a) The rate of 50-kHz calling was averaged 0–10 min after injection and is expressed as calls/min (mean + SEM). Each rat was tested under all conditions ($n=12$). Only AMPH and 0.75 mg/kg cocaine significantly increased the call rate. *$p<0.05$, ***$p<0.001$ *vs* corresponding saline (VEH) condition, $^{\#}p<0.001$ *vs* the corresponding AMPH condition (Tukey's test). (b) Time course of the call rate after AMPH (0.5 mg/kg, IV) or cocaine (0.25, 0.75, and 1.5 mg/kg, IV) administration. The *x* axis refers to the time after the end of the 10-s infusion. For visual clarity, only the VEH, AMPH, and the 0.75 mg/kg doses of cocaine (ie, the most effective dose of cocaine on the call rate) are illustrated.

Only AMPH and the 0.75 mg/kg dose of cocaine significantly increased the call rate compared with saline treatment, and cocaine was less effective than AMPH in this regard (Tukey's test: AMPH *vs* saline, $q=10.19$, $p<0.001$; 0.75 mg/kg cocaine *vs* saline, $q=4.08$, $p<0.05$; AMPH *vs* each cocaine dose, $q=6.11$–8.66, all $p<0.001$; Figure 10a). Analysis restricted to FM calls showed the same pattern of effects (Supplementary Figure S8). Under AMPH, the call rate increased detectably within the first 30 s after infusion (paired *t*-test *vs* saline, $t_{11}=3.12$, $p<0.01$), and this drug effect peaked between 180 and 210 s (Figure 10b). Cocaine (0.75 mg/kg) produced a significant increase in the call rate 60–90 s after the infusion (paired *t*-test *vs* saline, $t_{11}=2.27$, $p<0.05$), and this effect peaked between 120 and 150 s (Figure 10b).

Although cocaine only modestly affected call rate, it produced a highly significant shift in the call profile at all doses tested (Figure 11). In this respect, it closely mimicked the effect of AMPH, such that trill calls proportionally increased while flat calls decreased (Dunnett's tests *vs* saline: trills, $q=2.67$–4.35, $p<0.01$–0.05; flat calls, $q=3.25$–4.53, $p<0.01$ for each comparison). There was no change in the proportion of non-trill FM calls under AMPH or cocaine (Dunnett's test *vs* saline: $q=0.35$–2.05, NS).

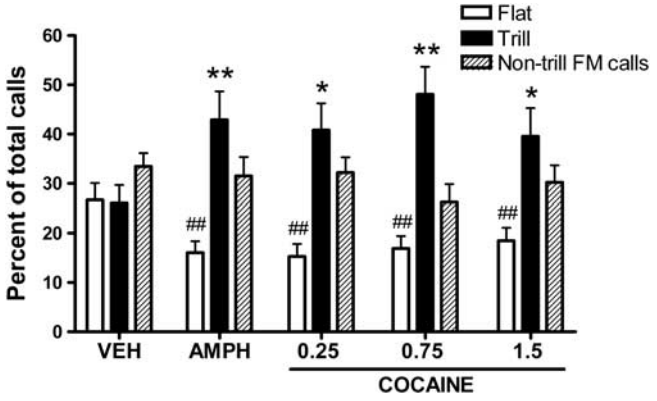

Figure 11 Experiment 7: AMPH (0.5 mg/kg, IV) and all doses of cocaine (0.25, 0.75, and 1.5 mg/kg, IV) promoted trill calls while suppressing flat calls. The *y* axis represents the percentage of the total calls that were trills, flat calls, and non-trill frequency-modulated calls for each drug/dose condition (mean + SEM, $n=12$). *$p<0.05$, **$p<0.01$, $^{\#\#}p<0.01$ *vs* corresponding VEH (ie, saline) condition (Dunnett's tests).

DISCUSSION

Previous investigations relating noradrenergic mechanisms to rat USV emission have almost exclusively focused on

adult 22-kHz USVs (McIntosh and Barfield, 1984) or pup calls (Blumberg *et al*, 2005); both types of call appear to be functionally distinct from the 50-kHz calls emitted by adult rats (Portfors, 2007). To our knowledge, the only previous report of a potential noradrenergic contribution to adult rat 50-kHz USVs was in the context of social stress (Tornatzky and Miczek, 1994). Hence, this study is the first to examine the association between NA and 50-kHz USV production in unstressed adult rats.

Pharmacological Considerations

As discussed below, the effects of prazosin, clonidine, and propranolol observed in this study are likely mediated through $\alpha1$, $\alpha2$, and $\beta1/\beta2$ adrenergic receptors, respectively. Doses of prazosin were based on the drug's potency in the following *in vivo* assays: $\alpha1$ radiotracer binding (Couch *et al*, 1988), antagonism of an $\alpha1$ agonist cue (Schechter, 1991), and inhibition the psychomotor stimulant effects of AMPH (Selken and Nichols, 2007; Vanderschuren *et al*, 2003). Prazosin, at the doses used in this study, is highly $\alpha1$-selective, with negligible affinity for $\alpha2$ or β adrenergic, DA, and serotonin receptors (Balle *et al*, 2003; Clineschmidt *et al*, 1979; Miach *et al*, 1980; Sanger, 1989) or for imidazoline sites (Angel *et al*, 1995). However, prazosin also binds to melatonin MT_3 receptors, although with significantly lower affinity than to $\alpha1$ receptors (Doxey *et al*, 1984; Molinari *et al*, 1996; Pickering and Niles, 1990). The function of the MT_3 receptor remains poorly characterized, except in the regulation of intraocular pressure (Pintor *et al*, 2001). Therefore, on present evidence it is not clear whether MT_3 antagonism would produce detectable behavioral effects.

Clonidine acts as a potent agonist at both $\alpha2$ adrenergic and I_1-imidazoline receptors (Edwards *et al*, 2001). In the dose range administered (0.01–0.1 mg/kg), clonidine would be expected to dose dependently stimulate $\alpha2$ autoreceptors (Drew *et al*, 1979), thereby inhibiting release and turnover of NA (Anden *et al*, 1970; Sacchetti *et al*, 2001). Moreover, within this dose range, clonidine (0.04 mg/kg) produced an $\alpha2$ receptor-mediated drug cue without detectable $\alpha1$- or β-receptor activity (Bennett and Lal, 1982). However, clonidine probably also activated I_1-imidazoline receptors. These receptors have been proposed to contribute to the CNS control of blood pressure (Holt, 2003) and to modulate aversive effects of opiate withdrawal (Georges *et al*, 2005). As the neuropharmacological and behavioral consequences of I_1-imidazoline receptor stimulation are largely unknown, we cannot exclude their possible role in USV inhibition by clonidine.

Propranolol selectively antagonizes $\beta1$, $\beta2$, and $5HT_{1A}$ receptors (Middlemiss and Tricklebank, 1992), while possessing much lower affinity for $\beta3$ receptors (Baker, 2005). Several observations suggest that $5HT_{1A}$ receptors did not contribute to the call profile-changing effect of propranolol under AMPH. First, the highly selective $5HT_{1A}$ antagonist NAD-299 (Ross *et al*, 1999) failed to affect USVs in this study, even when administered in a dose (0.2 mg/kg) beyond that required to inhibit *in vivo* responses to the $5HT_{1A}$ agonist 8-OH-DPAT (Arborelius *et al*, 1999; Johansson *et al*, 1997). Second, the highest dose of propranolol used here (ie, 10 mg/kg) did not inhibit 8-OH-DPAT effects on 5HT release (Sharp *et al*, 1989). Third, the effects of propranolol observed in this study were mimicked by co-administration of selective $\beta1$ and $\beta2$ antagonists (ie, betaxolol and ICI 118,551), neither of which interact significantly with the $5HT_{1A}$ receptor (Middlemiss *et al*, 1985). Finally, our negative finding with nadolol, a non-CNS penetrant β-adrenergic antagonist (Schiff and Saxey, 1984), suggests that propranolol's effects on ultrasonic calling depend on central $\beta1$ and/or $\beta2$ receptors.

Behavioral Considerations

Clonidine and prazosin. Both clonidine and prazosin, when administered alone, inhibited USV emission. An inhibitory effect of high-dose clonidine (ie, 0.1 mg/kg) is consistent with its known sedative effects (Drew *et al*, 1979). The inhibitory effects of lower doses of clonidine (ie, 0.01 and 0.02 mg/kg IP) are perhaps attributable to mild sedation, which has been seen in some (Carey *et al*, 2008; Drew *et al*, 1979; Sara *et al*, 1995) but not in all (De Luca *et al*, 1999; Skolnick *et al*, 1978) studies. Prazosin, in contrast, inhibited 50-kHz calling at doses that are clearly non-sedative (Drouin *et al*, 2002; Vanderschuren *et al*, 2003).

Both clonidine and prazosin dose dependently inhibited AMPH-induced calling, with partial-to-complete block even at low doses. It is unlikely that these drugs produced aversive effects, which might have inhibited 50-kHz calling. Clonidine, for example, is self-administered IV (Davis and Smith, 1977) and induces CPP (Asin and Wirtshafter, 1985; Cervo *et al*, 1993) in rats, whereas prazosin seems motivationally neutral (Forget *et al*, 2009; Zarrindast *et al*, 2002). The inhibitory effect of prazosin is potentially interesting in view of its reported failure to block either the discriminative stimulus effects of AMPH in rats (Arnt, 1996; West *et al*, 1995) or the acquisition of AMPH CPP (Hoffman and Donovan, 1995).

Although clonidine and prazosin, at doses used here, also suppress AMPH-induced locomotion (Drouin *et al*, 2002; Vanderschuren *et al*, 2003), the act of locomotion *per se* does not seem to cause rats to emit ultrasonic calls (Knutson *et al*, 2002).

Cirazoline and atipamezole. The $\alpha1$ agonist cirazoline failed to increase the call rate significantly or modify the call profile, when administered alone. However, cirazoline (0.5. and 1 mg/kg) produced major adverse side effects after injection, most likely due to its action on peripheral $\alpha1$ receptors (Micheletti *et al*, 1987). Thus, it remains unclear whether activation of central $\alpha1$ receptors without the peripheral side effects would elicit 50-kHz USVs. Surprisingly, comparable or even higher doses of cirazoline have been used in several other studies of conscious rats (Alsene *et al*, 2006; Sebban *et al*, 1999; Swerdlow *et al*, 2006).

In contrast, the highly selective $\alpha2$ antagonist atipamezole (Virtanen *et al*, 1989) did not produce any observable changes in behavior. Doses of atipamezole were chosen based on previous studies showing increased extracellular NA levels in the brain (Bondi *et al*, 2010; Wortley *et al*, 1999). The lack of effect of atipamezole on call rate suggests that increased NA release resulting from $\alpha2$ receptor antagonism is not sufficient to elicit USVs. Moreover, the effect of atipamezole on USVs under AMPH suggests that $\alpha2$

receptor inhibition does not affect AMPH-induced call rate, but may modestly contribute to AMPH's ability to modify the call profile.

Propranolol. This study reveals potentially novel psychostimulant effects that are mediated by CNS β-receptors. Propranolol profoundly altered the call profile in rats that were acutely challenged with AMPH. Thus, propranolol suppressed trill calls and promoted flat calls, effectively countering the profile-altering effects exerted by AMPH alone. Additional tests with betaxolol, ICI 118,551, nadolol, and NAD-299 implicated centrally located β-receptors. In contrast, propranolol did not inhibit the AMPH-induced enhancement of call rate, a result that may possibly be related to propranolol's inability to inhibit behavioral stimulant effects of AMPH (Simon *et al*, 1972; Vanderschuren *et al*, 2003). As both USVs and discriminative stimulus (cue) properties have been proposed to model subjective effects of drugs, it is of interest that propranolol antagonized AMPH's effects on call profile (this study) at doses that failed to inhibit the AMPH cue (West *et al*, 1995).

Remarkably, the effects of β-blockers on conventional measures of psychostimulant reward or aversion have received little attention in animals. For example, there seem to be no reports of CPP/aversion testing using propranolol. In an initial study, acute propranolol administration inhibited IV self-administration of AMPH in rats (Yokel and Wise, 1976). In addition, propranolol substantially reduced cocaine IVSA (Harris *et al*, 1996). Thus, in light of these findings, CNS β-adrenergic receptors warrant further attention in the context of psychostimulant reward and aversion.

50-KHz USVs in Relation to Subjective Drug Effects in Humans

In human subjects, there is considerable debate as to the relative importance of dopaminergic and noradrenergic mechanisms in the positive subjective effects of AMPH (Abi-Dargham *et al*, 2003; Brauer and de Wit, 1997; Dlugos *et al*, 2007; Jonsson, 1972; Leyton *et al*, 2007; Lott *et al*, 2005; Nurnberger *et al*, 1984; Rothman *et al*, 2001; Sofuoglu *et al*, 2009). For example, dopaminergic antagonists have failed to reduce psychostimulant euphoria in most studies (Brauer and de Wit, 1995, 1996, 1997; Gawin, 1986; but see Gunne *et al* (1972) and Jonsson (1972)). Moreover, human and animal studies suggest that DA transmission does not contribute to the hedonic impact of psychostimulants, but rather to the incentive salience of reward-related cues (Berridge and Robinson, 1998; Leyton *et al*, 2005, 2007). In contrast, several observations point to possible noradrenergic mediation of AMPH euphoria (Dlugos *et al*, 2007; Rothman *et al*, 2001; Sofuoglu *et al*, 2009); although preliminary studies using α- or β-receptor antagonists have been largely negative, only low antagonist doses were used (Brauer and de Wit, 1995; Jonsson, 1972; Nurnberger *et al*, 1984).

FM 50-kHz calls have been proposed as an index of positive affect in rats (Burgdorf *et al*, 2010). Accordingly, this study confirmed that AMPH selectively promotes trill calls (this study; Wright *et al*, 2010) at doses that are comparable to euphorigenic doses in human studies (Grilly and Loveland, 2001). Propranolol countered this call profile shift. In humans, the impact of β-receptor blockade on the euphoric effect of AMPH has been investigated in only two studies (Jonsson, 1972; Nurnberger *et al*, 1984), to our knowledge. Both studies used propranolol and were ostensibly negative. However, in the first of these, an unusually high dose of AMPH (200 mg, ie, $\sim$3 mg/kg IV) was combined with only moderate doses of propranolol (20 and 40 mg PO). In the second, the dose of AMPH was lower (0.3 mg/kg IV), but subjective effects were inferred only from the subjects' behavior; here, too, it is not clear whether propranolol (0.1 mg/kg IV) was administered in a sufficiently high dose. Therefore, our preclinical findings suggest that CNS β-receptor mechanisms would merit further study in humans under AMPH challenge.

Possible (Nor)Adrenaline–DA Interactions

USV emission by adult rats is not only influenced by (nor)adrenergic mechanisms (this study) but is also strongly DA dependent (see the 'Introduction' section). These neurotransmitter systems are extensively coupled (for review, see Weinshenker and Schroeder (2007)); for example, a number of studies have shown a critical role of noradrenergic transmission in AMPH-induced mesoaccumbens DA release (Darracq *et al*, 1998; Pan *et al*, 1996). However, it seems unlikely that clonidine, prazosin, or propranolol interfered with dopaminergic agonist actions of AMPH in this study. For example, prazosin (0.5 mg/kg IP) did not affect extracellular DA in the nucleus accumbens after systemic AMPH administration (Darracq *et al*, 1998). Similarly, clonidine failed to alter AMPH-induced extracellular DA levels (Florin *et al*, 1994; Tanda *et al*, 1996). Finally, propranolol administration did not inhibit several DA-dependent behavioral effects of AMPH, ie, locomotor stimulation (Simon *et al*, 1972; Vanderschuren *et al*, 2003), stereotypy (Simon *et al*, 1972), and cue properties (West *et al*, 1995).

Generalization to IV Cocaine and AMPH

Acute IP cocaine administration reportedly increases 50-kHz call rate (Williams and Undieh, 2010). Previous studies using IV cocaine have been performed in the context of self-administration (and its anticipation) and sensitization (Barker *et al*, 2010; Browning *et al*, 2011; Ma *et al*, 2010; Maier *et al*, 2010). Here, we provide the first report of the effects of non-contingent IV administration of cocaine on USVs. Cocaine increased the call rate at the 0.75 mg/kg dose, with a rapid onset (peak effect 120–150 s after infusion). Although this USV rate-enhancing effect of cocaine was less pronounced than that of AMPH, cocaine nevertheless produced a profound AMPH-like shift in the call profile at all doses tested (ie, 0.25–1.5 mg/kg). In this dose range, cocaine maintains self-administration (Roberts *et al*, 2007) and induces CPP (Nomikos and Spyraki, 1988; Sellings *et al*, 2006), but is also anxiogenic (Ettenberg, 2004); how these effects may relate to USV emission merits further investigation.

Information Gained from 50-kHz Call Subtype Analysis *vs* Call Rate

Several findings of this study highlight the importance of detailed call subtype analysis. First, both cocaine and

propranolol changed the propensity to emit different call subtypes at doses that did not significantly change the call rate. These results add to evidence that call rate and profile can be manipulated independently by drugs or lesions (Ciucci *et al*, 2009, 2007). Moreover, although several groups currently distinguish between FM and flat 50-kHz calls (Ahrens *et al*, 2009; Burgdorf *et al*, 2007, 2008a; Burgdorf and Panksepp, 2006; Simola *et al*, 2009; Wohr *et al*, 2008), only a few investigators have extended their analysis beyond those two classes (Ciucci *et al*, 2009; Kaltwasser, 1990; Takahashi *et al*, 2010; Vivian and Miczek, 1993; White *et al*, 1990; Wright *et al*, 2010). Importantly, our detailed analysis reveals that the prevalent trill call subtype (Wright *et al*, 2010) is not representative of all FM calls.

Human psychostimulant abusers cannot readily discriminate between cocaine and AMPH (Fischman *et al*, 1976). In this study, cocaine affected the FM call rate less than AMPH, yet produced an equivalent shift in the call profile (ie, preferentially promoting trills over flat calls). Therefore, insofar as FM 50-kHz calls convey information about positive affect in rats (as proposed by Burgdorf *et al* (2010)), the call profile might be more pertinent than the absolute FM call rate.

Limitations and Methodological Considerations

Adult rats vary considerably in their USV response to various stimuli including systemic AMPH (Burgdorf and Panksepp, 2006; Schwarting *et al*, 2007; Wohr *et al*, 2008; Wright *et al*, 2010). To study the effects of drugs on AMPH-induced calling, it was necessary to exclude low responders based on an initial test screen. However, it is important to bear in mind that low- and high-calling rats may differ in other behavioral or neurochemical respects (Burgdorf *et al*, 2008b). The test screen likely explains why we did not subsequently observe sensitization with repeated exposure to AMPH during the experiment, as USV sensitization seems to occur mainly within the first three exposures to cocaine or AMPH (Ahrens *et al*, 2009; Meyer *et al*, 2011; Mu *et al*, 2009).

These findings strongly indicate a (nor)adrenergic role in AMPH-induced 50-kHz USVs. However, the evidence for α1 receptor mediation rests on the use of a single drug—prazosin. Although prazosin is a well-characterized and selective α1 receptor antagonist (see above), it would have been desirable to test other drugs of the same class. However, other currently available α1 antagonists are either less α1-selective (eg, phentolamine), α1 subtype selective (eg, tamsulosin), brain impenetrant (eg, doxazosin), or little characterized in the rat (eg, HEAT).

In this study, only prazosin significantly inhibited 50-kHz calling when administered alone. However, rates of spontaneous calling were generally low, making it hard to detect potential suppressive effects of other drugs. To determine whether noradrenergic transmission has a wider role in USV production, it would be informative to test these drugs in combination with non-pharmacological stimuli that evoke high rates of 50-kHz calling (Ciucci *et al*, 2007; Panksepp and Burgdorf, 2000).

Conclusions

These findings provide the first evidence of (nor)adrenergic involvement in the elicitation of adult rat 50-kHz USVs by AMPH. Furthermore, USV emission seems to be differentially associated with α1- *vs* β-receptor mechanisms, whereby (nor)adrenergic transmission through α1 receptors principally modulates the call rate, whereas NA (or adrenaline) acting on β-receptors affects the acoustic subtypes of 50-kHz calls emitted.

In the context of drug addiction, psychostimulants reinforce self-administration behavior and acutely promote positive affect. At present, it is not clear how these two effects are related. Dopaminergic transmission in the brain seems critical to motivation, but has not been convincingly linked to psychostimulant euphoria. Preliminary evidence points to a noradrenergic contribution to euphorigenic effects of AMPH, but receptor mechanisms have not been identified. These findings suggest that CNS β-adrenergic receptors merit further attention in this regard.

ACKNOWLEDGEMENTS

This research was supported by a Natural Science and Engineering Research Council of Canada discovery grant (155055 to PBSC) and a Canadian Institutes of Health Research of Canada operating grant (MOP-10516 to PBSC). We thank Annie Constantin for performing IV catheterization surgeries and Tina Scardochio for constructive comments on the manuscript. JMW holds a NSERC PGSD.

DISCLOSURE

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)

Psychopharmacology (2012) 224:477–487
DOI 10.1007/s00213-012-2776-z

ORIGINAL INVESTIGATION

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

Jennifer M. Wright · Lan Deng · Paul B. S. Clarke

Received: 14 February 2012 / Accepted: 9 June 2012 / Published online: 3 July 2012

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

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

Electronic supplementary material The online version of this article (doi:10.1007/s00213-012-2776-z) contains supplementary material, which is available to authorized users.

J. M. Wright · L. Deng · P. B. S. Clarke (✉)
Department of Pharmacology and Therapeutics, McGill University,
McIntyre Medical Building Rm. 1320,
3655 Promenade Sir William Osler,
Montreal, QC H3G 1Y6, Canada
e-mail: paul.clarke@mcgill.ca

Introduction

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

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

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

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

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

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

Methods

Subjects

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

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

Locomotor activity and CPP apparatus

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

Acoustic data acquisition

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

Drugs

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

Experimental protocol

Experiment 1

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

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

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

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

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

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

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

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

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

Analysis and classification of ultrasonic vocalizations

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

Data analysis and statistics

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

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

Results

Experiment 1

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

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

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

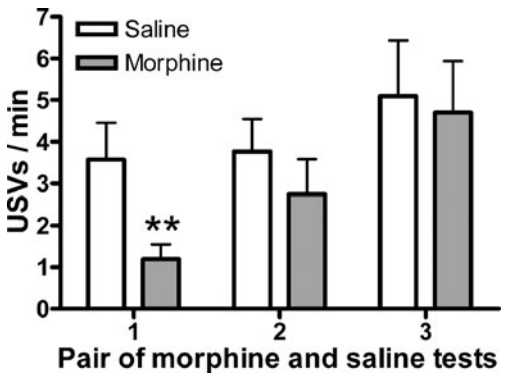

Fig. 1 Experiment 1.1: morphine (1 mg/kg, SC) initially suppressed 50-kHz calling, but rats became tolerant to this effect with repeated testing. The y-axis represents mean + SEM 50-kHz USVs per minute under saline (*open bars*) and 1 mg/kg morphine (*gray bars*). **p < 0.01 (n=24)

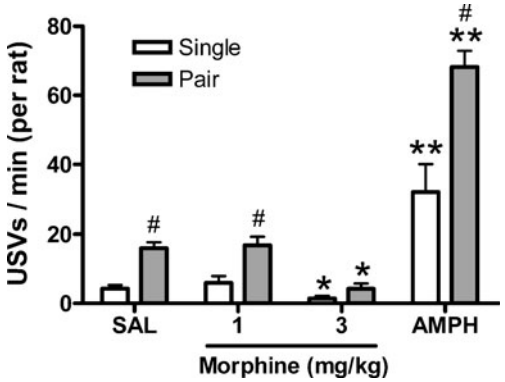

Fig. 2 Experiment 1.2: morphine dose-dependently inhibited calling in both singly- (*open bars*) and pair-tested (*gray bars*) rats. The y-axis represents mean+SEM USVs per minute (on a per rat basis). Each rat was tested under all eight conditions (n=11 rat pairs). *$p < 0.05$, **$p <$ 0.01 vs. respective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

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

Experiment 2

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

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

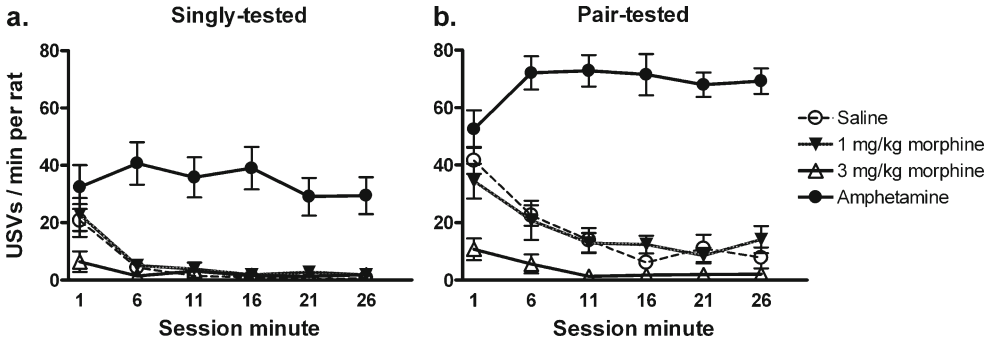

Fig. 3 Experiment 1.2: time course of USV emission following saline (*open circles*), morphine (1 or 3 mg/kg, SC; *downward/solid* and *upward/open triangles*, respectively) or AMPH (1 mg/kg, IP; *solid circle*) when rats were singly tested (**a**) and pair-tested (**b**). The *y*-axes represent mean+SEM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

Experiment 3

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

Analysis of USV subtypes

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

Discussion

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

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

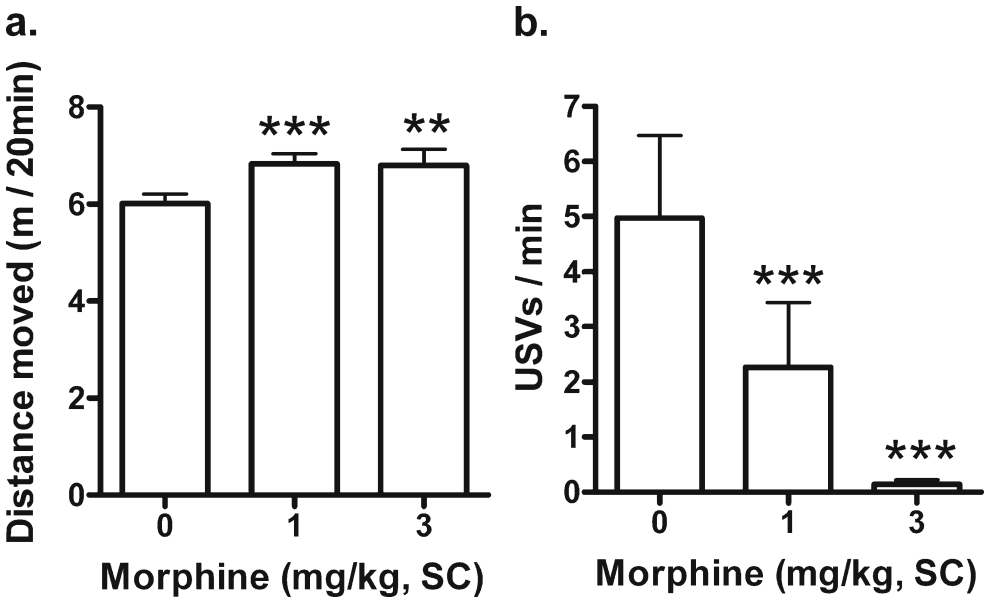

Fig. 4 Experiment 1.3: morphine (1 and 3 mg/kg, SC) significantly increased locomotor activity (**a**), while dose-dependently decreasing emission of 50-kHz calls (**b**). Each rat was tested under all conditions (n=24), **$p < 0.01$, ***$p < 0.001$ vs. saline condition

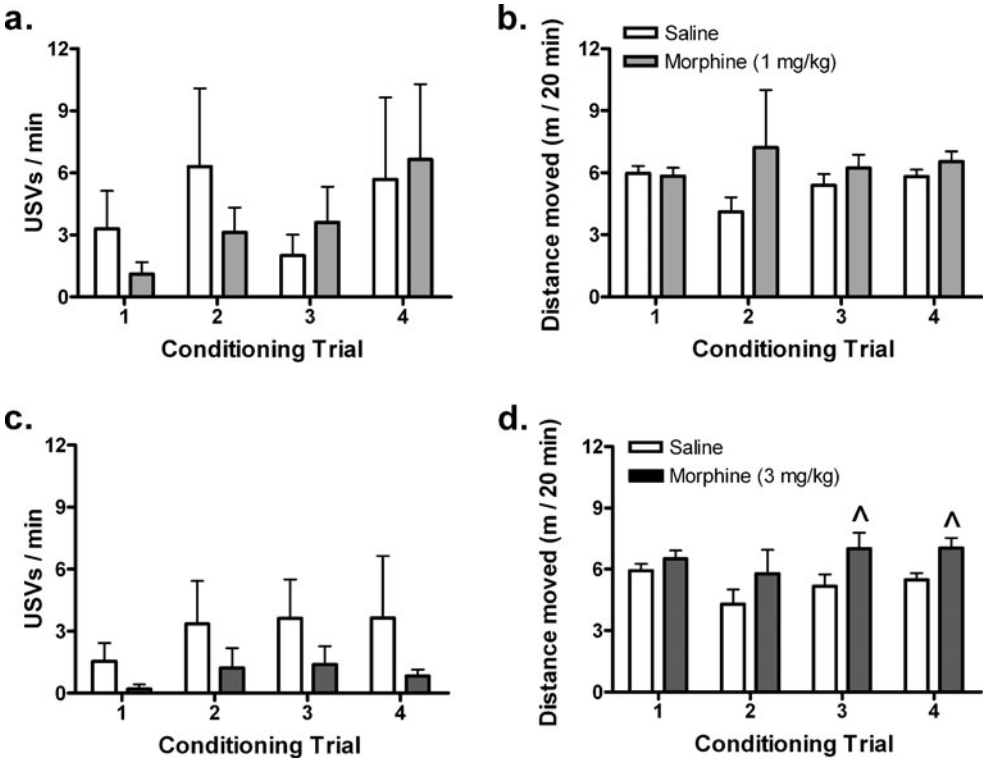

Fig. 5 Experiment 2: call rate was unaffected during morphine conditioning. Each rat received saline (*open bars*) and morphine, given at 1 mg/kg (**a**, **b**) (n=12, *light gray bars*) or 3 mg/kg (**c**, **d**) (n=12, *dark gray bars*). Locomotor activity during conditioning with 1 mg/kg (**b**) and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

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

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

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

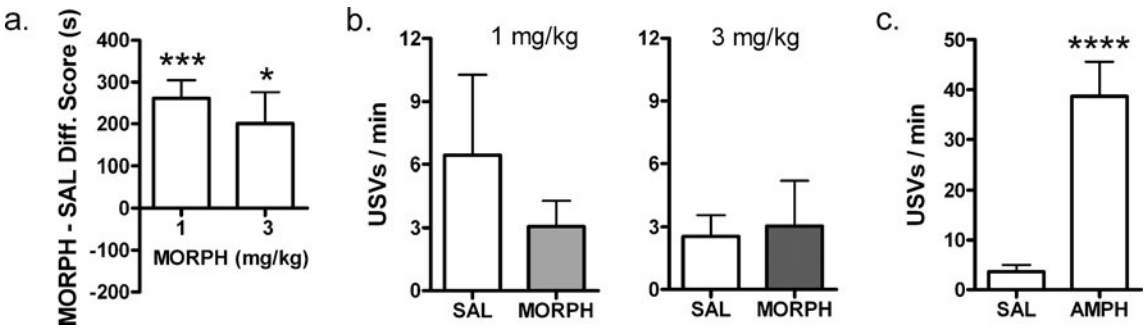

Fig. 6 Experiment 2: post-conditioning and AMPH tests. **a** On the CPP test day (day 10), both doses of morphine produced a significant place preference. **b** On days 11 and 12, there was no significant difference in USV emission when rats were confined and when drug-free to the morphine-paired vs.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

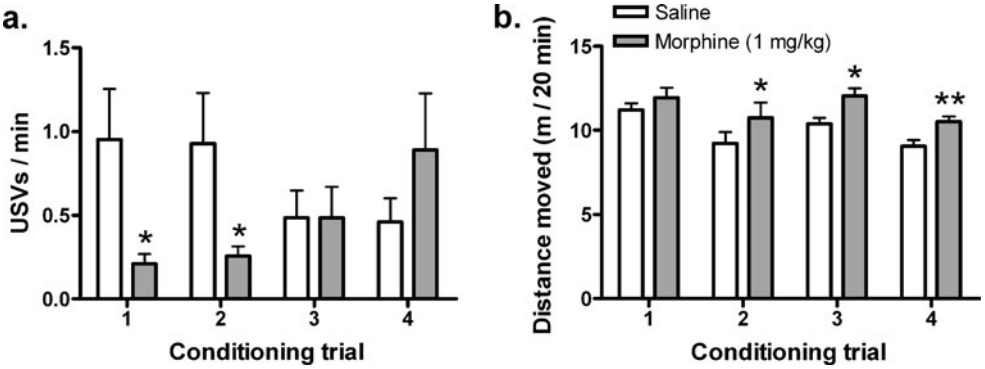

Fig. 7 Experiment 3: during conditioning trials, morphine initially decreased the call rate (**a**). However, morphine significantly increased locomotor activity during the last three trials (**b**). *$p < 0.05$, **$p < 0.01$ ($n=16$)

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

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

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

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

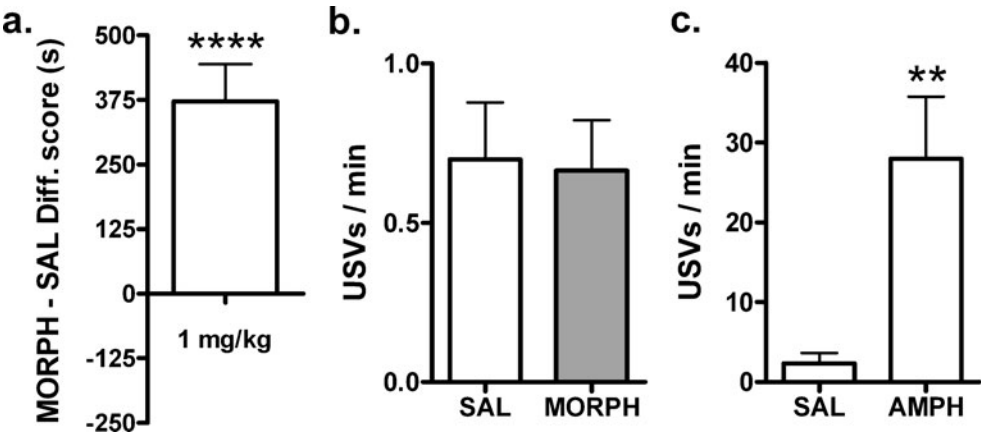

Fig. 8 Experiment 3: **a** On the CPP test day, morphine produced a significant place preference. **b** There was no significant difference in USV emission when rats were on the morphine- vs. saline-paired floor textures during the CPP test (*open and gray bars*, respectively, mean+SEM USVs per minute).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

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

Limitations

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

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

Conclusion

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

Acknowledgments This study was supported by a Natural Science and Engineering Research Council of Canada (NSERC) discovery grant (155055, to P.B.S.C), and an NSERC Postgraduate Scholarship D (to J.M.W). The authors thank Tina Scardochio for constructive comments on the manuscript. P.B.S.C. is a member of the Center for studies in Behavioral Neurobiology at Concordia University in Montreal. The authors have no financial relationship with the organizations that sponsored this research. All experiments comply with the current laws of Canada.

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Psychopharmacology (2013) 225:853–868
DOI 10.1007/s00213-012-2871-1

ORIGINAL INVESTIGATION

The role of dopaminergic transmission through D1-like and D2-like receptors in amphetamine-induced rat ultrasonic vocalizations

Jennifer M. Wright · May R. S. Dobosiewicz · Paul B. S. Clarke

Received: 20 July 2012 / Accepted: 2 September 2012 / Published online: 28 September 2012

Abstract
Rationale Systemic amphetamine (AMPH) administration increases the rate of 50-kHz ultrasonic vocalizations (USVs) in adult rats and preferentially enhances the 'trill' subtype; these effects of AMPH critically depend on noradrenergic transmission, but the possible contributions of dopamine are unclear.
Objective To assess the role of dopamine in 50-kHz USVs emitted drug-free and following systemic AMPH administration.
Methods Adult male Long–Evans rats pre-selected for high AMPH-induced calling rates were tested with AMPH (1 mg/kg, intraperitoneal (IP)) and saline following pretreatment with the following dopamine receptor antagonists: SCH 23390 (0.005–0.02 mg/kg, subcutaneous (SC)), SCH 39166 (0.03–0.3 mg/kg, SC), haloperidol (0.1, 0.2 mg/kg, IP), sulpiride (20–80 mg/kg, SC), raclopride (0.1–0.5 mg/kg, SC), clozapine (4 mg/kg, SC), risperidone (0.5 mg/kg, SC), and pimozide (1 mg/kg, IP). The dopamine and noradrenaline reuptake inhibitors (GBR 12909 and nisoxetine, respectively) were also tested, alone and in combination.
Results SCH 23390, SCH 39166, haloperidol, and raclopride dose-dependently inhibited vocalizations under AMPH and suppressed the proportion of trill calls. Sulpiride, however, had no discernable effect on call rate or profile, even at a high dose that reduced locomotor activity. Single doses of clozapine, risperidone, and pimozide all markedly decreased calling under saline and AMPH. Finally, GBR 12909 and nisoxetine failed to promote 50-kHz USVs detectably or alter the subtype profile, when tested alone or in combination.
Conclusions The rate of 50-kHz USVs and the call subtype profile following systemic AMPH administration depends on dopaminergic neurotransmission through D1-like and D2-like receptors. However, inhibiting dopamine and/or noradrenaline reuptake appears insufficient to induce calling.

Keywords Ultrasonic vocalization · Amphetamine · Dopamine · Noradrenaline · Atypical antipsychotic · Dose–response · D1 receptor · D2 receptor · Hedonia · Affect

Introduction

Higher-frequency ultrasonic vocalizations (USVs) emitted by adult laboratory rats, generally termed "50-kHz calls" (for review, see Brudzynski 2009; Wohr and Schwarting 2010), are frequently associated with appetitive stimuli (Burgdorf et al. 2010; Knutson et al. 2002) and have been proposed to reflect positive affect (Brudzynski 2007; Burgdorf and Moskal 2009; Burgdorf et al. 2010). However, 50-kHz USVs are acoustically diverse, with many identified subtypes including flat (i.e., constant frequency) calls and at least 12 types of frequency-modulated (FM) calls (Wright et al. 2010). The relative prevalence of the different call subtypes, which we have termed the "call profile" (Wright et al. 2010), can be experimentally modified independently of the

Electronic supplementary material The online version of this article (doi:10.1007/s00213-012-2871-1) contains supplementary material, which is available to authorized users.

J. M. Wright · M. R. S. Dobosiewicz · P. B. S. Clarke (✉)
Department of Pharmacology and Therapeutics,
McGill University,
McIntyre Medical Building Rm. 1320 3655 Promenade Sir William Osler,
Montreal, QC H3G 1Y6, Canada
e-mail: paul.clarke@mcgill.ca

overall rate of 50-kHz call emission (Ciucci et al. 2007, 2009; Wright et al. 2012b).

Dopaminergic (DAergic) neurotransmission appears to play a key role in USV emission. Notably, acute systemic injection of the dopamine (DA) agonist apomorphine promoted 50-kHz calls (Williams and Undieh 2010), and intra-accumbens administration of the D_2/D_3 agonist quinpirole modulated USV production in a dose-related triphasic fashion (Brudzynski et al. 2012). Conversely, DA receptor antagonists are reported to inhibit 50-kHz USVs elicited by several natural and artificial rewarding stimuli, namely systemic cocaine (Williams and Undieh 2010), intracerebral amphetamine (AMPH) and glutamate (Thompson et al. 2006; Wintink and Brudzynski 2001), tickling (Burgdorf et al. 2007), electrical brain stimulation (Burgdorf et al. 2007), and copulation-related contexts (Bialy et al. 2010; Ciucci et al. 2007, 2009).

The psychostimulant AMPH, which enhances both DAergic and noradrenergic transmission (McKittrick and Abercrombie 2007), exerts two principal effects on 50-kHz vocalizations: It increases the overall call rate (Ahrens et al. 2009; Simola et al. 2009; Wintink and Brudzynski 2001; Wright et al. 2010, 2012b), and in relative terms, it shifts the "call profile," thereby enhancing the trill subtype while suppressing flat calls (Wright et al. 2010, 2012b). These rate-enhancing and call profile-altering effects of AMPH are critically dependent on α_1 and β adrenergic receptor function, respectively (Wright et al. 2012b). To our knowledge, however, it has not been determined whether the effects of systemic AMPH administration on 50-kHz USV emission are also dependent on DAergic transmission.

The first main aim of the present study was therefore to test the hypothesis that DAergic neurotransmission is required for 50-kHz calls that are emitted when tested drug-free or following systemic AMPH administration. The second, related, aim was to determine whether either D1-like or D2-like DA receptors (Le Foll et al. 2009) play a role. These questions were addressed in Experiments 1–7, in which we tested the effects of acute pretreatment with several D1- or D2-like DA receptor antagonists in combination with systemic saline or AMPH challenge (see Table 1). During testing, it emerged that the atypical antipsychotic drug sulpiride (Rama Rao et al. 1981) did not inhibit AMPH-induced calling, in striking contrast to two classical D2 antagonists (i.e., haloperidol and raclopride). Therefore, as a third aim, we assessed whether sulpiride's lack of effect reflected its atypical antipsychotic profile, by testing two other atypical neuroleptic drugs (clozapine and risperidone) and one additional classical D2 antagonist (pimozide). We also recorded USVs and locomotor activity simultaneously (Experiment 7), in order to confirm that sulpiride was behaviorally active, despite its failure to influence 50-kHz calling.

A final aim was to address whether enhancing DA or noradrenaline (NA) transmission is *sufficient* to induce 50-kHz USVs or affect the call profile (Experiments 8–10—see Table 1). To this end, rats were acutely challenged with the selective DAT inhibitor GBR 12909 and the selective NET inhibitor nisoxetine, given alone and in combination.

Methods

Subjects

Subjects were 114 male Long–Evans rats (Charles River Laboratories, St Constant, Quebec, Canada), weighing 376 ±50 g (mean±SD) at the start of the experiment. They were housed two or three per cage ($25\times48\times20$ cm^3) in a temperature- and humidity-controlled colony room (19–20 °C, 50–60 %) at the McGill University Animal Research Center. Rats were maintained on a reverse 12:12 light/dark cycle, with lights off at 0700 h. All behavioral testing took place during the dark phase of the cycle. Food and water were available ad libitum, except during testing sessions. In all experiments, rats were initially drug- and experimentally naïve, with the following exceptions: In Experiments 3 and 4, rats had received four prior systemic injections of AMPH (0.25, 0.5, 1, and 2 mg/kg, IP), and in Experiment 7, rats had received four prior administrations of morphine (1 mg/kg, SC). All procedures were approved by the McGill Animal Care Committee in accordance with the guidelines of the Canadian Council on Animal Care.

Overview of experiments

Ten experiments were performed, as summarized in Table 1. Briefly, Experiments 1–7 tested the effects of antagonist pretreatment on the USV response (i.e., call rate and subtype profile) to systemic AMPH. Experiment 7 additionally examined locomotor activity during the USV recording. The acute USV responses to the DA and NA reuptake inhibitors (i.e., GBR 12909 and nisoxetine), given alone or in combination, were examined in Experiments 8–10.

Experimental protocol

AMPH screen A significant minority of rats emit few calls in response to systemic AMPH (Wright et al. 2010). Therefore, subjects in most experiments were initially screened for AMPH-induced calling. Exceptionally, in order to reduce pre-experiment drug exposure, subjects in Experiments 3, 4, and 7 were not screened since they had already received prior AMPH or morphine administration (see above). The AMPH screening method was as described previously (see Wright et al. 2012b for further details). Briefly, rats

Table 1 Summary of experiments

Experiment	Pretreatment	Doses, mg/kg	Route	Time before saline/AMPH, min	*n*
1	SCH23390	0.005, 0.01, 0.02	SC	20	10
2	SCH39166	0.03, 0.1, 0.3	SC	30	12
3	Haloperidol	0.1, 0.2	IP	60	12
4	Sulpiride	20, 40	SC	60	12
5	Raclopride	0.1, 0.2, 0.5	SC	30	12
	Sulpiride	40, 80	SC	30	
6	Clozapine	4	SC	30	12
	Risperidone	0.5	SC	30	
	Pimozide	1	IP	30	
7	Sulpiride	80	SC	30	16
Experiment	Drug	Doses, mg/kg	Route	Time before testing, min	*n*
8	GBR 12909	5, 10, 20	IP	20	8
9	Nisoxetine	4, 8, 16	IP	15	8
10	GBR 12909	10	IP	20	12
	Nisoxetine	12	IP	15	

received three administrations of AMPH (1 mg/kg, IP) spaced 2 days apart; rats with the lowest rate of calling on the third AMPH test were excluded from subsequent testing. Only the third AMPH test session was analyzed because the first two sessions are not necessarily indicative of a rat's subsequent USV response to AMPH (unpublished observation). In total, 52 rats (out of 126 rats that underwent screening) were excluded on this basis.

Drug testing All experiments featured a fully parametric within-subject design, whereby each rat was tested once under each drug/dose condition (see Table 1 for details). Thus, in Experiments 1–7, rats received all combinations of pretreatment and treatment drugs including all vehicle controls. After the pretreatment time interval had elapsed, each rat was injected with saline or AMPH (1 mg/kg, IP) and immediately placed in a test chamber and recorded for 20 min. Similarly, in Experiments 8–10, every rat was tested under the following conditions: vehicle, AMPH (1 mg/kg—positive control), and each dose of the drug(s) being tested. Here, recording sessions were of 20-min duration except for the GBR 12909 dose–response study (Experiment 8), where rats were tested for 40 min. Within each experiment, the order of testing was counterbalanced as far as possible given the number of subjects. Test sessions were always spaced 2 days apart in order to minimize possible carry-over effects of the drugs.

Drugs

All test drugs, doses, routes of administration, and pretreatment/treatment time intervals are shown in Table 1. Drugs were: D-amphetamine sulfate (Sigma-Aldrich, Poole, UK); haloperidol and S(−)-sulpiride (both from Sigma-Aldrich, St. Louis, MO); pimozide, R(+)-SCH-23390 HCl, SCH 39166 HBr (i.e., Ecopipam), raclopride, and risperidone (all from Tocris Bioscience, Ellisville, MO); clozapine, GBR 12909 2HCl, and (±)-nisoxetine HCl (all from the NIMH Chemical Synthesis and Drug Supply Program). Doses of the different compounds refer to the form indicated above. GBR 12909 was administered in a volume of 2 ml/kg; all other drugs were administered in a volume of 1 ml/kg. Sulpiride was dissolved in a few drops of glacial acetic acid and diluted with sterile saline. Clozapine, GBR 12909, haloperidol, pimozide, and risperidone were dissolved in a 0.1 M tartaric acid solution. All other drugs were dissolved in sterile saline. Drug vehicles were used for control injections. The pH of GBR 12909 could not be raised beyond 4.5 (with NaOH) without precipitation. In case the lower pH affected call emission, each rat was tested twice with AMPH in Experiment 10, once with the standard drug solution and once with the same solution acidified with HCl to pH 4.5. Since there was no difference in call rate or profile between the two AMPH tests, data from these tests were pooled for the remainder of the analysis.

Behavioral recording

USV recordings were conducted as previously described (Wright et al. 2012b). With the exception of Experiment 7 (see below), recordings took place in four clear Plexiglas experimental chambers (ENV-007CT, Med Associates, St Albans, VT), each of which was enclosed in a melamine compartment lined with sound-attenuating acoustic foam (Primacoustic, Port Coquitlam, British Columbia). A condenser ultrasound microphone (CM16/CMPA, Avisoft Bioacoustics, Berlin, Germany) was securely inserted through a small (5-cm diameter) hole located centrally in the top panel

of each experimental chamber. Consequently, the microphones were 15–30 cm from rats during testing. Microphone signals were fed into an UltraSoundGate 416 H data acquisition device (Avisoft Bioacoustics) with a sampling rate of 250-kHz and 16-bit resolution.

For Experiment 7, USV recordings were made in rectangular, open-topped chambers (58 cm long×29 cm wide× 53 cm high) to allow simultaneous recording of USVs and locomotor activity, as previously described (Wright et al. 2012a). Two ultrasound microphones were secured inside each chamber at opposite corners, approximately 10 cm from the top (i.e., 40 cm above the floor). Sound-attenuating acoustic foam enveloped the walls and extended 20 cm above the top of each chamber. A video tracking system (EthoVision v 3.0, Noldus Information Technology, Leesburg, VA, USA) measured locomotor activity (expressed as the total horizontal distance moved) during the second half (i.e., min 11–20) of the session to allow AMPH to take effect.

All lights were off during behavioral testing, except for Experiment 7, where far-red (wavelength>650 nm) illumination using a Kodak GBX-2 safelight filter (Vistek, Toronto, Ontario, Canada) provided darkroom lighting.

Analysis and classification of ultrasonic vocalizations

Acoustical analysis was performed using Avisoft SASLab Pro (version 5.1, Avisoft Bioacoustics), as previously described (Wright et al. 2012b). Calls were selected manually from spectrograms by an individual who was masked to the treatment condition. Each identified 50-kHz call was classified into 1 of 14 distinct categories: complex, upward ramp, downward ramp, flat, short, split, step-up, step-down, multi-step, trill, flat–trill combination, trill with jumps, or composite (see Wright et al. (2010) for criteria for call identification and classification, several examples of each call type, as well as descriptive statistics relating to acoustic parameters). A few representative 50-kHz calls are shown in Fig. 1. This method of manual call selection has been validated by surgical devocalization, and classification is associated with high inter- and intra-rater reliability (Wright et al. 2010). The 22-kHz calls were not analyzed since they were rarely observed in this study (specifically, one rat made two calls under sulpiride 40 mg/kg plus AMPH 1 mg/kg and another rat made 20 calls under sulpiride 80 mg/kg plus AMPH 1 mg/kg).

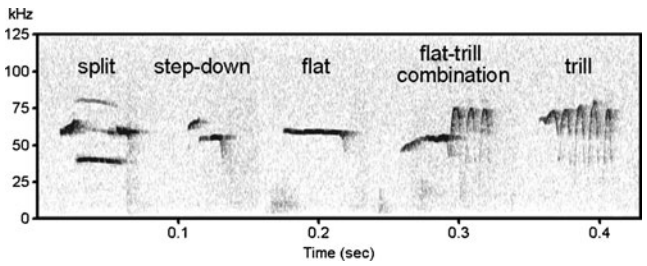

Fig. 1 Spectrogram containing individual 50-kHz calls representative of the following subtypes (*left to right*): split, step-down, flat, flat-trill combination, and trill. See Wright et al. (2010) for additional examples of all fourteen 50-kHz call subtypes

Data analysis and statistics

Data were analyzed using commercial software (Systat v11, SPSS, Chicago, IL; GraphPad Prism 4, GraphPad Software, La Jolla, CA). For Experiments 1–7, USVs that occurred during minutes 12, 14, and 16 of the 20-min session were counted and classified. These minutes were chosen since AMPH-induced calling becomes most pronounced within the 10–20 min time interval following AMPH administration (Wright et al. 2010). In Experiment 8, data throughout the entire 40 min session were analyzed. Finally, for Experiments 9 and 10, USV analysis was performed for minutes 3, 8, 13, and 18 of the 20-min session (i.e., we chose 4 min of time-sampling and spread it evenly across the session). One rat was removed for the call *subtype* analysis in Experiment 2 (SCH 39166) because it only emitted one call at the highest dose, making it an extreme outlier when evaluating the percentage data. Repeated-measures ANOVA was performed to determine the effect of the within-subjects factors "pretreatment" and "treatment," where appropriate. Pairwise comparisons were performed using paired *t*tests or Wilcoxon tests; the choice of test depended on the distribution of the raw data. ANOVA *p* values were subject to the Huynh–Feldt correction, where appropriate. Multiple comparisons relating to the call rate data were subject to Holm–Bonferroni corrections, except where stated. However, for the call subtype analysis, pairwise comparisons were performed using uncorrected tests, in order to maintain statistical power. For all analyses, a two-tailed p value<5 % (after any correction) was considered significant.

Results

Note that statistically significant results were found for certain of the less frequent call subtypes, but they were not consistently observed across doses or drugs of the same class and are likely to be false-positives; hence, these results are not reported here.

Experiments 1 and 2: effects of the D1 antagonists SCH 23390 and SCH 39166

As expected, AMPH given alone (i.e., with vehicle pretreatment) greatly increased the rate of 50-kHz calling (Wilcoxon Z=2.80 and 3.06, both p<0.01; Fig. 2a, b). The call rate under

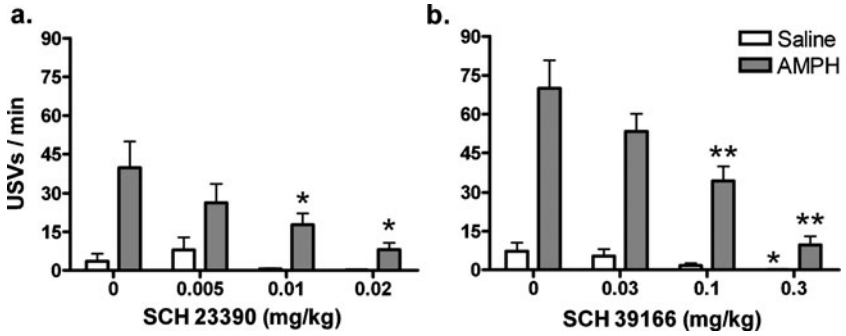

Fig. 2 Experiments 1 and 2: The D_1 antagonists SCH 23390 (**a**) and SCH 39166 (**b**) dose-dependently inhibited the call rate under AMPH. The *y* axes represent mean+SEM calls/min. Each rat was tested under all pretreatment/treatment conditions (SCH 23390 group $n=10$; SCH 39166 group $n=12$). SCH 39166 alone also decreased the call rate at the highest dose tested (i.e., 0.3 mg/kg). *$p<0.05$, **$p<0.01$ versus corresponding vehicle pretreatment condition

AMPH was dose-dependently reduced by both SCH 23390 and SCH 39166, with significant effects at the two higher doses (SCH 23390, Wilcoxon $Z=2.70$ and 2.70, $p<0.05$; SCH 39166, Wilcoxon $Z=2.90$ and 3.06, $p<0.01$; Fig. 2). Each antagonist, given alone, tended to suppress calling below the already-low baseline call rate, but a statistically significant inhibitory effect only occurred at the highest dose of SCH 39166 (Wilcoxon $Z=2.80$, $p<0.05$; Fig. 2b).

Higher doses of the D1-like antagonists also significantly affected the call profile. More specifically, the proportion of trill calls under AMPH was dose-dependently suppressed by both SCH 23390 (0.01 and 0.02 mg/kg versus vehicle, Wilcoxon $Z=2.29$ and 2.19, $p<0.05$; Fig. 3a) and SCH 39166 (0.3 mg/kg versus vehicle, Wilcoxon $Z=2.52$, $p<0.05$; Fig. 3c). In addition, SCH 39166 significantly enhanced the proportion of flat calls under AMPH at the highest dose tested (i.e., 0.3 mg/kg) (Wilcoxon $Z=2.38$, $p<0.05$; Fig. 3d). Although the proportion of flat calls appeared to be enhanced by SCH 23390, this failed to reach statistical significance (Fig. 3b). No other call subtype was significantly altered.

Experiments 3–5: effects of the D_2 antagonists haloperidol, sulpiride, and raclopride

Call rate under AMPH Haloperidol, at both doses tested (0.1 and 0.2 mg/kg), significantly inhibited calling following AMPH administration (respectively, Wilcoxon $Z=2.31$, $p<0.05$, and 3.06, $p<0.01$; Fig. 4a); sulpiride (20 and 40 mg/kg), in contrast, had no effect (Fig. 4b). Sulpiride was tested again at a higher dose (Experiment 5), this time in parallel with raclopride (Fig. 4c). Sulpiride again failed to affect the rate of calling after AMPH treatment, whereas raclopride behaved similarly to haloperidol, inhibiting 50-kHz calling at all doses tested (Wilcoxon $Z=3.06$, 2.98, and 3.06, $p<0.01$; Fig. 4c).

Call rate after antagonist alone Haloperidol did not alter the call rate after saline challenge (Fig. 4a); here, however, control call rates were very low (i.e., <3 calls/min). Sulpiride, tested alone, significantly reduced calling at only one dose (40 mg/kg Wilcoxon $Z=2.28$, $p<0.05$; Fig. 4c), and this apparent effect was not replicated across experiments

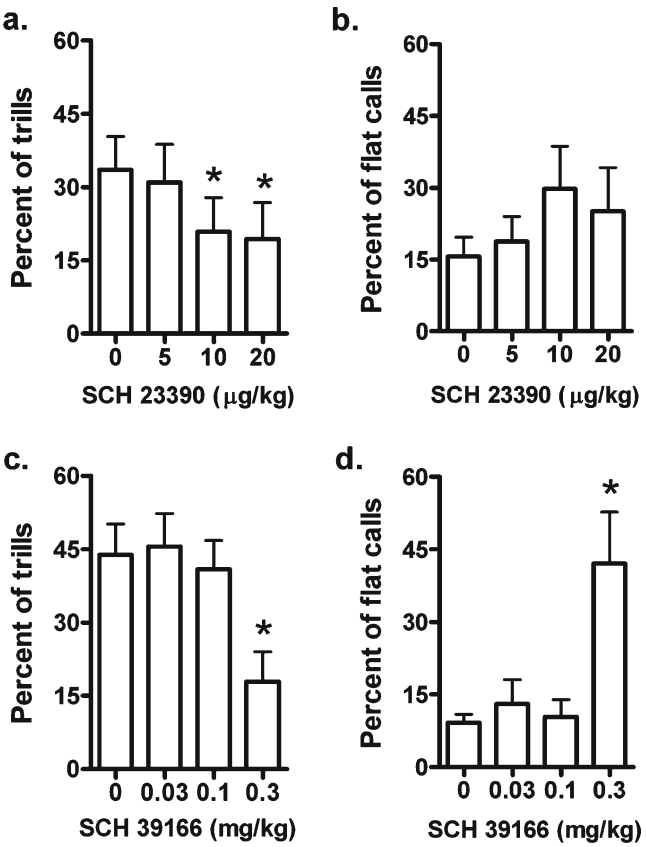

Fig. 3 Experiments 1 and 2: Pretreatment with the D_1 antagonists SCH 23390 (**a**) and SCH 39166 (**c**) before AMPH dose-dependently reduced the percent of trill calls. SCH 39166 also increased the percent of flat calls at the highest dose tested (0.3 mg/kg) (**d**). The apparent increase in the percent of flat calls with SCH 23390 was statistically non-significant (**b**). Each rat was tested under all pretreatment/treatment conditions (SCH 23390 group $n=10$; SCH 39166 group $n=12$). *$p<0.05$ versus vehicle control

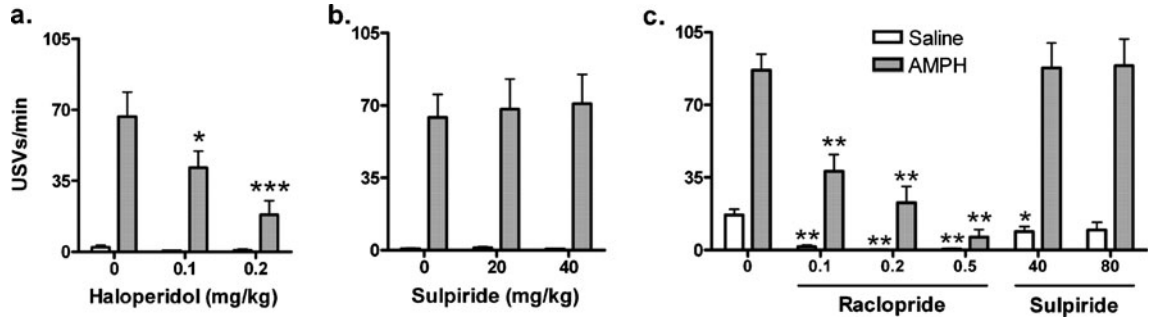

Fig. 4 Experiments 3–5: Haloperidol (**a**) and raclopride (**c**) dose-dependently inhibited USV emission under AMPH (*grey bars*) at all doses tested, while sulpiride (**b**, **c**) was ineffective. Raclopride (**c**) also reduced the call rate following saline treatment (*open bars*). Sulpiride only modestly reduced the drug-free call rate at 40 mg/kg in Experiment 5 (**c**). Each rat was tested under all pretreatment/treatment conditions (n=12 rats per experiment). *p<0.05, **p<0.01, ***p<0.001 versus corresponding vehicle pretreatment

(i.e., Experiment 4 versus 5; see Fig. 4b versus c). In contrast, raclopride tested alone significantly inhibited the call rate at all doses tested (0.1, 0.2, and 0.5 mg/kg versus vehicle, Wilcoxon Z=2.85, 3.06, and 3.06, p<0.01; Fig. 4c).

Call profile Haloperidol and raclopride dose-dependently suppressed the proportion of trill calls following AMPH challenge (haloperidol 0.2 mg/kg versus vehicle, Wilcoxon Z=2.51, p<0.05; raclopride 0.5 mg/kg versus vehicle, Wilcoxon Z=2.1, p<0.05; Fig. 5a, c). This effect appeared less potent than the rate-inhibiting effect (Fig. 4a, c). Raclopride (0.2 mg/kg) also increased the proportion of flat calls under AMPH (mean±SEM percent of flat calls following pretreatment with vehicle versus 0.2 mg/kg raclopride, 12.7±2.9 versus 32.8±4.5; Wilcoxon Z=2.5, p<0.05). In contrast, sulpiride marginally *increased* the proportion of trill calls at 40 mg/kg in Experiment 5 (Wilcoxon Z=2.19, p<0.05; Fig. 5c) but not in Experiment 4 (Fig. 5b).

Experiment 6: effects of pimozide and the atypical antipsychotics clozapine and risperidone

Pimozide, clozapine, and risperidone were all tested at a single, high dose. All three antagonists markedly inhibited both USV after saline treatment and AMPH-induced USV production (see Fig. 6). Despite low rates of calling, call subtype analysis revealed that pimozide significantly reduced the proportion of trill calls under AMPH (mean± SEM percent of trills: vehicle versus pimozide, 36.4±6.5 versus 13.3±11.4, respectively; Wilcoxon Z=2.37, p<0.05).

Experiment 7: effect of high-dose sulpiride on 50-kHz USVs and locomotor activity

Sulpiride (80 mg/kg) significantly decreased AMPH-induced locomotor activity (ANOVA pretreatment×treatment interaction, $F_{1,15}$=14.85, p<0.01; Fig. 7). Sulpiride also reduced locomotor activity when given alone (t_{15}= 3.39, p<0.01; Fig. 7a). In contrast, sulpiride exerted no detectable effect on either the call rate (Fig. 7b) or profile (not shown).

Experiments 8–10: effect of GBR 12909 and nisoxetine, alone and in combination

Unlike AMPH, neither GBR 12909 nor nisoxetine significantly promoted 50-kHz calling at any dose tested; all comparisons were statistically non-significant after Holm–

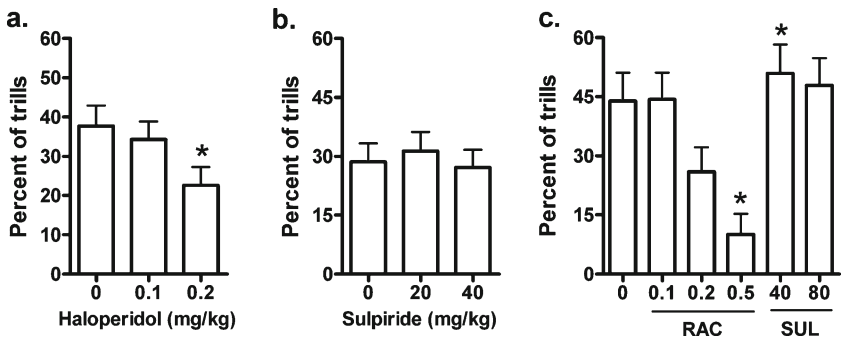

Fig. 5 Experiments 3 and 5: Haloperidol (**a**) and raclopride (*RAC*) (**c**) suppressed trills (as a proportion of all 50-kHz calls) following AMPH administration at the highest doses tested. Sulpiride (*SUL*), in contrast, was largely ineffective (**b**, **c**), except for an increase in the proportion of trills at 40 mg/kg in Experiment 5 (**c**). Each rat was tested under all pretreatment/treatment conditions (n=12 rats per experiment). *p< 0.05 versus vehicle control

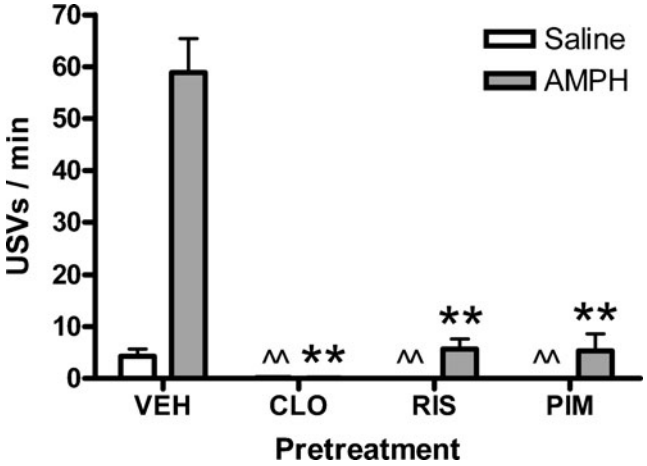

Fig. 6 Experiment 6: Single doses of clozapine (4 mg/kg, SC; *CLO*), risperidone (0.5 mg/kg, SC; *RIS*), and pimozide (1 mg/kg, IP; *PIM*), all markedly reduced the 50-kHz call rate under saline (*open bars*) and AMPH 1 mg/kg IP (*grey bars*). Each rat was tested under all pretreatment/treatment conditions (n=12 rats). ^^p<0.01 versus vehicle/saline control, **p<0.01 versus vehicle/AMPH control

Bonferroni correction (Experiments 8 and 9, respectively; Fig. 8a, b). In Experiment 8, GBR 12909 tended to increase the call rate at 10 mg/kg, especially in the first half of the 40-min session, i.e., time 20–40 min post-injection (Supplemental Fig. S1). Accordingly, this shorter post-injection interval was used when this drug was retested in Experiment 10. Here, selected doses of GBR 12909 (i.e., 10 mg/kg) and nisoxetine (i.e., 12 mg/kg) were administered, not only alone but also in combination; there was still no significant enhancement (or suppression) of call rate (Fig. 8c). Notably, the 10 mg/kg dose of GBR 12909 which appeared to increase calling in Experiment 8 no longer showed such a trend (Fig. 8c).

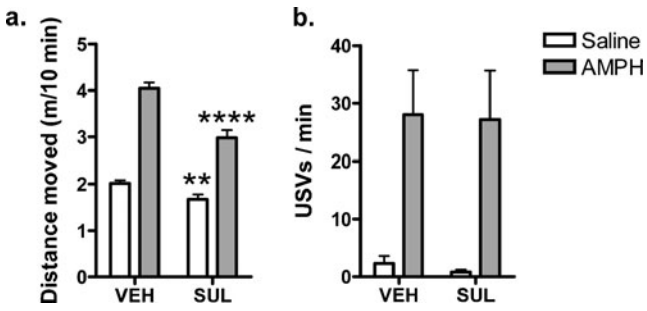

Fig. 7 Experiment 7: Sulpiride (*SUL*; 80 mg/kg, SC) significantly inhibited AMPH-induced locomotor activity (panel **a**) (ANOVA pretreatment×treatment interaction—$F_{1,15}$=14.85, p<0.01) but produced no detectable effect on the rate of USV emission (panel **b**). The y axes represent mean+SEM total horizontal distance (meters) travelled (panel **a**) or the 50-kHz call rate (panel **b**), following administration of saline (*open bars*) or AMPH (*grey bars*). **p<0.01, ****p<0.0001 versus corresponding vehicle (*VEH*) control

The reuptake inhibitors, given alone or in combination, failed to mimic the effect of AMPH on the call profile. For example, in Experiment 10, AMPH significantly increased the relative prevalence of trill calls, but neither GBR 12909, nor nisoxetine, or their combination showed this effect (mean±SEM percent trills: vehicle versus AMPH, 22.9± 5.2 versus 46.3±6.7, respectively; Wilcoxon Z=2.58, p< 0.01). Conversely, a significant reduction in the proportion of flat calls was observed following the co-administration of GBR 12909 and nisoxetine, yet AMPH unexpectedly did not reduce the proportion of flat calls in this particular experiment (mean±SEM percent flat calls: vehicle versus GBR 12909+nisoxetine, 21.9± 6.3 versus 10.3±6.0, respectively; Wilcoxon Z=2.67, p<0.01).

Discussion

The present study provides the first evidence that D1-like and D2-like receptor antagonists modulate the effects of systemic AMPH administration on the 50-kHz call rate and profile. Exceptionally, sulpiride, which is a D2-like antagonist with atypical antipsychotic features, consistently failed to affect USV emission. In addition, neither GBR 12909 (DAT inhibitor) nor nisoxetine (NET inhibitor), or their combination, mimicked the effects of AMPH on USV production. Below, we argue that both D1-like and D2-like DA receptors play a critical role in 50-kHz USV emission, and we suggest mechanisms contributing to sulpiride's lack of effect. We subsequently review antagonist-induced USVs suppression in the context of other behavioral and clinical effects of the same drugs. Finally, we discuss whether enhanced DA or NA transmission is sufficient to promote USV emission.

D1 dopaminergic receptor antagonism

The D1-like antagonists SCH 23390 and SCH 39166 dose-dependently inhibited the 50-kHz call rate and the percentage of trill calls following AMPH challenge; both antagonists also tended to reduce the call rate below control (i.e., drug-free) levels, although a significant reduction was only seen at the highest dose of SCH 39166. SCH 23390 and SCH 39166 both bind with high affinity to D1 and D5 receptors, with negligible affinity for D2-like receptors (i.e., D2, D3, and D4) (Tice et al. 1994). While SCH 23390 also has considerable affinity for serotonin receptors, namely $5HT_2$ and $5HT_{1C}$ (Bischoff et al. 1986; Nicklaus et al. 1988), SCH 39166 does not (Alburges et al. 1992; McQuade et al. 1991a, b; Wamsley et al. 1991). To our knowledge, these drugs do not have any other significant off-target effects. Thus, DA D1-like receptors appear critical to the USV-altering effects of systemic AMPH and may also regulate USV emission in the absence of this drug.

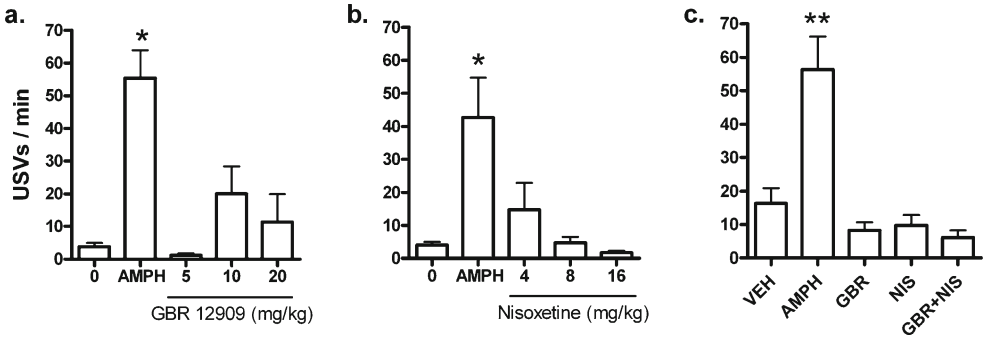

Fig. 8 Experiments 8–10: GBR 12909 (**a**) and nisoxetine (**b**) failed to significantly promote 50-kHz calling at any dose tested. Panel **c** shows that single doses of GBR 12909 (*GBR*, 10 mg/kg IP) and nisoxetine (*NIS*, 12 mg/kg IP), given either alone or even in combination (*GBR+NIS*), still failed to modify the call rate detectably (**c**). *$p<0.05$, **$p<0.01$ versus vehicle control (*VEH*)

D2 dopaminergic receptor antagonism

All six D2-like antagonists, with the notable exception of sulpiride, markedly inhibited or abolished the stimulatory effect of AMPH on call rate. Additionally, haloperidol and raclopride dose-dependently decreased the proportion of trill calls under AMPH. The latter finding is in line with previous studies showing a reduction in the proportion of FM calls in response to sexual odors following systemic haloperidol pretreatment (Ciucci et al. 2007, 2009). It appears likely that DA transmission through D2-like receptors is critical for both the call rate and profile following AMPH, since several possibilities exist as to why sulpiride is anomalous:

1. Sulpiride may exert an additional (as yet unidentified) action which functionally counteracts D2 receptor blockade. Indeed, studies with muscarinic cholinergic and adenosine A2A receptor antagonists have provided such a precedent, in that these drugs can reverse the behavioral effects of DA receptor blockade (Collins et al. 2012; Morpurgo and Theobald 1964).
2. The phenomenon of D2-like receptor heteromerization (Maggio et al. 2009) suggests another plausible mechanism by which sulpiride might exert functional effects that are distinct from those of other D2-like antagonists.
3. It is unlikely that our doses of sulpiride were insufficient to antagonize USV emission, since comparable or even lower doses have proven effective in a number of DA-dependent behavioral assays, i.e., apomorphine hyperactivity and stereotypy (de Paulis et al. 1985), the AMPH cue (Nielsen and Andersen 1992; Nielsen and Jepsen 1985), conditioned place preference (CPP) induced by food or testosterone (Guyon et al. 1993; Schroeder and Packard 2000), and intravenous self-administration of nicotine or cocaine (Sorge and Clarke 2009). Importantly, a high dose of sulpiride that failed to affect the call rate did, at the same time, reduce AMPH-induced hyperactivity (present study—Experiment 7); the latter effect is consistent with previous findings (Ljungberg and Ungerstedt 1985; Moore and Kenyon 1994; Sharp et al. 1986; White et al. 1992).
4. Sulpiride, in contrast to many D2-like antagonists, possesses considerably lower affinity at D4 compared with D2 and D3 receptors (Rondou et al. 2010; Seeman et al. 1997; Seeman and Van Tol 1994). However, it is unlikely that D4 receptors are critical to USV emission since raclopride (Experiment 5) markedly reduced USVs despite also having very low affinity at D4 receptors (Seeman and Van Tol 1994).
5. The "atypical" antipsychotic properties of sulpiride do not appear related to its lack of effect on USV emission, since the atypical drugs clozapine and risperidone clearly inhibited calling.
6. Since D2-like antagonists tend to be pharmacologically non-selective (Jafari et al. 2012), it is conceivable that all the D2-like antagonists tested, except for sulpiride, fortuitously suppressed calling through some shared non-DAergic mechanism. However, this possibility seems remote since the compounds were drawn from multiple, structurally heterogeneous chemical classes (Jafari et al. 2012), and we are unaware of any such shared receptor candidate. Notably, α1 adrenergic receptor blockade abolishes AMPH-induced calling (Wright et al. 2012b), but some DA antagonists (e.g., raclopride) lack significant affinity for this receptor (Hall et al. 1986; Ishiwata et al. 2001; Ogren et al. 1986).

Behavioral mechanisms

The USV-related effects produced by the DA-like antagonists in the present study are summarized in Table 2, together with several other behavioral effects of the same drugs reported in the literature. Antagonist doses that inhibited saline- or AMPH-induced USVs frequently overlapped with those affecting other behavioral measures. However, as discussed below, no particular behavioral measure matched our USV findings completely.

Table 2 Effects of antagonists on USVs and other behavioral measures

Pretreatment		USV results (present study)			Other behavioral effects					
Drug	Dose	USV rate under saline[a]	USV rate under AMPH	Trills under AMPH	Spontaneous LMA	Catalepsy	AMPH[b]-induced LMA	CPA/CPP	AMPH[c] CPP	AMPH[d] cue
SCH 23390	0.005	–	–	–	– (Cervo and Samanin 1996; Hoffman and Beninger 1985; Menzaghi et al. 1997; Sacaan et al. 1996; Salmi et al. 1998)	– (Christensen et al. 1984; Morelli and Di 1985; Ouagazzal et al. 1993)	?	– (Acquas et al. 1989; Leone and Di Chiara 1987)	– (Hiroi and White 1991)	– (Callahan et al. 1991; Nielsen and Andersen 1992; Nielsen and Jepsen 1985)
	0.01	–	↓	↓	– (Cervo and Samanin 1996; Hoffman and Beninger 1985; Sacaan et al. 1996),↑ (Meyer et al. 1993), ↓ (Menzaghi et al. 1997; Salmi et al. 1998)	– (Christensen et al. 1984; Morelli and Di 1985; Ouagazzal et al. 1993)	↓ (Ouagazzal et al. 1993)	– (Acquas et al. 1989; Leone and Di Chiara 1987), CPA (Shippenberg and Herz 1987; Shippenberg and Herz 1988)	– (Hiroi and White 1991)	– (Callahan et al. 1991; Nielsen and Andersen 1992; Nielsen and Jepsen 1985; Nielsen et al. 1989), ↓ (Arnt 1988)
	0.02	–	↓	↓	– (Cervo and Samanin 1996; Shen et al. 2010),↓ (Menzaghi et al. 1997; Salmi et al. 1998)	– (Christensen et al. 1984; Morelli and Di 1985; Ouagazzal et al. 1993)	↓ (Ouagazzal et al. 1993)	– (Acquas et al. 1989; Leone and Di Chiara 1987), CPA (Shippenberg and Herz 1987; Shippenberg and Herz 1988)	– (Hiroi and White 1991), ↓ (Acquas and Di Chiara 1994)	↓ (Arnt 1988; Exner et al. 1989; Nielsen and Andersen 1992; Nielsen and Jepsen 1985; Smith et al. 1989)
SCH 39166	0.03	–	–	–	– (Batsche et al. 1994)	– (Hietala et al. 1992), yes (Prinssen et al. 1993)	?	?	↓ (Acquas and Di Chiara 1994)	?
	0.1	–	↓	–	– (Batsche et al. 1994)	– (Hietala et al. 1992), yes (Prinssen et al. 1993)	?	?	↓ (Acquas and Di Chiara 1994)	↓ (West et al. 1995)
	0.3	↓	↓	↓	– (Batsche et al. 1994),↓ (Collins et al. 2010)	– (Hietala et al. 1992), yes (Prinssen et al. 1993)	?	?	↓ (Acquas and Di Chiara 1994)	↓ (West et al. 1995)
Haloperidol	0.1	–	↓	–	– (Sanchez et al. 1991)	– (Christensen et al. 1984; Liao et al. 1999), yes (Hoffman and Donovan 1995b; Morelli and Di 1985)	↓ (Arnt 1995; Hoffman and Donovan 1995a; Hoffman and Donovan 1995b; Poncelet et al. 1987)	– (Hoffman and Donovan 1995a; Spyraki et al. 1982)	↓ (Hoffman and Donovan 1995a)	↓ (Exner et al. 1989; Nielsen and Jepsen 1985; Nielsen et al. 1989)
	0.2	–	↓	↓	↓ (Sanchez et al. 1991)	– (Christensen et al. 1984; Liao et al. 1999), yes (Hoffman and Donovan 1995b; Morelli and Di 1985; Sanchez et al. 1991)	↓ (Arnt 1995; Hoffman and Donovan 1995a; Hoffman and Donovan 1995b; Mithani et al. 1986; Poncelet et al. 1987)	– (Spyraki et al. 1982)	↓ (Hoffman and Donovan 1995a; Mithani et al. 1986; Spyraki et al. 1982)	↓ (Arnt 1996; Exner et al. 1989; Nielsen and Jepsen 1985; Nielsen et al. 1989)
(-)Sulpiride	20	–	–	–	– (Ferrari and Giuliani 1995; Morgenstern et al. 1983)	– (Imperato and Di Chiara 1985; Tagliamonte et al. 1975)	↓ (Ljungberg and Ungerstedt 1985; Moore and Kenyon 1994; Poncelet et al. 1987; White et al. 1992)	– (Shippenberg and Herz 1988)	– (Hiroi and White 1991)	– (Nielsen and Andersen 1992; Nielsen and Jepsen 1985)
	40	– or ↓	–	– or ↑	– (Ferrari and Giuliani 1995; Morgenstern et al. 1983)	– (Imperato and Di Chiara 1985; Tagliamonte et al. 1975)	↓ (Ljungberg and Ungerstedt 1985; Moore and Kenyon 1994; Poncelet et al. 1987; White et al. 1992)	– (Shippenberg and Herz 1988)	↓ (Hiroi and White 1991)	– (Nielsen and Jepsen 1985), ↓ (Nielsen and Andersen 1992)

Table 2 (continued)

Pretreatment		USV results (present study)			Other behavioral effects					
Drug	Dose	USV rate under saline[a]	USV rate under AMPH	Trills under AMPH	Spontaneous LMA	Catalepsy	AMPH[b]-induced LMA	CPA/CPP	AMPH[c] CPP	AMPH[d] cue
	80	–	–	–	↓ (Cervo and Samanin 1996; present study)	– (Imperato and Di Chiara 1985; Tagliamonte et al. 1975)	↓ (present study; Ljungberg and Ungerstedt 1985; Moore and Kenyon 1994; Poncelet et al. 1987; Sharp et al. 1986; White et al. 1992)	?	↓ (Hiroi and White 1991)	↓ (Nielsen and Andersen 1992; Nielsen and Jepsen 1985)
Raclopride	0.1	↓	↓	–	– (Garcia Horsman and Paredes 2004; Hillegaart and Ahlenius 1987; Salmi et al. 1998; Shen et al. 2010), ↓ (Millan et al. 2004)	– (Hillegaart and Ahlenius 1987; Hoffman and Donovan 1995b; Ouagazzal et al. 1993; Wadenberg et al. 2000a; Wadenberg et al. 2000b)	– (Ouagazzal et al. 1993), ↓ (Hoffman and Donovan 1995a; Hoffman and Donovan 1995b)	– (Garcia Horsman and Paredes 2004; Hoffman and Donovan 1995a)	?	– (Furmidge et al. 1991; Nielsen and Andersen 1992), ↓ (Varty and Higgins 1997)
	0.2	↓	↓	–	– (Hillegaart and Ahlenius 1987; Salmi et al. 1998), ↓ (Millan et al. 2004; Shen et al. 2010)	– (Hillegaart and Ahlenius 1987; Hoffman and Donovan 1995b; Wadenberg et al. 2000b), yes (Ouagazzal et al. 1993)	↓ (Hoffman and Donovan 1995a; Hoffman and Donovan 1995b; Ouagazzal et al. 1993)	– (Hoffman and Donovan 1995a)	↓ (Garcia Horsman and Paredes 2004)	– (Furmidge et al. 1991; Nielsen and Andersen 1992),↓ (Nielsen et al. 1989)
	0.5	↓	↓	↓	↓ (Garcia Horsman and Paredes 2004; Hillegaart and Ahlenius 1987; Millan et al. 2004; Salmi et al. 1998; Shen et al. 2010)	– (Hillegaart and Ahlenius 1987), yes (Hoffman and Donovan 1995b; Ouagazzal et al. 1993; Wadenberg et al. 2000b)	↓ (Hoffman and Donovan 1995a; Hoffman and Donovan 1995b; Ouagazzal et al. 1993)	– (Hoffman and Donovan 1995a)	↓ (Garcia Horsman and Paredes 2004; Hoffman and Donovan 1995a)	↓ (Furmidge et al. 1991; Nielsen and Andersen 1992; Nielsen et al. 1989)
Clozapine	4	↓	↓	–	↓ (Arnt 1995; Sanchez et al. 1991)	– (Hoffman and Donovan 1995b; Liao et al. 1999; Sanchez et al. 1991)	↓ (Arnt and Skarsfeldt 1998; Hoffman and Donovan 1995a; Hoffman and Donovan 1995b)	– (Hoffman and Donovan 1995a)	– (Hoffman and Donovan 1995a)	↓ (Arnt 1996; Nielsen and Andersen 1992; Nielsen and Jepsen 1985)
Risperidone	0.5	↓	↓	–	– (Arnt 1995)	– (Arnt and Skarsfeldt 1998), yes (Hoffman and Donovan 1995b)	↓ (Arnt 1995; Hoffman and Donovan 1995a; Hoffman and Donovan 1995b)	– (Hoffman and Donovan 1995a)	↓ (Hoffman and Donovan 1995a)	↓ (Arnt 1996)
Pimozide	1	↓	↓	–	↓ (Agmo and Soria 1999; Horvitz and Ettenberg 1991; Schaefer and Michael 1984; Spivak and Amit 1986)	– (McMillen et al. 1980), yes (Christensen et al. 1984)	↓ (Poncelet et al. 1987; Schaefer and Michael 1984)	?	?	↓ (Ho and Huang 1975)

Minus sign no significant change, *question mark* currently no published data (to our knowledge), *arrow up* or *arrow down* significant increase or decrease, respectively

[a] Particularly for SCH 23390, haloperidol, and sulpiride, inhibitory effects might have been masked by the low rate of drug-free calling

[b] 0.5–3.5 mg/kg AMPH

[c] 1–2 mg/kg AMPH

[d] 0.3–1 mg/kg AMPH

USVs versus motor function Several DA antagonists (i.e., haloperidol, clozapine, risperidone, pimozide) inhibited USV emission at doses expected to markedly suppress drug-free or AMPH-associated locomotion (Table 2). In general, however, there was no consistent relationship between motor impairment and USV emission. In particular, raclopride inhibited drug-free USV production even at low doses which tend not to inhibit locomotion, and conversely, sulpiride inhibited drug-free and AMPH-induced locomotion without detectably affecting USV production (Table 2).

AMPH cue The discriminative stimulus effects of AMPH are of particular interest since they serve to model the drug's subjective effects in humans (Brauer et al. 1997). The USV-stimulatory and cue effects of AMPH appear similarly affected by our D1 and D2 antagonists, but only the latter is attenuated by sulpiride (see Table 2 for references).

USVs versus reward/aversion Since 50-kHz USVs have been proposed as a measure of drug reward, it is potentially informative to compare our results with published work using the conventional reward measure of CPP, while acknowledging that the latter reflects conditioned rather than unconditioned drug effects. Both D1 antagonists appeared to inhibit 50-kHz calling under saline treatment, allowing for the low rate of drug-free calling. However, it is unclear whether D1 receptor blockade reliably produces a conditioned place aversion (CPA) in rats (Table 2), since D1 antagonist effects are either mixed (SCH 23390) or unreported (SCH 39166). In contrast, D2-like antagonists consistently fail to produce a CPP or CPA in adult rats (Tzschentke 1998). The lack of D2 antagonist-induced CPP or CPA does not appear to reflect a learning or memory deficit, since D2 receptor blockers do not inhibit the acquisition of all types of CPP or CPA (Tzschentke 1998). Thus, D2 receptor antagonists appear neutral in the CPP/CPA test, yet all our D2 receptor antagonists (with the exception of sulpiride) tended to inhibit calling under saline treatment.

The acquisition of AMPH CPP is inhibited by D1 and D2 receptor antagonists, according to most reports (Table 2). However, our USV findings reveal two striking differences: (1) sulpiride did not inhibit AMPH-induced calling (present study), whereas it inhibited AMPH CPP (Hiroi and White 1991), and (2) clozapine abolished AMPH-induced calling, yet failed to inhibit AMPH CPP (Hoffman and Donovan 1995a). Importantly, these studies employed comparable doses of antagonist and AMPH.

USVs versus affect Although classic antipsychotics (e.g., haloperidol) do not produce a CPA in rats (see above), they often produce dysphoria in human subjects (Emerich and Sanberg 1991; Voruganti and Awad 2004). Atypical antipsychotics, in contrast, appear far less commonly associated with dysphoria, as evidenced by sulpiride, clozapine, and risperidone (Mehta et al. 1999; Potvin et al. 2003; Voruganti et al. 2000). Although the latter two drugs produced profound alterations in USV emission in the present study, circulating levels of these three DA antagonists probably far exceeded the clinical range.

USVs versus AMPH euphoria The dose of AMPH employed in the present study (i.e., 1 mg/kg) appears comparable to euphorigenic doses in human studies (Grilly and Loveland 2001). FM 50-kHz ultrasonic vocalizations (USVs) have been proposed to reflect hedonia (Burgdorf and Moskal 2009), and the trill subtype in particular appears most closely associated with rewarding doses of AMPH and cocaine (Wright et al. 2010, 2012b). Although trill calls following AMPH were preferentially inhibited by both D1-like and some D2-like (i.e., haloperidol, raclopride, and pimozide) antagonists in the present study, it is important to note that animal and human studies do not strongly support a role for DA in hedonia but rather in incentive salience or "wanting" (Brauer and de Wit 1997; Leyton et al. 2005, 2007; Smith et al. 2011). Therefore, in view of the present findings, we speculate that emission of FM 50-kHz calls, and trills in particular, may relate to incentive salience rather than hedonia. Flat calls, in contrast to trill calls, were significantly increased in relative terms by certain doses of SCH 39166 and raclopride, possibly as a consequence of trill call suppression. Flat calls have been proposed to have a social-coordinating function unrelated to positive affect (Wohr et al. 2008).

Dopamine and noradrenaline reuptake inhibitors

AMPH and cocaine, which increase both DA and NA transmission (McKittrick and Abercrombie 2007), enhance USV production and modulate the call profile (Wright et al. 2010, 2012b). The results of the present study together with previous findings (Wright et al. 2012b) suggested that both DA and NA transmission are *necessary* for the observed effects of AMPH on USV emission. The question of *sufficiency* was addressed by subsequently examining whether the selective DAT and NET inhibitors GBR 12909 (Andersen 1989) and nisoxetine (Wong et al. 1982; Wong and Bymaster 1976), respectively, could mimic the USV effects of AMPH or cocaine. Neither GBR 12909 nor nisoxetine, alone or in combination, mimicked the effect of AMPH on the call rate or profile in the present study. At doses tested here, GBR 12909 would be expected to elevate extracellular DA, and co-administration of a NET blocker would likely potentiate this increase (Carboni et al. 2006). To our knowledge, there are no studies directly examining extracellular NA following nisoxetine administration in rats. Instead, the doses of nisoxetine were chosen based on their ability to generalize to the cues produced by the non-selective β-adrenergic

agonist isoproterenol (Crissman and O'Donnell 2002) and the NET blocker reboxetine (Millan and Dekeyne 2007); the latter drug produces a marked increase in extracellular levels of NA (Dekeyne et al. 2001).

GBR 12909 and nisoxetine, unlike AMPH, appear to exert their behavioral effects solely through transmitter reuptake inhibition. We have previously found that the DA/NA reuptake blocker cocaine moderately stimulated 50-kHz calling, while mimicking AMPH's ability to promote trill calls preferentially (Wright et al. 2012b). It is unclear why GBR 12909 and nisoxetine failed to exert either of these effects; here, cocaine's ability to inhibit the 5-HT reuptake transporter (Wall et al. 1995) or enhance exocytotic DA release (Ramsson et al. 2011) may be relevant.

Limitations

Adult rats exhibit large variability in their USV response to systemic AMPH (Taracha et al. 2012; Wright et al. 2010). In order to examine drug effects on AMPH-induced calling, we identified low responders using an initial AMPH screen in most experiments. A substantial number of subjects were then excluded, resulting in a selected population that may differ in other behavioral or neurochemical respects (Burgdorf et al. 2008). Notably, the failure of sulpiride to modify the call rate or profile was independent of whether rats were screened or not (compare Experiment 5 with Experiments 4 and 7). The present method of selecting adult rats based on their acute response to AMPH helps to address the issue of low baseline call rates and high individual differences. Other approaches include selective breeding (Burgdorf et al. 2008) and possibly through prior social manipulations (Vivian and Miczek 1991).

Due to the labor-intensive nature of this type of USV analysis, only a small fraction of the entire session (i.e., 3 or 4 min) was time-sampled for most experiments. It is possible that USV effects outside our chosen time intervals were missed. This method of time-sampling therefore limits interpretation of the present findings.

Finally, certain drugs, namely sulpiride, GBR 12909, and nisoxetine, exerted no discernable effects on USV emission. In the case of sulpiride, we performed an additional experiment (Experiment 7) where USVs and locomotion were assessed simultaneously. However, the negative findings with GBR 12909 and nisoxetine (or their combination) were not followed up with additional behavioral testing. While GBR 12909 would be expected to stimulate locomotor activity at all the doses tested (Hooks et al. 1994; Powell et al. 2001), nisoxetine does not appear to affect this measure in adult rats (Davids et al. 2002; Powell et al. 2001). The lack of positive controls in Experiments 8–10 is a limiting factor when interpreting these results.

Conclusion

USVs are a potentially rich source of information about the rat's subjective state. The present study furthers our understanding of the neurochemical substrates regulating USV production in adult rats. DA transmission appears critical for the 50-kHz USV response to systemic AMPH, since antagonism of either D1-like or D2-like receptors (with the notable exception of sulpiride) reversed the effects of AMPH on the call rate and profile. DA transmission also appears to modulate drug-free call emission. It appears that, although both DA and NA are required, inhibition of DA and NA reuptake per se is not sufficient to elicit an AMPH-like USV response.

Acknowledgments Supported by a Natural Science and Engineering Research Council of Canada (NSERC) discovery grant (155055, to P.B.S.C) and an NSERC Postgraduate Scholarship D (to J.M.W). The authors would like to acknowledge the NIMH Chemical Synthesis and Drug Supply Program for generously providing clozapine, GBR 12909 2HCl, and (±)-nisoxetine HCl for this study. P.B.S.C. is a member of the Center for Studies in Behavioral Neurobiology at Concordia University in Montreal. The authors have no financial relationship with the organization that sponsored this research. All experiments comply with the current laws of Canada.

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