



# The antagonistic relationship between aversive and appetitive emotional states in rats as studied by pharmacologically-induced ultrasonic vocalization from the nucleus accumbens and lateral septum

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## ABSTRACT

Rats can emit 22-kHz or 50-kHz ultrasonic vocalizations (USVs) in negative, as well as positive contexts which index their emotional state. 22-kHz USVs are emitted during aversive contexts and can be initiated by activation of the ascending cholinergic pathways originating from the laterodorsal tegmental nucleus or initiated pharmacologically by injection of cholinergic agonists into target areas of these pathways (medial cholinceptive vocalization strip). Conversely, 50-kHz USVs are emitted during positive pro-social contexts and can be initiated by stimulation of ascending dopaminergic pathways originating from the ventral tegmental area or by injection of dopamine agonists into target areas of these pathways (nucleus accumbens shell). Recently, we have shown an inhibitory effect a positive emotional state has on the emission of carbachol-induced 22-kHz USVs from the anterior hypothalamic/medial preoptic area. However, this structure is a fragment of that cholinceptive vocalization strip. We wanted to examine if we could observe similar effect when the aversive state is induced from the lateral septum, the most rostral division of the cholinceptive vocalization strip. The results supported previous findings. First, microinjection of the dopamine agonist R(-)-apomorphine into the nucleus accumbens shell resulted in increased emission of frequency modulated (FM) 50-kHz USVs that are regarded as signals expressing a positive emotional state in rats. Second, FM 50-kHz USVs and not flat (F) 50-kHz USVs were able to decrease 22-kHz USVs induced by microinjections of carbachol into the lateral septum. This research provides further support to the hypothesis that the initiation of a positive emotional state functionally antagonizes initiation of a negative emotional state in rats.

## 1. Introduction

Adolescent and adult rats communicate by producing two different categories of ultrasonic vocalizations (USVs) identified as 22-kHz USVs and 50-kHz USVs, which can be further subdivided into flat (F) and frequency modulated (FM) USVs (Knutson et al., 2002; Brudzynski, 2007, 2009, 2013; Burgdorf et al., 2008). In sonographic analyses, 22-kHz USVs have a flat temporal pattern, a long call duration between 100 and 3000 ms, a peak frequency between 19 and 27 kHz and bandwidth of 3–5 kHz, while 50-kHz USVs have a duration ranging from 20 to 100 ms, a peak frequency ranging from 48 to 70 kHz and a bandwidth of 7–25 kHz (Brudzynski, 2001; Wright et al., 2010).

Both types of USVs have been hypothesized to be an inseparable component in signaling emotional states (Brudzynski, 2007, 2013). Ethological and pharmacological experiments provided evidence that 22-kHz USVs reflect a negative emotional state that can be initiated

either conditionally or unconditionally. For example, emission of 22-kHz USVs can be initiated by presence of fox or lion urine (Fendt et al., 2018), by presentation of a cat to rats living in a visible burrow system (Blanchard et al., 1991), by social defeat (Kroes et al., 2007), or by anticipation of foot-shock (Jelen et al., 2003).

Pharmacological evidence supporting the thesis that 22-kHz USVs are associated with negative emotional state comes from investigations using anxiolytics and anxiogenics. Anxiolytics, drugs that decrease the self-reported measure of anxiety in humans, can decrease the emissions of 22-kHz USVs in rats (Cullen and Rowan, 1994; Jelen et al., 2003; Miczek et al., 1995; Sánchez and Meier, 1997; Sun et al., 2010; Vivian et al., 1994). Likewise, pentylenetetrazole, an anxiogenic drug, has been shown to increase the number of cue-emitted 22-kHz USVs (Jelen et al., 2003) as well as increase the duration of immobility in rats (Willadsen et al., 2018). Further evidence that supports the argument that 22-kHz USVs reflect a negative emotional state comes from studies

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reporting emission of 22-kHz USVs during withdrawal from drugs of abuse or in the absence of expected rewards (Barker et al., 2015; Covington III and Miczek, 2003; Vivian and Miczek, 1991). These contexts are associated with self-reported measures of negative affect in humans (Barr et al., 2002; Corr, 2002; Pelchat, 2002).

Conversely, the emission of 50-kHz USVs by rats has been argued to signal a positive emotional state. Rats will increase the number of FM 50-kHz USVs in play behaviour with other conspecifics (Burke et al., 2017) or with the experimenter (heterospecific play or ticking) (Burgdorf et al., 2008; Panksepp and Burgdorf, 2000). 50-kHz USVs can also be initiated both pharmacologically and by anticipation of delivery of rewarding electrical brain stimulation (Burgdorf et al., 2000; Scardochio et al., 2015), in response to intravenous amphetamine application (Ahrens et al., 2009), or in anticipation of cocaine consumption (Browning et al., 2011).

The anatomical systems that innervate brain structures important for the initiation of 50-kHz USVs or 22-kHz USVs belong to two separate ascending pathways utilizing two distinct neurotransmitters. Cholinergic cell bodies within the laterodorsal tegmental nucleus (LTDg) that are innervating key limbic structures along the mesencephalon and diencephalon, can initiate 22-kHz USVs upon microinjection of cholinergic agonists into terminal fields or by glutamatergic stimulation of the LTDg neurons (for review see Brudzynski, 2007, 2014). The terminal fields of this ascending mesolimbic cholinergic system that specifically initiate the production of 22-kHz USVs have been termed the medial cholinceptive vocalization strip. In the most caudal extent, this system innervates rostral brain stem and the hypothalamus and terminates in the lateral septum in the most rostral division (Brudzynski, 2007). Conversely, dopamine cell bodies located in the ventral tegmental area (VTA) that are innervating the nucleus accumbens shell are important for the initiation of 50-kHz USVs. This pathway has been termed the ascending mesolimbic dopamine system and has been extensively studied for its contribution to reward and motivation (Ikemoto, 2007).

There is indirect evidence that supports an antagonistic relationship between the action of acetylcholine and dopamine during the initiation of an emotional state. Systemic injection of the dopamine agonist amphetamine, a condition that reliably initiates the production of 50-kHz USVs (Mulvihill and Brudzynski, 2019) decreases brain acetylcholine levels (Domino and Olds, 1972; Vasko et al., 1974). Likewise, systemic injection of morphine, which can induce conditioned place-preference (a measure of an appetitive state) in mice (Cole et al., 2013), increases the extracellular levels of dopamine in the nucleus accumbens (Leone et al., 1991) while decreasing basal acetylcholine levels in the nucleus accumbens (Rada et al., 1991). The morphine effect on basal dopamine and acetylcholine levels in the nucleus accumbens is reversed upon pre-treatment with the opioid antagonist naloxone (Rada et al., 1991). Pre-treatment with naloxone also induces withdrawal-like symptoms, conditioned place-aversion (a measure of anxiety) (Lin et al., 2018) and emission of 22-kHz USVs (Vivian and Miczek, 1991). Thus, the initiation of 22-kHz and 50-kHz USVs are not only indirect measures of emotional states in the signaler, but they are also an indirect measure of dopamine/acetylcholine ratios in forebrain areas.

Recently, we have also shown an antagonistic interaction of dopamine on acetylcholine-induced initiation of vocal expression of an emotional state. Our result showed decreased emissions of carbachol-induced 22-kHz USVs from the anterior hypothalamic-medial preoptic (AH-MPO) area after R(-)-apomorphine was injected in the medial shell of the nucleus accumbens (Silkstone and Brudzynski, 2019). However, the AH-MPO is only one of the nuclei in the medial cholinceptive vocalization strip (Brudzynski, 2010). This strip, which stretches from the LTDg to the most rostral extent of the basal forebrain is involved in the initiation of aversive vocalization in both cats and rats (Brudzynski, 2007).

The purpose of the current experiment was to investigate if the injection of the dopamine agonist R(-)-apomorphine into the nucleus

accumbens could decrease carbachol-induced 22-kHz USVs from the LS, a nucleus located in the most rostral extent of the medial cholinceptive vocalization strip (Bihari et al., 2003; Brudzynski et al., 2011). This will help clarify if the reduction in emission of 22-kHz USVs in response to R(-)-apomorphine injection into the medial shell of the nucleus accumbens is a response localized to AH-MPO stimulation, or if the reduction in the expression of a negative emotional state is a general response across the medial cholinceptive vocalization strip.

## 2. Methods and procedures

### 2.1. Stereotaxic implantation of cannulae into the left nucleus accumbens shell and left LS

Twenty-five Long-Evans rats were used for double injections of pharmacological agents into two different brain areas that induce either 50-kHz USVs or 22-kHz USVs. R(-)-apomorphine was injected into the medial shell of the nucleus accumbens to induce positive emotional arousal signaled by the emission of FM 50-kHz USVs, while carbachol was injected into the LS to induce negative emotional arousal reflected by the emission of 22-kHz USVs. The intensity, or magnitude, of emotional states was measured by the number of emitted USVs.

#### 2.1.1. Subjects and surgery

Twenty-five adult male Long-Evans rats (Charles River) with body weight ranging from 280 to 320 g at the time of surgery served as the experimental subjects. All animals were housed in polycarbonate cages (48 cm × 25 cm × 20 cm high) with constant room temperature (23 °C ± 1 °C), controlled humidity conditions and in a 12:12 h light-dark cycle. Animals were housed in pairs with a dust-free corn cob bedding (Fisco Enterprises, Bolton, ON) with black polyvinyl tubing for hiding, wooden blocks for play with ad libitum access to water and pelleted Rodent Lab Diet (#5001, Ren's Feed & Supplies Limited, Oakville, ON). After five days of acclimation, rats underwent stereotaxic surgery.

Rats underwent stereotaxic surgery for unilateral implantation of guide cannula into the left hemisphere. Briefly, rats were anesthetized with gaseous isoflurane at a concentration of 3% and placed in a Kopf stereotaxic apparatus (Model 900, David Kopf Instruments, Tujunga, CA) in a flat skull position. While in the apparatus, burr holes were drilled into the skull and two guide cannula (constructed from 23 G syringe needles with O.D. = 650 μm, Beckton-Dickinson Canada, Mississauga, ON) was implanted according to the coordinates from the Paxinos and Watson (2005) stereotaxic atlas. One cannula was implanted into the lateral septum (LS, stereotaxic parameters from the interaural line ranged from A-P: 9.12–8.6; L: 0.6–1.2 from the midline, and D-V: -4 to -4.6 mm from the surface of the skull), and the other cannula was implanted into the left shell of the nucleus accumbens (parameters from the interaural line ranged from A-P: 10.4–10.8; L: 0.8–1.8; D-V: 5.8–6.4). Cannulae were permanently secured to the skull by stainless steel jeweler's screws and methyl methacrylate resin (Perm Resin, Hygenic Corporation of Canada Inc., St. Catharines, ON). For further details see Fornari et al. (2012). Rats were placed in the study upon five days of recuperation from surgery and subsequent inspection of their condition by the veterinarian.

#### 2.1.2. Drugs and injection order

Carbachol (carbamylcholine chloride, Sigma Chemical Co., St. Louis MO.) was dissolved in 0.9% sterile saline and was injected unilaterally into the LS by a constant rate Hamilton® CR 700 micro-syringe (Hamilton Company, Reno, NV) in a dose of 1.0 μg/0.3 μl at a rate of ~4.5 nl/s. R(-)-apomorphine hydrochloride (Sigma, St. Louis, MO) was dissolved in vehicle and injected in a concentration of 3.0 μg/0.3 μl at the same rate of carbachol. The vehicle was prepared by adding 0.1% ascorbic acid to sterile saline and buffered to a pH around 5. The vehicle (veh) served as a control for apomorphine, while saline (sal) was

the control for carbachol.

R(-)-apomorphine or vehicle was first injected into the shell of the nucleus accumbens. After injection of the drug or vehicle was finished, the injection cannula was left in place for an additional 60 s to allow for proper drug diffusion. After 60 s the injection cannula was slowly withdrawn and a sterile plug-pin was used to seal off the cannula, the rat was then placed in its home cage for 60 s. After 60 s, the rat was taken out of his cage, and carbachol or saline was injected into the LS at the same rate and volume as R(-)-apomorphine. After the injection was finished, the injection cannula was left in place for an additional 60 s to allow for proper diffusion. After 60 s, the injection cannula was removed; the guide cannula was then closed using a sterile plug-pin. The rat was then immediately placed in the recording chamber and recorded for 10-min.

### 2.1.3. Recording of vocalizations

Recording of ultrasonic vocalizations took place in a Plexiglass recording chamber (25 cm × 18 cm × 18 cm). On top of the recording chamber, an Avisoft® CM16/CPMA condenser microphone (frequency range 2–250 kHz, Avisoft® Bioacoustics, Berlin, Germany) was placed with an average distance of 25 cm to the rat's head. Recording of the USVs was done in real time and stored in a 16-bit format for later analysis. Analysis of USVs was done off-line using Avisoft® SAS LabPro program. Spectrograms were created using Fast Fourier transform (length: 552; Frame: 100%; Window: Hamming; Overlap: 75%).

Identification of 22-kHz and 50-kHz USVs was followed as described in previous studies (Brudzynski et al., 1991; Brudzynski, 2007; Thompson et al., 2006). Briefly, USVs that had a peak frequency that fell between 19 and 29 kHz and had a duration longer than 100 ms were classified as 22-kHz USVs while calls that had a peak frequency that fell between 39 and 80 kHz and had a duration less or equal to 100 ms were classified as 50-kHz USVs. USVs with peak frequency from 30 to 40 kHz were very rare and were not taken for analysis. Subsequent classification of 50-kHz into frequency modulated (FM) and flat (F) calls, i.e., unmodulated USVs, was based on morphological characteristics of calls on the sonograms consistent with the study by Burgdorf et al. (2008). Recording of USVs took place for 10 min. After that time, the rat was placed back into its home cage. Each rat received a clean cage for recording time, and each soiled cage was removed from the test room. Before each additional rat was tested, the table was wiped down with Virox® (Virox Technologies Inc., Oakville, ON) then further cleaned with a diluted ethyl alcohol solution followed by distilled water.

After the rat had received the final injection, it was anesthetized with an overdose of sodium pentobarbital. Before removal of the brain, an India-ink solution was prepared (1:100 dilution) and injected into the brain to aid histological determination of injection sites.

### 2.1.4. Histology and localization

After the experiment, animals underwent transcardial perfusion

with 10% solution of formalin. Brains were removed, fixed with formalin for 24 h, and coronally sectioned on a freezing microtome (Cryo-Histomat, Hacker Instruments and Industries, Fairfield, NJ) to a thickness of ~40 μm. Sections were placed on 1% poly-lysine coated slides, then underwent Nissl staining and were coated with Permount™ mounting medium (Fisher Scientific Co., Ottawa, ON) and coverslipped. For further details of the histological procedure see Lindroos and Leinonen (1983).

Localizations were performed using a projection microscope. Small depositions of India ink before perfusion were used to confirm injection sites. After marking localization of all injection sites, they were transferred according to their stereotaxic coordinates on a selected medial stereotaxic section. The medial stereotaxic section was used as a composite diagram of all injection sites for a given group.

### 2.1.5. Statistics

A non-parametric repeated measures ANOVA (Friedman's ANOVA) followed by Sign-ranked post hoc test was used to assess the statistical difference between the number of 50-kHz USVs induced by R(-)-apomorphine or vehicle from the nucleus accumbens shell. Analysis of sonographic features (call duration and peak frequency) was done using repeated measures ANOVA. A Shapiro-Wilks test was used to assess the normality of sonographic features to ensure the appropriate statistical procedure. All statistics were done using SPSS v 17.0 (SPSS Inc., Chicago, U.S.A.). Multiple comparisons were corrected with Bonferroni method. Reported means are followed by the standard error of the mean (S.E.M). A Pearson correlation was used to assess the relationship between the change in recorded 22-kHz USVs between injection conditions, and the number of recorded F or FM 50-kHz USVs.

## 3. Results

### 3.1. R(-)-apomorphine was able to increase the mean number of recorded F and FM 50-kHz USVs compared to control injection

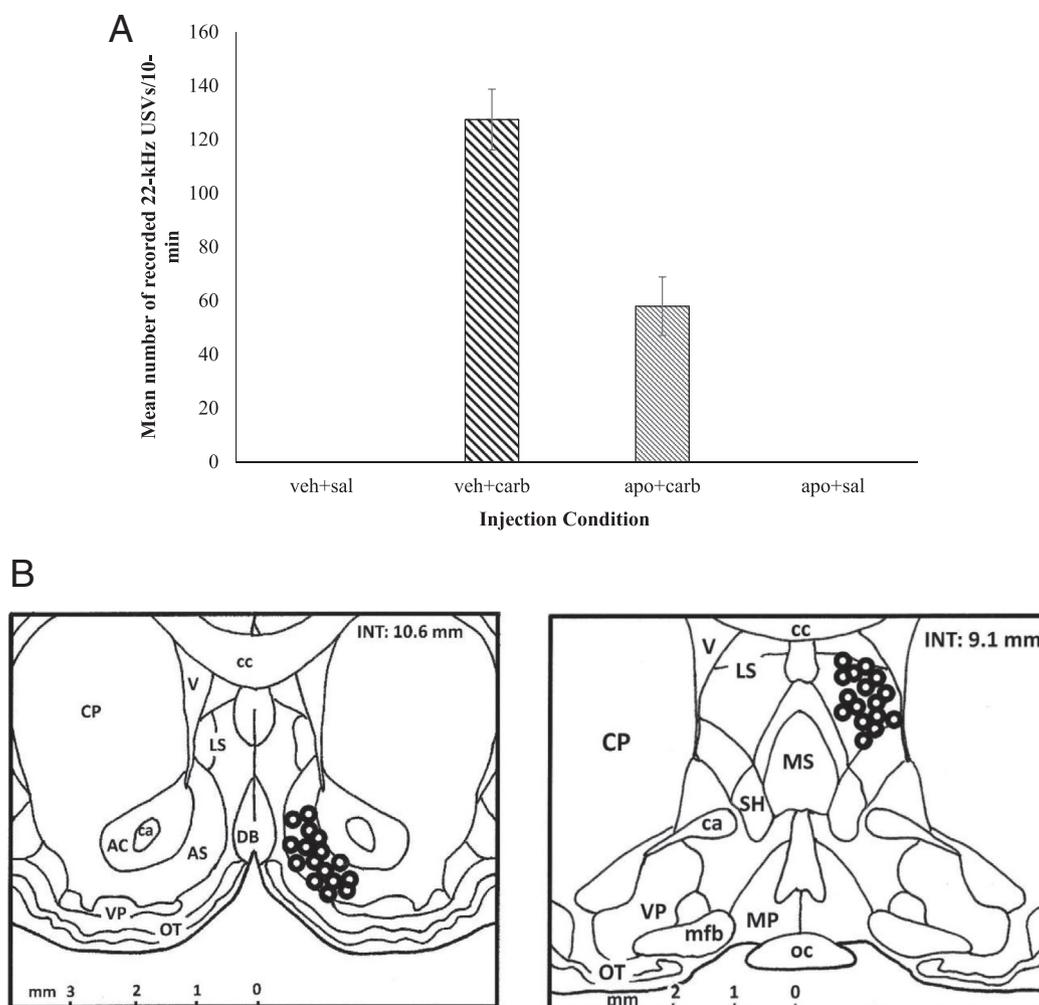
Injection of R(-)-apomorphine into the medial shell of the nucleus accumbens, followed by injection of saline into the LS (apo + sal), significantly increased the mean number of FM 50-kHz USVs ( $\chi^2[3] = 32.9$ ,  $p < 0.001$ ) and F 50-kHz USVs ( $\chi^2[3] = 20.5$ ,  $p < 0.008$ ) compared to veh + sal controls (see Table 1,  $n = 16$ ). However, when R(-)-apomorphine was injected outside the medial shell of the nucleus accumbens, the emission of 50-kHz USVs was very low and sporadic, and there was no difference in the number of recorded FM or F 50-kHz USVs as compared to veh + sal condition (see, Table 1,  $n = 9$ ). Thus, apomorphine was effective in initiating 50 kHz USVs but only from the medial division of the nucleus accumbens shell.

**Table 1**

Mean number ( $\pm$  S.E.M) of recorded 22-kHz, F 50-kHz and FM 50-kHz USVs across different injection conditions for R(-)-apomorphine injected into the medial shell of nucleus accumbens (left side of the Table) or outside of the medial shell of accumbens (right side of the Table).

USVs	Medial nucleus accumbens shell + LS (n = 16)				Outside medial nucleus accumbens shell + LS (n = 9)			
	veh + sal	veh + carb	apo + carb	apo + sal	veh + sal	veh + carb	apo + carb	apo + sal
FM 50-kHz	2.1 $\pm$ 0.8 <sup>a</sup>	N.A	N.A	33.8 $\pm$ 4.8 <sup>a</sup>	2.6 $\pm$ 1.1	N.A	N.A	4.4 $\pm$ 1.5
F 50-kHz	3.5 $\pm$ 1.0 <sup>b</sup>	N.A	N.A	23.6 $\pm$ 4.1 <sup>b</sup>	6.2 $\pm$ 1.4	N.A	N.A	5.5 $\pm$ 0.9
22-kHz	0	127.4 $\pm$ 11.3 <sup>c</sup>	57.9 $\pm$ 10.8 <sup>c</sup>	0	0	109.7 $\pm$ 12.3	89.1 $\pm$ 15.2	0

Note: Statistically significant differences in the post-hoc analysis for the number of emitted F or FM 50-kHz USVs during different injection conditions are denoted by the superscript letters. *Superscript letters and significance*: a:  $p < 0.001$ ; b:  $p = 0.008$ ; c:  $p = 0.037$ . *Abbreviations*: veh + sal, rats received vehicle injection into the medial shell of the nucleus accumbens followed by vehicle injection into the LS; veh + carb, vehicle injection into accumbens shell followed by carbachol injection into the LS; apo + carb, R(-)-apomorphine into the medial shell of the nucleus accumbens, followed by carbachol injection into the LS, apo + sal, R(-)-apomorphine injection into the medial shell of the nucleus accumbens followed by vehicle injection into the LS. N.A - Not Applicable.



**Fig. 1.** A. Mean ( $\pm$  S.E.M) number of 22-kHz USVs recorded during four different injection conditions. Injection of R(-)-apomorphine into the shell of the nucleus accumbens and followed by carbachol in the LS (apo + carb) was able to significantly decrease the mean number of recorded 22-kHz USVs when compared to veh + carb injection condition ( $\chi^2[3] = 44.7$ ,  $p = 0.037$ ,  $n = 16$ ). There was no recorded 22-kHz USVs under the veh + sal or the apo + sal injection conditions. *Abbreviations:* veh + sal – injection of vehicle into the accumbens followed by saline in the LS; veh + carb – injection of vehicle into the accumbens followed by carbachol in LS; apo + carb – injection of apomorphine into the accumbens followed by carbachol in LS; apo + sal – injection of apomorphine into the accumbens followed by saline in LS. For localization of injection sites, see Fig. 1B.

B. Localization of injection sites (dark circles) in the medial shell of the nucleus accumbens ( $n = 16$ ) and corresponding localization of injection sites in the LS ( $n = 16$ ). Each site was injected four times with different combination of vehicles or drugs. Coronal sections of the rat brain at the interaural (INT) stereotaxic planes 10.6 and 9.1, respectively, have been based on the stereotaxic atlas by Paxinos and Watson (2005). *Abbreviations:* AC – core of the nucleus accumbens; AS – shell of the nucleus accumbens; ca – anterior commissure; cc – corpus callosum; CP – caudate-putamen; DB – diagonal band; LS – lateral septum; mfb – medial forebrain bundle; MP – medial preoptic area; MS – medial septum; oc – optic chiasm; OT – olfactory tubercle; SH – striohypothalamic nucleus; V – lateral ventricle; VP – ventral pallidum. Scale in mm.

### 3.2. Intracerebral injection of R(-)-apomorphine into the medial shell of nucleus accumbens decreased carbachol-induced 22-kHz USVs from the LS

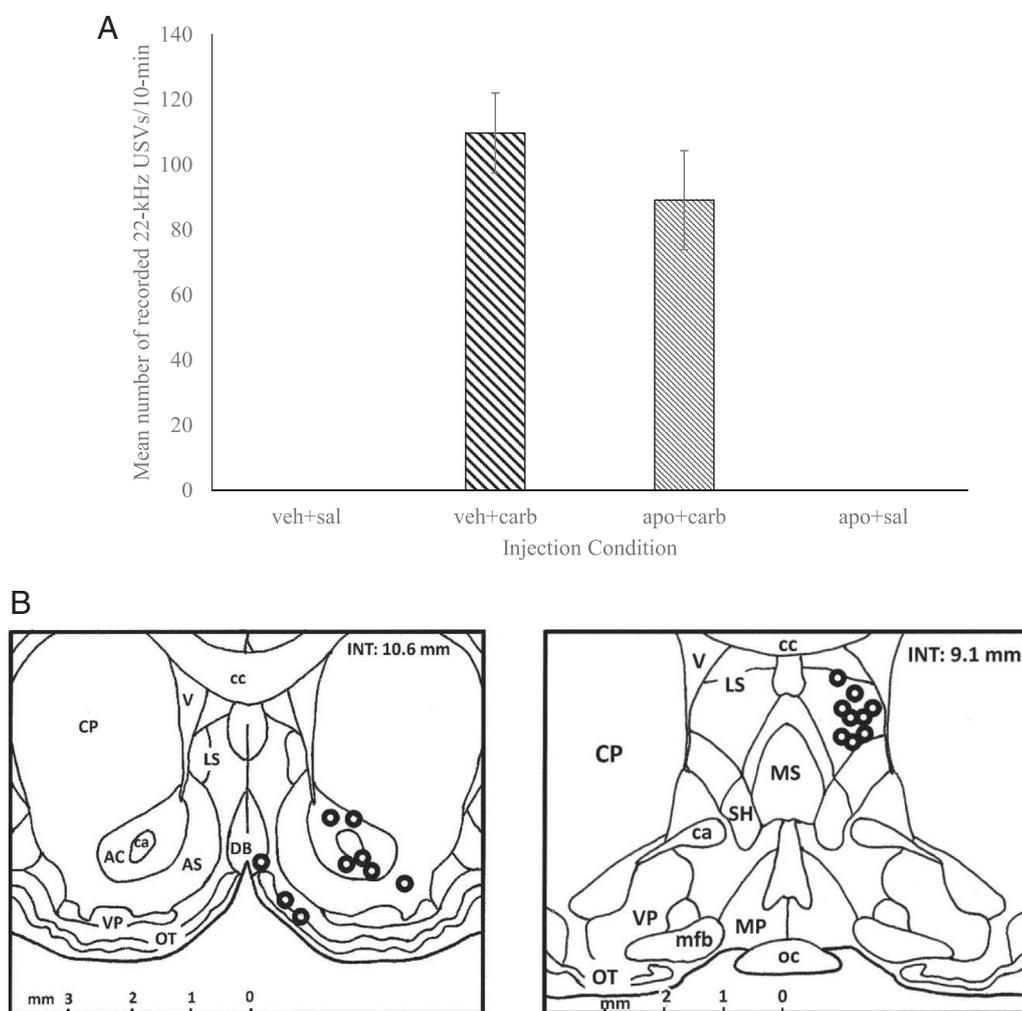
This set of experiments was designed to investigate if R(-)-apomorphine injections into the nucleus accumbens shell could decrease subsequent carbachol-induced 22-kHz USVs from the LS. Injection of the vehicle to the shell of the accumbens followed by carbachol into the LS (condition veh + carb) induced robust emission of 22 kHz USVs with  $127.4 \pm 11.3$  per 10-min recording (Fig. 1A, left bar). Injection of R(-)-apomorphine into the accumbens shell followed by carbachol injected to LS (condition apo + carb) significantly attenuated the mean number of recorded 22-kHz USVs as compared to vehicle condition ( $\chi^2[3] = 44.7$ ,  $p = 0.037$ , see Table 1 and Fig. 1A, right bar, and see localizations of injection sites in Fig. 1B;  $n = 16$ ). There were no 22-kHz USVs recorded during the veh + sal or the apo + sal injection conditions (Fig. 1B).

Thus, R(-)-apomorphine injection into the medial shell region of

the nucleus accumbens significantly attenuated emission of 22 kHz USVs. However, when R(-)-apomorphine was injected outside the medial shell of the nucleus accumbens (see localizations, Fig. 2B), there was no difference in the mean number of recorded 22-kHz USVs between conditions veh + carb vs. apo + carb ( $\chi^2[3] = 24.9$ ,  $p > 0.95$ ; Table 2B; Fig. 2A,  $n = 9$ ). Again, no 22-kHz USVs were recorded during the veh + sal or the apo + sal injection conditions. Thus, apomorphine injection into the medial shell of the nucleus accumbens was effective at attenuating emissions of 22 kHz USVs.

### 3.3. Increased emission of FM 50-kHz USVs during apo + sal injection condition is correlated with a reduction in the number of recorded 22-kHz USVs during the apo + carb injection condition

To investigate the relationship between the number of emitted F and FM 50-kHz USVs and the subsequent decrease in the number of emitted 22-kHz USVs, emitted F and FM 50-kHz calls were plotted against the



**Fig. 2.** A. Mean ( ± S.E.M) number of 22-kHz USVs recorded during four different injection conditions. Injection of R(-)-apomorphine outside the medial nucleus accumbens shell, followed by carbachol injection into the LS was unable to significantly reduce the mean number of recorded 22-kHz USVs ( $\chi^2[3] = 24.9, p > 0.95$ ). There were no recorded 22-kHz USVs during the veh + sal and the apo + sal injection conditions. For sequence of injection conditions and abbreviations, see Fig. 1A and for localization of injection sites, see Fig. 2B.

B. Localization of injection sites (dark circles) outside of the medial shell of the nucleus accumbens (n = 9) and corresponding localization of injection sites in the LS (n = 9). Coronal sections of the rat brain at the interaural (INT) stereotaxic planes 10.6 and 9.1, respectively, have been based on the stereotaxic atlas by Paxinos and Watson (2005). For list of abbreviations, see legend to Fig. 1B.

magnitude of the subsequent decrease in the number of 22-kHz USVs. The x-axis in Fig. 3 and Fig. 4 are the total number of F or FM 50-kHz USVs, respectively, that were recorded per rat (n = 16) during the apo + sal injection condition. The y-axis in Fig. 3 and Fig. 4 depict the standardized change (z-score) in the number of emitted 22-kHz USVs between veh + carb and apo + carb injection conditions.

No correlation between the number of recorded F 50-kHz USVs and the change in the number of recorded 22-kHz USVs between veh + carb and apo + carb injection conditions was observed ( $r_s[14] = -0.39, p = 0.133, \text{Fig. 3}$ ). However, there was a significant negative correlation between the number of FM 50-kHz USVs emitted and the subsequent decrease in recorded 22-kHz USVs ( $r_s[14] = -0.54, p = 0.030, \text{Fig. 4}$ ). Thus, the stronger the positive emotional arousal signaled by emission of FM 50 kHz USVs, the greater the suppression of the negative emotional arousal reflected by the smaller number of emitted 22 kHz USVs.

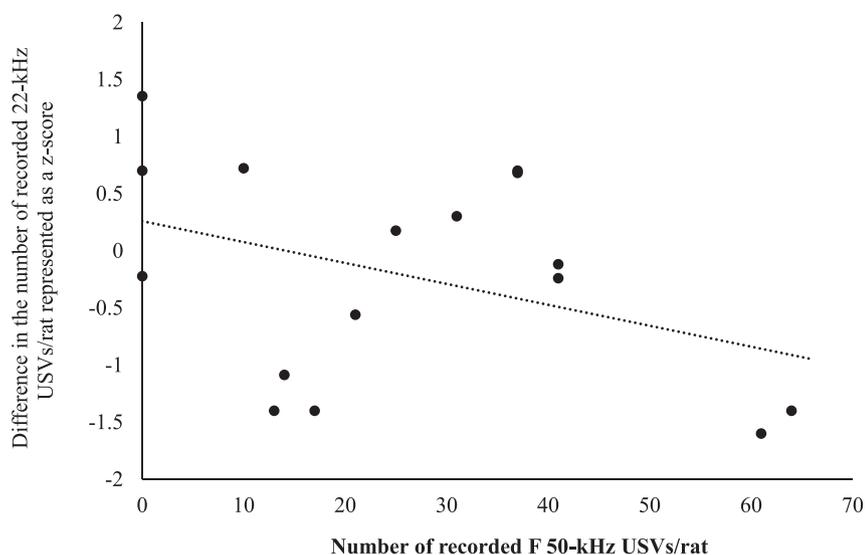
**Table 2**  
Pooled sonographic features of recorded USVs during injection conditions.

USVs	Duration (ms) of recorded USVs				Peak frequency (kHz) of recorded USVs			
	veh + sal	veh + carb	apo + carb	apo + sal	veh + sal	veh + carb	apo + carb	apo + sal
22-kHz	0	683.1 ± 33.8	662.9 ± 23.3	0	0	22.8 ± 0.34	23.3 ± 0.26	0
F 50-kHz	40.1 ± 1.1	N.A	N.A	32.6 ± 3.1	47.1 ± 1.4	N.A	N.A	53.2 ± 1.8
FM 50-kHz	28.5 ± 1.6	N.A	N.A	30.8 ± 2.1	58.2 ± 1.3	N.A	N.A	56.1 ± 2.9

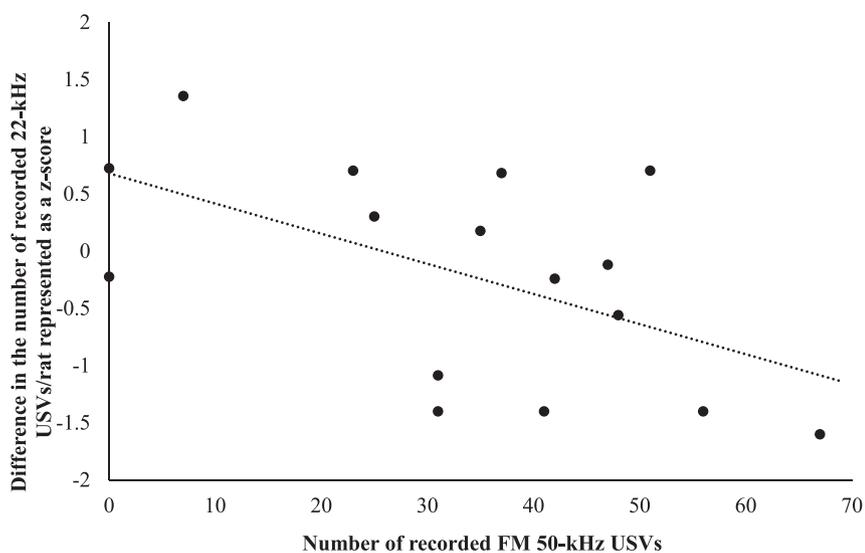
Note: Mean ( ± S.E.M) spectrographic parameters of recorded 22-kHz and 50-kHz USVs. There were no statistically significant differences between the duration or peak frequency of recorded 22-kHz USVs or 50-kHz USV across injection conditions. For abbreviations, see note to Table 1.

**3.4. Time-course of recorded 22-kHz USVs during veh + carb, and apo + carb injection conditions**

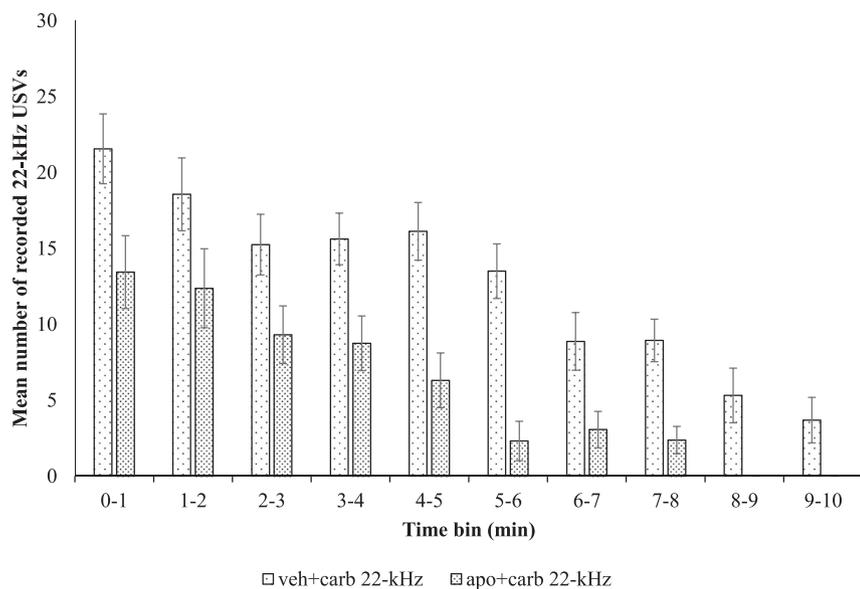
Plotting the number of recorded 22-kHz USVs as a function of time displays a slow decline in the number of emitted 22-kHz USVs over the duration of recording for both veh + carb and apo + carb injection conditions. During the veh + carb injection condition, the maximal responses occurred within the first three minutes then declined to a minimum response between the 9–10-min mark (see Fig. 5, n = 16). The overall pattern of recorded 22-kHz USVs was similar during the apo + carb injection with two notable differences. The first was the decreased magnitude of the response during each epoch, and the second difference was the cessation of vocalizations ending between 7 and 8 min epoch (see Fig. 5, n = 16).



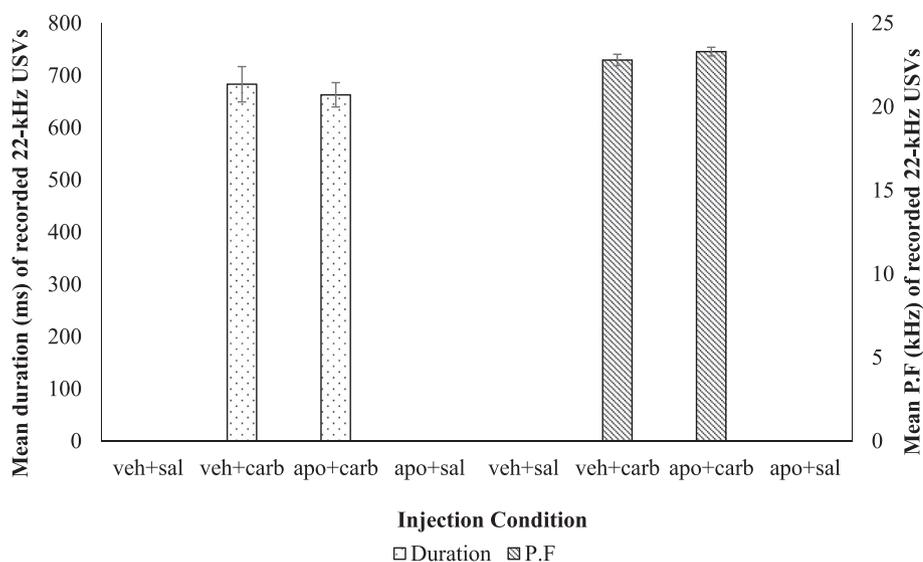
**Fig. 3.** Difference in the number of recorded 22-kHz USVs between veh + carb and apo + carb injection conditions expressed as a function of z-score (y-axis) and plotted as a function of the number of recorded F 50-kHz USVs during a 10-min recording session (x-axis). There was no significant correlation between the number of F 50-kHz USVs and the change in number of recorded 22-kHz USVs between veh + carb and apo + carb injection conditions ( $r_s[14] = -0.392, p = 0.133$ ). Each data point represents an individual rat. For abbreviations, see legend to Fig. 1A.



**Fig. 4.** Difference in the number of recorded 22-kHz USVs (standardized, y-axis) as a function of the number of FM 50-kHz USVs recorded during 10-min (x-axis). There was a statistically significant correlation between the number of emitted FM 50-kHz USVs during the apo + sal injection condition and the change in the number of recorded 22-kHz USVs between veh + carb and apo + carb conditions ( $r_s[14] = -0.541, p = 0.030; n = 16$ ).



**Fig. 5.** Time-course of the responses shown in 1 min bins for the number of recorded 22-kHz USVs during the veh + carb and apo + carb injection conditions. Overall, injection of apomorphine into the shell of the nucleus accumbens prior to injection of carbachol into LS decreased the quantity of 22-kHz USVs and terminated the response 120 s sooner.



**Fig. 6.** Mean ( $\pm$  S.E.M) values of spectrographic features of recorded 22-kHz USVs across injection conditions. There was no statistical difference in the single call duration (Duration) of recorded 22-kHz USVs ( $F[1.9, 46.5] = 0.368$ ,  $p = 0.11$ , partial  $\eta^2 = 0.015$ ) or in the peak frequency (P.F.) of recorded 22-kHz USVs ( $F[1.9, 46.8] = 1.5$ ,  $p = 0.32$ , partial  $\eta^2 = 0.059$ ).

### 3.5. Acoustic parameters of recorded USVs

A one-way repeated measures ANOVA was conducted to determine if there was a statistically significant difference in the call duration or peak frequency of recorded 22-kHz USVs across injection conditions. There was no statistical difference in the call duration of recorded 22-kHz USVs ( $F[1.9, 46.5] = 0.368$ ,  $p = 0.11$ , partial  $\eta^2 = 0.015$ ) or the peak frequency of recorded 22-kHz USVs ( $F[1.9, 46.8] = 1.5$ ,  $p = 0.32$ , partial  $\eta^2 = 0.059$ ) (Fig. 6; Table 2).

A one-way repeated measures ANOVA was also used to assess if there were any statistically significant differences in the duration or the peak frequency between F and FM 50 kHz calls. There was no difference for any injection condition to significantly alter the duration of F 50-kHz USVs ( $F[2.6, 63.8] = 4.83$ ,  $p = 8.84$ , partial  $\eta^2 = 0.168$ ) or FM 50-kHz USVs ( $F[2.4, 58.7] = 8.4$ ,  $p = 0.97$ , partial  $\eta^2 = 0.259$ ). There was also no difference in the peak frequency of F 50-kHz USVs ( $F[2.3, 56.4] = 3.0$ ,  $p = 0.61$ , partial  $\eta^2 = 0.11$ ) or FM 50-kHz USVs ( $F[2.6, 62.2] = 2.4$ ,  $p = 0.53$ , partial  $\eta^2 = 0.091$ ) across the injection conditions (Figure not shown; see Table 2 for values). These results confirmed that all emitted 22 kHz USVs and 50 kHz USVs were species-typical calls and were not modified by intracerebral injections.

## 4. Discussion

The purpose of the experiment was to determine if initiation of a positive emotive state, reflected by the emission of 50-kHz USVs, could decrease the number of emitted aversive 22-kHz USVs initiated via microinjection of carbachol into the LS.

### 4.1. Injection of R(-)-apomorphine into the nucleus accumbens shell increased the number and F and FM 50-kHz USVs

Injection of apomorphine into the medial shell of the nucleus accumbens produced species-typical F and FM 50-kHz USVs. These results were consistent with our previous reports, and with other publications, showing increased F and FM 50-kHz USVs after intracerebral injection of dopamine agonists into the medial shell of the nucleus accumbens, or systemic injection of dopamine agonists like amphetamine (Burgdorf et al., 2001; Wintink and Brudzynski, 2001; Thompson et al., 2006; Ahrens et al., 2009; Burgdorf and Moskal, 2010; Brudzynski et al., 2011, 2012; Brudzynski, 2015; Mulvihill and Brudzynski, 2019). Emission of 50-kHz USVs, and particularly FM 50-kHz calls seem to be indicative of the initiation of a positive emotional state since rats will emit these types of vocalizations during amphetamine-induced

conditional place preference (Ahrens et al., 2013; Knutson et al., 1999), during the anticipation of consumption of cocaine or sucrose (Browning et al., 2011), or during mating, or during juvenile play in rats (Burke et al., 2017).

Other studies using emotional modulation of the startle reflex, which expresses a negative state, have also reported that maximal startle amplitudes were decreased by systemic apomorphine (Martin-Iverson and Stevenson, 2005). Our results corroborate their findings and further provide evidence that suggests an antagonistic influence that a positive emotional state has on the expression of a negative emotional state.

### 4.2. Injection of carbachol into the lateral septum increased species-typical 22-kHz USVs

Our results indicated that injection of carbachol into the LS was able to significantly increase the mean number of 22-kHz USVs compared to control injections. Anatomically, the LS is located rostr dorsally to the anterior commissure and caudally to the nucleus accumbens and is involved in the expression of defensive behaviours, anxiety, and fear (Treit and Menard, 1997; Ouagazzal et al., 1999; Singewald et al., 2003; Reis et al., 2010). It is the most rostral portion of the medial cholinceptive vocalization strip (Brudzynski, 2001, 2010, 2013, 2014), i.e., the area from which cholinergic agonists can induce aversive arousal with the emission of 22 kHz USVs.

The medial cholinceptive vocalization strip is a strip of neural tissue that originates from the rostral division of the LDTg (Brudzynski, 2010, 2014, 2015). The cholinergic neurons within the LDTg form ascending pathways through the brain (mesolimbic cholinergic system) and terminate in extensive areas of the midbrain and forebrain. The release of acetylcholine from a limited subset of these pathways that terminates in the medial cholinceptive vocalization strip will initiate aversive arousal and emission of 22-kHz USVs (Brudzynski, 2010, 2014). Pharmacological activation of the areas of the strip by muscarinic acetylcholine agonists induced species-typical aversive 22-kHz USVs in rats. In the current study, intracerebral injection of carbachol into the LS, as the rostral part of the medial cholinceptive strip, produced species-typical 22-kHz USVs that did not differ from those reported in previous studies (Bihari et al., 2003; Brudzynski et al., 2011).

### 4.3. R(-)-apomorphine significantly decreased carbachol-induced 22 kHz USVs from the lateral septum

An interesting finding in the experiment was that FM 50-kHz USVs,

and not F 50-kHz USVs were correlated with a reduction in the emission of carbachol-induced 22-kHz USVs. This result suggests that, unlike F 50 kHz calls, emission of FM 50 kHz USVs are more indicative of positive emotive states than F 50-kHz USVs. In support of these results, it has been hypothesized that F 50 kHz vocalizations are primarily used as a social coordination signal (Brudzynski and Pniak, 2002; Snoeren and Ågmo, 2014; Wöhr et al., 2008). Since 50-kHz USVs are indirectly reflective of increased dopamine concentration in the shell of the nucleus accumbens and 22-kHz USVs are indirectly reflective of acetylcholine concentration along the medial cholinceptive vocalization strip, decreased vocal expression of 22-kHz USVs in response to apomorphine microinjections could indirectly reflect decreased levels of acetylcholine along the medial cholinceptive vocalization strip.

Despite apomorphine's antagonism of the aversive state induced by carbachol, apomorphine was unable to entirely eliminate emitted 22-kHz USVs. This could be a result of the pharmacokinetic profile of apomorphine or the efficacy of the drugs. Pharmacokinetic data in humans demonstrated a half-life time of apomorphine lasting approximately five minutes with clearance time around 4 h (Gancher et al., 1989). Given the design of the current experiment, there is a 120 s delay between the injection of apomorphine and carbachol. Thus it is possible there was a decay of apomorphine action before carbachol was injected into the LS. Another explanation could simply be attributed to the fact that carbachol could be more efficacious at initiating a negative emotional state than apomorphine is at initiating a positive emotional state.

## 5. Conclusions

It has been postulated that rat may signal only one of the emotional states at any given time and emissions of 22-kHz or 50-kHz calls are mutually exclusive (Brudzynski, 2007). Thus, the positive and negative emotional states remain in a mutually antagonistic relationship during the expression of an emotional state. The main purpose of the present experiment was to determine if the initiation of positive emotional state by apomorphine microinjection into the medial nucleus accumbens shell could decrease the magnitude of a subsequent negative emotional state induced by microinjection of carbachol into the LS. Our previous research has shown that inducing a positive emotional state by injecting R(-)-apomorphine into the shell of the nucleus accumbens decreased the magnitude of expression of a subsequent negative emotional state induced by carbachol injection into the AH-MPO, the main part of the medial cholinceptive vocalization strip. Our current findings extend this observation to the LS suggesting a broader antagonistic relationship between the mesolimbic dopamine system and the ascending mesolimbic cholinergic system terminating in the medial cholinceptive vocalization strip.

## Declaration of interests

None.

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