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Effect of microinjections of dopamine into the nucleus accumbens shell on emission of 50 kHz USV: Comparison with effects of D-amphetamine

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ABSTRACT

Systemic pharmacological manipulation of dopamine (DA) signaling has been central to many investigations of 50 kHz ultrasonic vocalizations (USVs) in the rat. In particular, the indirect DA releaser D-amphetamine (AMPH) has been used extensively in many such investigations. The possible unique character of the native transmitter relative to DA-stimulating drugs such as AMPH in inducing and modulating emission of 50 kHz USVs has not been investigated. Adult male Long Evans rats were tested with intracerebral application of DA into the nucleus accumbens shell at several doses (3.75 μg–120 μg) to determine its capacity to induce 50 kHz USV emission. Additionally, the call profile characteristics of intracerebral DA injections were compared with those of intracerebral application of AMPH. Results indicated that local increases in DA signaling within the nucleus accumbens shell are sufficient to increase 50 kHz call rate, reduce latency to call, and increase the degree of frequency modulation of emitted USVs. However, our results found that microinjections of DA were not as efficacious in either inducing 50 kHz USVs or increasing frequency modulation without antagonism of the dopamine reuptake transporter when compared with AMPH. In summary, these results support the notion that the native transmitter DA is driving the increase in frequency modulation seen after administration of DA stimulating drugs. These results also suggest that drugs affecting dopamine may be altering the 50 kHz call profile in a distinct manner from the native transmitter and thus caution should be used in interpreting their effects.

The behaviour of emitting ultrasonic vocalizations (USVs) by *Rattus norvegicus* has been established as a reliable marker of underlying emotional states (Brudzynski, 2009, 2015; Wöhr and Schwarting, 2013; Barker et al., 2015). The two primary categories of USVs emitted by adult rats are the 22 kHz and 50 kHz USV types. These broad call categories are defined by both sonographic character (including call duration, sound frequency and bandwidth) and the different behavioural situations (and accompanied emotional states) associated with their emission (Brudzynski, 2009, 2013). There is abundant evidence which relates these 22 and 50 kHz call categories with two distinct neurochemical brain systems (for review see Brudzynski et al., 2018). The ascending mesolimbic cholinergic system is responsible for initiating the aversive emotional arousal expressed by 22 kHz USV emission (Brudzynski and Bihari, 1990; Brudzynski, 1994, 2001, 2010; Brudzynski and Barnabi, 1996). In contrast, the ascending mesolimbic dopaminergic (DAergic) system appears responsible for initiating the positive emotional arousal expressed by 50 kHz USV emission (Burgdorf et al., 2007; Ciucci et al., 2007, 2009; Brudzynski, 2009; Scardochio et al., 2015). This system is characterized by projections of dopamine (DA) fibers, from neurons located in the ventral midbrain (e.g., in the ventral tegmental area; VTA) to rostral brain regions associated with emotional processing (Alcaro et al., 2007; Ikemoto, 2007). The most highly innervated rostral structures by ascending VTA DA fibers are the nucleus accumbens (NAc) and olfactory tubercle, which together form the ventral striatum (Swanson, 1982; Voorn et al., 2004; Ikemoto, 2007; Yetnikoff et al., 2014).

The emission of 50 kHz USVs occurs in a variety of positive appetitive contexts associated with the activity of this DA system, including both social and non-social rewarding situations (Knutson et al., 1998, 1999; Burgdorf et al., 2008; Brenes and Schwarting, 2015; Wöhr, 2018). For instance, Hori et al. (2013) found that 50 kHz USV emission in response to tickling (heterospecific play) was dependent on DA signaling within the NAc. Direct pharmacologically-induced release of DA at the terminal ends of these ascending mesolimbic pathways, such as via microinjections of amphetamine (AMPH) into the NAc, unconditionally elicited 50 kHz calling (Burgdorf et al., 2001; Thompson et al., 2006). Conversely, microinjections of AMPH into the caudate-putamen failed to influence rates of 50 kHz USV emission (Burgdorf et al., 2001). Beyond the use of AMPH, a range of agonistic DA agents have been linked with 50 kHz USV induction. The indirect agonist

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actions of systemic cocaine (Barker et al., 2010; Williams and Undieh, 2010, 2016), methylphenidate (Simola et al., 2012), and methamphetamine (Mahler et al., 2013), all similarly increased 50 kHz USV emission. All these drugs increase the concentration of DA within the synaptic cleft via altering the function of the plasma membrane transporter for DA (henceforth DAT; Zhu and Reith, 2008).

The dependence of 50 kHz USV emission on DA is further evidenced by studies disrupting the function of the ascending mesolimbic DA system. Depletion of DA levels in the rostral forebrain of animals by neurotoxic lesions of the ascending DA fibers greatly reduced the capacity of a rat to emit 50 kHz USVs to a previously rewarding stimulus (Burgdorf et al., 2007; Ciucci et al., 2007, 2009; Grant et al., 2015). While systemic (i.p.) antagonism of D_1-like and D_2-like DA receptors, both alone and in combination, significantly reduced 50 kHz USV production in response to an estrous female (Ringel et al., 2013). Similarly, systemic administration of DA antagonists was found to attenuate 50 kHz USV emission induced by cocaine or amphetamine (Williams and Undieh, 2010, 2016; Scardochio and Clarke, 2013; Wright et al., 2013). These findings indicate a necessary role of both D_1-like and D_2-like receptor subtypes for 50 kHz USV emission.

The use of non-psychostimulant drugs (including direct DA receptor agonists) to induce 50 kHz USV emission in a comparable manner to psychostimulants, however, has proved to be more complicated. Drugs that increase synaptic concentrations of DA (e.g., the DAT inhibitor GBR-12909) have failed to significantly induce 50 kHz USV emission in a manner comparable to AMPH when both were systemically applied (Wright et al., 2010; Wright et al., 2013). Systemic administration of the relatively non-selective D_1/D_2 agonist apomorphine was found by Williams and Undieh (2010) to significantly induce 50 kHz USV emission while systemic administration of D_1 agonist SKF38393 or D_2 agonist quinpirole alone did not. Moreover, administration of direct receptor-selective agonists alone and in certain combinations was found to inhibit 50 kHz calling (Scardochio and Clarke, 2013). However, this avenue of pharmacological research is limited by the fact that systemic administration affects all aspects of the underlying neural circuitry associated with 50 kHz USV emission. This is especially important in consideration of the contributing presence of D_2-autoreceptors at both somatodendritic and axonal terminal areas (Ford, 2014). Within the shell of the NAc, direct administration of quinpirole was found to induce 50 kHz USVs at a comparable rate to the response observed following 7 μg of AMPH (Brudzynski et al., 2012). This quinpirole-induced calling could be antagonized by either raclopride (D_2 antagonist) or U-99194A (D_3 antagonist), indicating contributions from both D_2 and D_3 receptors.

The various pharmacological agents used to induce 50 kHz USVs, including AMPH, or to antagonize specific DA receptors, appear to produce differences in the acoustic parameters of individual calls in addition to affecting the proportion of specific subtypes of USVs emitted (Wright et al., 2010, 2013; Brudzynski et al., 2012). It was found that AMPH selectively increased the proportion of trill calls (Wright et al., 2010), while the DAT blocker GBR-12909 was found to have failed to alter the 50 kHz USV profile (Wright et al., 2013). These differences may reflect the wide array of effects AMPH produces beyond increasing synaptic DA. AMPH is known to affect the noradrenergic and serotonergic systems as well as to inhibit the function of degradative enzymes (i.e., monoamine oxidase, MAO; Sulzer et al., 2005; Fleckenstein et al., 2007). Both the noradrenergic and serotonergic systems have themselves been implicated in both the induction of 50 kHz USV emission and the alteration of call profile (Wright et al., 2012; Wöhr et al., 2015).

Despite these off-target effects of AMPH, antagonism of D_1-like or D_2-like receptors was found to alter call profile and acoustic parameters of 50 kHz USVs, indicating differential contributions of receptor subtypes (Wright et al., 2013). Frequency-modulation of 50 kHz USVs appears particularly reliant on DA signaling, although, when drugs are given systemically it is difficult to rule out their effects on cranial nerve control of vocal fold function (Ciucci et al., 2009; Ringel et al., 2013). General system-wide antagonism of DA receptors may disrupt the animal's ability to articulate frequency-modulated vocalization by altering laryngeal function (Feng et al., 2009; Ringel et al., 2013).

The purpose of the present study was to investigate the capacity of using the native transmitter of DA as a pharmacological agent to induce 50 kHz USV emission when applied directly into the NAc. Given the complex landscape of off target actions that typical DAergic agents possess (particularly psychostimulants such as AMPH), using DA itself to increase the available concentration of transmitter capable of acting at post-synaptic receptors appears advantageous. Use of DA directly into the terminal field of the ascending mesolimbic DA system and comparing it with AMPH may reveal the character of 50 kHz USV emission primarily contributed by DA receptor action.

We hypothesized that microinjections of DA directly into the shell of the NAc would induce 50 kHz USV emission at a higher rate and with a decreased latency to call compared with vehicle. Additionally, the effects of DA on 50 kHz calling would differ as a function of increased dose and be susceptible to antagonism of D_2 receptors within the NAc shell. We further expected that the acoustic parameters of average call duration and bandwidth measured from individual 50 kHz USVs induced by DA microinjections would be greater than those following vehicle. It is this character of calling that appears most dependent on DA signaling (Ciucci et al., 2009; Ringel et al., 2013). In line with this, we expected DA microinjections to significantly increase the ratio of frequency-modulated USVs (FM calls) compared with flat calls. We did not expect that the acoustic character of 50 kHz USV calling or the ratio of FM calls would differ between DA and AMPH microinjections on the assumption that the calls induced by AMPH result primarily from increased DA concentration in the synaptic cleft. To test these hypotheses and predictions, we employed direct microinjections of DA at varying doses into the shell of the NAc and compared results with those after AMPH with or without pre-treatment of D_2 antagonist raclopride, DAT antagonist GBR-12909, or vehicle on the emission of 50 kHz USVs.

1. Methods

1.1. Subjects

All subjects were male Long Evans rats (obtained from Charles River Laboratories, Saint-Constant, QC, Canada). Subjects were given 5 days of acclimation time upon arrival into the animal facility. Before entry into the study, all animals were housed in pairs but following cannula implantation, they were housed singly. In accordance with Brock University protocols for laboratory handling, all animals were housed in polycarbonate cages (48 × 27 × 20 cm) with a plastic tube (polyvinyl) inside for hiding, an aspen block of wood for gnawing, and two paper towels for environmental enrichment. Cages were lined with dust-free corn cob bedding. The housing room had controlled room temperature (21 °C ± 2 °C) and humidity (40–60%). Subjects were housed with a maintained 12:12 h light/dark cycle and ad libitum access to standard rat chow (Harlan Laboratories, Wisconsin, USA) and water except while in the post-operative recovery period. During post-operative recovery from the stereotaxic surgical procedure, subjects were given access to wet chow and a purified dietary supplement (DietGel Boost®, ClearH20, Maine, USA). For the first day post-operation, housing cages were lined with paper-towels in place of bedding. All research protocols were approved by Brock University Animal Care and Use Committee and complied with guidelines and policies set forth by the Canadian Council on Animal Care.

1.2. Procedural overview

To investigate the possible dose-dependent effects of injected DA on 50 kHz USV emission, all animals received surgical implantation of guide cannulae through the skull into the brain. This cannulation

allowed for intracerebral microinjection of drugs directly into the shell of the nucleus accumbens (NAcSh). A total of 25 rats were used for NAcSh injections with 15 subjects receiving injections of vehicle and three of six possible doses of DA (experiment 1; 3.75 μg, 7.5 μg, 15 μg, 30 μg, 60 μg, or 120 μg) and 10 subjects receiving a pre-treatment injection followed by either AMPH or DA into the NAcSh (experiment 2 and 3, five animals each). These latter experimental groups were used to better characterize the pharmacological response to DA microinjections and determine USV profile. Experiments 2 and 3 consisted of paired-injections (intracerebral or subcutaneous pre-treatment + intracerebral microinjection treatment). Experiment 2 involved intracerebral injections of vehicle + dopamine (Veh + DA), raclopride + dopamine (Rac + DA), and vehicle + AMPH (Veh + AMPH). Experiment 3 involved subcutaneous pretreatment with GBR-12909 + intracerebral dopamine (GBR + DA) and subcutaneous pretreatment with vehicle + intracerebral AMPH (Veh + AMPH). GBR-12909 was used because it is a potent dopamine reuptake inhibitor (antagonist to the plasmolemmal DAT) (Andersen, 1989). Systemic application of this DAT antagonist has been employed in investigations of USVs previously (Wright et al., 2013).

Recordings (10 min in duration) of USVs were carried out for all subjects immediately following any given microinjection. Number of calls and sonographic parameters of individual calls were than analyzed for all recordings. The testing order for experiment 1 involved randomized order of all micro-injections (three DA doses as well as vehicle). In addition to this injection-order counterbalance each injection was separated by 7 days to provide a washout period and reduce the likelihood of sensitization. For experiment 2 the injection order for Rac + DA and Veh + DA was randomly counterbalanced across subjects, however, Veh + AMPH was the last injection for all subjects. This was done to prevent sensitization to AMPH from contaminating the other injections, although, again each injection was separated by 7 days. For experiment 3, a fixed injection schedule was employed with GBR + DA received first followed by Veh + AMPH after a 7 day washout period.

1.3. Stereotaxic surgeries

At the time of surgery, all subjects weighed approximately 300–350 g. All animals underwent stereotaxic surgery to receive implantations of stainless-steel guide cannulae (640 μm outer diameter, made from 23 gauge needles) bilaterally into the shell of the nucleus accumbens (NAc). In preparation for the surgical procedure the rats were anesthetized with isoflurane (5% induction, 2% maintenance) and mounted on a stereotaxic frame (Model 900, David Kopf Instruments, Tujunga, CA, USA). Upon achievement of appropriate anesthetic sleep depth, a dose of enrofloxacin antibiotic (enrofloxacin, Baytril®, Bayer DVM, at a dose of 5 mg/kg) was administered to reduce risk of infection and a dose of analgesic (meloxicam, 2 mg/kg, Metacam, Boehringer Ingelheim Vetmedica, GmbH) was administered to reduce pain and discomfort upon waking. The surgical site was prepared by application of a 7% iodine scrub solution, followed by 70% isopropanol, and finally locally treated with 10% iodine.

Implantation coordinates for the NAcSH were 9.96 to 10.2 mm anterior to interaural line (AP), 1.0 mm lateral from midline (L), and 6.0 to 7.0 mm ventral from brain surface (V) according to coordinates from a stereotaxic atlas (Paxinos and Watson, 1986). Guide cannulae were secured to the skull using small stainless-steel screws and dental acrylic (DenPlus, Longueuil, QC) and were plugged with removable stainless-steel wires. No procedures took place on rats until five days post-surgery and only on healthy subjects, behavioural procedures (starting with habituation) began no later than 7 days post-surgery. Surgical procedures and post-operative care of the animals was always done with the supervision of an appointed veterinarian.

1.4. Drug injections and preparation

Stainless steel injection cannulae (30 gauge needles; 310 μm outer diameter, Beckton-Dickinson Canada, Mississauga, ON) connected to a Hamilton constant rate microsyringe (CR-700-20, Hamilton Company, Reno, NV) were used for intracerebral injections for all subjects. Injections were carried out at a rate of 0.2 μl/min with a volume of 0.3–0.5 μl injected at one side of the brain with hemisphere of injection counterbalanced across subjects. After injecting the given experimental substance, the injection cannula was left inside the guide cannula for at least 30 s to allow time for diffusion of the agent away from the cannula tip.

All drug solutions were prepared fresh the day of injection using sterile isotonic saline and were buffered to a pH of 5.5. Dopamine hydrochloride (Sigma-Aldrich Canada Ltd., Oakville, ON) was dissolved in varying concentrations for intracerebral application in several different doses (3.75 μg, 7.5 μg, 15 μg, 30 μg, 60 μg, or 120 μg) in 0.5 μl vehicle for animals in experiment 1. Vehicle and injectable solutions of DA contained 0.5% ascorbic acid as an antioxidant. Raclopride L-tartrate (Sigma-Aldrich, ON) was prepared for an intracerebral pre-treatment dose of 6.8 μg in 0.2 μl vehicle. D-Amphetamine sulfate (dextroamphetamine, Sigma-Aldrich, Great Britain) was prepared for an intracerebral dose of 7 μg in 0.3 μl vehicle. GBR-12909 (Sigma-Aldrich, ON) was prepared for a systemic pre-treatment dose of 5 mg/kg s.c. in 0.5 ml vehicle. Each brain site was injected no more than four times.

The pre-treatment and treatment injections were separated by 10 min (with the animal in the home cage) and were given in a volume of 0.5 ml (s.c.) or 0.2–0.5 μl (intracerebral microinjection, see below) respectively. The dose of DA used for comparison with AMPH and raclopride microinjections into the NAcSh was 6 μg in 0.2 μl volume of vehicle. The dose of DA used for comparison with AMPH and GBR-12909 was 15 μg in 0.5 μl volume of vehicle. The dose of raclopride used for microinjections was 6.8 μg in 0.2 μl vehicle. The dose of AMPH for intracerebral microinjections was 7 μg in 0.3 μl vehicle. Doses were chosen for comparable molarity of microinjections across groups but also for half equimolar amount of raclopride relative to DA and near equimolar amount of DA and AMPH.

1.5. Histological procedure

At the end of the study, all animals were anesthetized by an overdose of barbiturate (sodium pentobarbital, Euthanyl, Vetoquinol N-A, Quebec, Canada). Brains were transcardially perfused and postfixed in a 10% formalin solution before being sectioned with a freezing microtome (Cryo-Histomat, Hacker Instruments and Industries, Fairfield, NJ). Histological sections (40–50 μm thick) were stained with thionine according to the original staining method by Windle et al. (1943), and looked at under a light microscope to localize sites of injections. Localized sites were mapped onto coronal sections of the rat brain using a stereotaxic atlas (Paxinos and Watson, 1986). For a representative visual composite of localized injection sites see Fig. 9.

1.6. Recording and analysis of ultrasonic vocalizations

All subjects received habituation to the injection and recording procedure for three days following recovery from the surgical procedure. This habituation involved gentle handling and exposure to the recording environment. The recording environment was dimly lit with a single table-top direct-current lamp. The recording procedure itself consisted of the subject being transported to the recording room in its home cage, it was taken out of the cage, then gently handled and given an intracerebral injection before being placed back into its home cage for 1 min. The animal was then placed into a recording chamber (25 cm wide × 18 cm deep × 18 cm height polycarbonate cage) where USV production was recorded for 10 min. The recording chamber was filled with fresh corn cob bedding and was not re-used across rats; every

subject received a new chamber for each recording.

All recordings were made using an UltraSoundGate CM16/CMPA (Avisoft Bioacoustics, Glienicke, Germany) condenser microphone (working frequency range 2–250 kHz) located on top of the recording apparatus on a metal grate (approximately 25 cm from the animal). The microphone was connected via an UltraSoundGate 416 USB audio device (Avisoft Bioacoustics) to a computer (Dell PC) and recordings were made using multi-channel triggering hard-disk software (Avisoft RECORDER version 4.40). Acoustic data were recorded at a sampling rate of 250 kHz in 16-bit format. Analysis of USVs was done off-line using Avisoft SASLab Pro (version 4.40) and Sonotrack™ (Metris BV, The Netherlands) software (version 4.40).

USVs were analyzed and the identification and characterization of USVs was accomplished in a manner as described and used previously in several papers (Brudzynski, 2009, 2015; Mulvihill and Brudzynski, 2018a, 2018b). Briefly, 50 kHz USVs had peak frequencies between 35 and 90 kHz, were typically < 100 ms in duration, and had varying degrees of frequency modulation. 22 kHz USVs were rare or absent but would be identified by having a low peak frequency (20–30 kHz), long call duration, and with constant frequency. Given the virtual absence of 22 kHz USVs this call type was omitted from the analysis. The two analysis programs were used for distinct and non-overlapping analyses in separate groups of animals. Reliability analysis for determining number of 50 kHz calls between the two programs indicated that performance of the Sonotrack program is comparable to a competent experimenter performing manual detection using Avisoft program (Intraclass correlation coefficient = 0.945, 95% CI: 0.758, 988). Avisoft SASLab generated spectrograms were manually screened for 50 kHz USVs and were used to calculate sonographic parameters of peak frequency (in kHz), call duration (in ms), and bandwidth (in kHz) of individual calls for experiment 1 only. Additionally, Avisoft SASlab was used to determine numbers of 50 kHz USV subtypes, which were used to calculate the FM ratio for all experiments. Sonotrack generated spectrograms were utilized for preprogrammed automatic screening which determined number of USVs, mean call duration, and mean sound frequency of USVs for the Pre-treatment group only. For automatic detection of 50 kHz calls by Sonotrack, a bandpass filter was employed to reduce background noise (low and high cut-off frequencies of 35 and 90 kHz, respectively). All spectrograms were generated using a fast Fourier transform (512 FFT-length, 100% frame, Flat Top window, and 75% time window overlap), at 488 Hz of frequency resolution.

USV subtype determination was based on sonographic shape. 50 kHz calls were classified into the flat subtype if they appeared to have a relatively constant frequency (bandwidth < 6 kHz). If the calls were frequency-modulated (FM) they were classified as either trill or non-trill subtypes. For calculating FM to flat call ratios all subtypes with frequency modulation (both trill and non-trill FM calls) were counted and divided by the number of flat 50 kHz USVs. Manual screening of 50 kHz USVs was accomplished by one trained experimenter.

1.7. Statistics

All statistical analyses were performed using SPSS Statistics (version 20, IBM Corporation). For experiment 1 comparisons, any variables with violations of the assumption of normality were corrected using nonlinear transformations. To accomplish this correction scores were all logarithmically transformed using the natural base of *e* following a linear transformation of +1. To initially establish an effect of DA independent of dose, the subject's DA injection scores were averaged and compared with vehicle using a paired *t*-test. For investigating the effect of doses, a repeated-measures ANOVA was used across DA injection dose-bins for the given within-subjects variable. DA dose-bins were constructed to account for the unequal n across each objective dose by converting the DA dose data into within-subjects variables. This was accomplished by categorizing injections within each individual subject into low, medium, or high relative doses. Given the low valid sample sizes for analyses involving the Pre-treatment group only non-parametric tests were employed. Wilcoxon signed-rank tests were used for comparisons between two paired variables in the Pre-treatment group analyses.

A total of 41 animals were subjected to experimental manipulation (experiment 1: 27, experiment 2 and 3: 14), however, for analysis of USV call induction only subjects with valid injection site localization were used (16 subjects excluded). For any analysis on parameters of individual calls any non-callers were excluded in addition to these localization invalid subjects. The associated n of any analysis is reported with its respective figure, where appropriate.

Where suitable, bias corrected accelerated (BCa) confidence intervals are reported utilizing 1000 bootstrap samples.

2. Results

2.1. Induction of 50 kHz USV emission by intracerebral DA microinjections

Collapsed across doses and compared with vehicle for experiment 1, there was evidence that DA induced 50 kHz call emission ($t_{(11)} = 2.55$, $p = .027$, BCa 95% CI [0.200, 1.170]). The recorded response to DA ($M = 6.65$, $SD = 8.11$ USVs/min, range (difference between highest and lowest) = 28.1) had significantly elevated USV emission relative to vehicle ($M = 4.55$, $SD = 7.21$ USVs/min, range = 17.8; see Fig. 1a). Call rate in the two conditions was found to be significantly correlated ($r = 0.70$, $p = .011$). This effect of DA compared with vehicle was not found in subjects where the injection site was localized outside of the NAcSH ($t_{(7)} = 1.87$, $p = .104$, BCa 95% CI [−1.29, −0.018]). To investigate any difference in call rate across doses of DA a repeated measures ANOVA was conducted on calls calculated as a percent change of vehicle across dose bins (low, medium, and high). No significant difference was found in call emission as a percent change of vehicle across these dose bins ($F_{(2, 20)} = 2.14$, $p = .143$), although the difference between low and medium doses trended towards significance ($F_{(1, 10)} = 4.59$, $p = .058$; see Fig. 1b).

Additionally, there was an effect of DA (collapsed across dose) to decrease latency to call (measured in seconds) compared with vehicle ($t_{(7)} = 2.55$, $p = .038$, BCa 95% CI [10.50, 57.12]). On average across DA recordings, there was a latency of 22 s (SD = 19.43) to emit 50 kHz USVs which was significantly shorter than the average latency of 55 s (SD = 47.54) observed after vehicle (see Fig. 2). There was no significant difference found in latency to emit calls among particular doses of DA ($F_{(2, 14)} = 2.14$, $p = .155$).

2.2. Effect of intracerebral DA microinjections on acoustic parameters of individual calls

No significant difference was found in measured acoustic parameters (call duration, peak frequency, and bandwidth) between DA-induced USVs (collapsed across dose) and calls after vehicle microinjection recordings ($F_{(1, 7)} = 0.58$, $p = .472$; see Fig. 3). There was also no evidence of any significant difference across doses of DA on average call duration ($F_{(2, 14)} = 0.89$, $p = .432$) or peak frequency ($F_{(2, 14)} = 1.78$, $p = .204$). For the average bandwidth of recorded calls however, there was a significant difference found across DA doses ($F_{(2, 14)} = 4.10$, $p = .040$). Pairwise comparisons found this difference resulted from the average bandwidth of calls recorded in low-dose conditions being significantly higher than those recorded under medium dose conditions ($p = .030$), but not under high dose conditions (see Fig. 4). An analysis of the relationship between 50 kHz call rate induced by DA and average bandwidth induced by DA found a significant positive correlation ($r = 0.613$, $p = .026$). Individual rats that produced more USVs in response to DA microinjections appeared to have a greater likelihood of frequency modulation among individual calls (see Fig. 5).

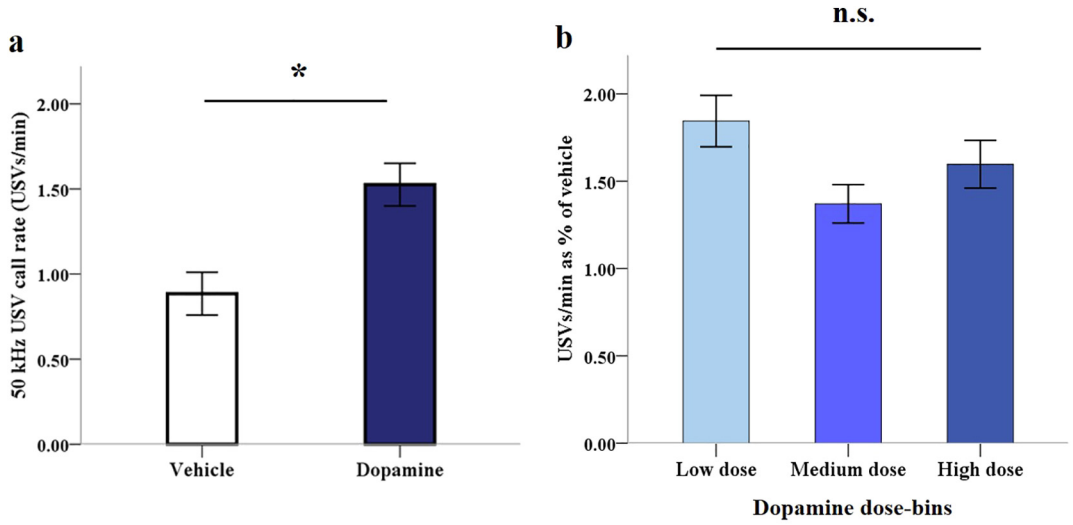

Fig. 1. (**a**) Comparison of recorded 50 kHz USV call rate measured as number of USVs per min between vehicle and dopamine microinjections (n = 12) for experiment 1. Dopamine call rate represents average call rate collapsed across dose. Dopamine induced significantly higher call rate compared with vehicle at $p < .05$. Results are represented as means ± SEM following logarithmic transformation using the natural base of *e*. (**b**) Comparison of recorded 50 kHz USV call rate measured as number of USVs per min across dopamine dose-bins as a function of percent of vehicle (n = 11) for experiment 1. No significant effect of dose-bin was found, although low dose trended towards statistical difference from medium dose. Results are represented as means ± SEM as percent of vehicle following logarithmic transformation using the natural base of *e*. * = $p < .05$. Use of (**n.s.**) denotes no significant differences across dose bins (at $p > .05$).

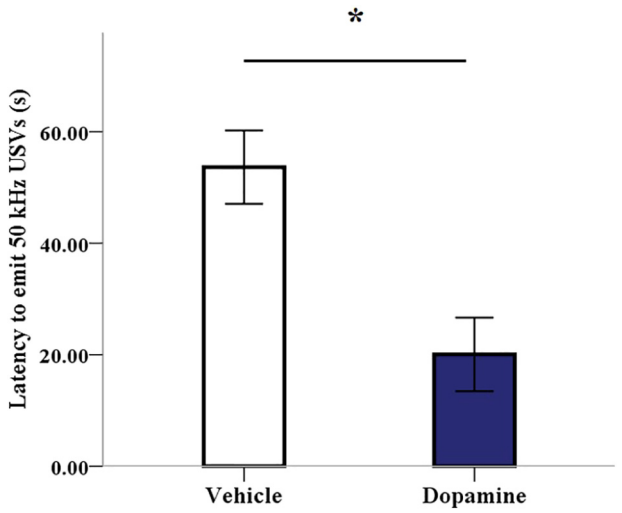

Fig. 2. Comparison of latency to emit 50 kHz USVs measured in seconds between vehicle and dopamine microinjections (n = 8) for experiment 1. Dopamine data represents average latency collapsed across doses. Dopamine microinjection recordings had a significantly lower latency to emit calls. Results are represented as means ± SEM. * = $p < .05$.

2.3. Effect of intracerebral DA microinjections on 50 kHz USV FM/flat ratio

Consistent with the finding of a positive correlation between DA-induced call rate and bandwidth, there was evidence found that DA altered the ratio of frequency modulated (FM) over flat 50 kHz USVs (FM/flat) when collapsed across dose and compared with FM/flat ratio after vehicle ($t_{(7)} = 2.40$, $p = .047$, BCa 95% CI [0.186, 1.009], see Fig. 6). Recordings following DA microinjections had a greater average FM/flat USV ratio ($M = 2.57$, $SD = 1.28$) relative to vehicle ($M = 1.96$, $SD = 1.39$). No significant difference was found for FM/flat ratio among particular doses of DA ($F_{(2, 14)} = 0.87$, $p = .440$).

2.4. Comparison of intracerebral DA microinjections with AMPH and GBR-12909

In a separate group of animals (experiment 2, n = 5), microinjections of vehicle into the NAcSh followed by AMPH (Veh + AMPH) was found to increase 50 kHz USV call rate compared to vehicle followed by DA (Veh + DA; *Medians* of 1.1 and 0.2 USVs/min, ranges of 8.4 and 0.8 respectively; see Fig. 7a). This median difference was found to be statistically significant (Wilcoxon signed-ranks test, $Z = 2.03$, $p = .042$, $r = 0.91$). In addition to increasing call rate, Veh + AMPH increased average duration of individual USVs ($Z = 2.02$, $p = .043$, $r = 0.91$; see Fig. 7b), though had no detectable effect on average sound frequency of individual USVs compared with Veh + DA condition ($Z = 0.67$, $p = .500$; see Fig. 7c). In contrast, intracerebral pre-treatment with the D_2 receptor antagonist raclopride (6.8 µg in 0.2 µl vehicle) prior to DA, produced no difference in median call rate ($Z = 1.07$, $p = .285$), average call duration ($Z = 1.83$, $p = .068$) or sound frequency ($Z = 0.36$, $p = .715$) of individual calls compared with Veh + DA condition.

In an additional group of animals (experiment 3, n = 5), blocking systemic DA re-uptake via systemic pre-treatment with GBR-12909 (GBR + DA, 5 mg/kg s.c. + 15 µg/0.5 µl intracerebrally) appeared to mitigate the difference observed between DA and AMPH induction of USVs. The 50 kHz USV call rate following GBR + DA (*Median* = 1.9 USVs/min, range = 2.5) was lower than call rate after AMPH microinjections following s.c. vehicle pre-treatment (*Median* = 2.8 USVs/min, range = 8.9). This difference however, although trending towards it, did not reach statistical significance ($Z = 1.83$, $p = .068$; see Fig. 8a). There was also no significant difference found in latency to call (measured in s) between these injection conditions ($Z = 0.73$, $p = .465$).

GBR + DA was also not found to significantly differ in average duration ($Z = 0.94$, $p = .345$, Fig. 8b) or sound frequency ($Z = 1.21$, $p = .225$, Fig. 8c) of individual 50 kHz USVs when compared with calls after systemic vehicle paired with microinjections of AMPH into the NAcSh. Investigating the ratio of FM relative to flat 50 kHz USVs between GBR + DA and AMPH induction found a significant difference ($Z = 2.02$, $p = .043$). Microinjections of AMPH into the NAcSh appeared to induce a greater number of FM calls relative to flat when compared with DA microinjections following GBR-12909 pretreatment

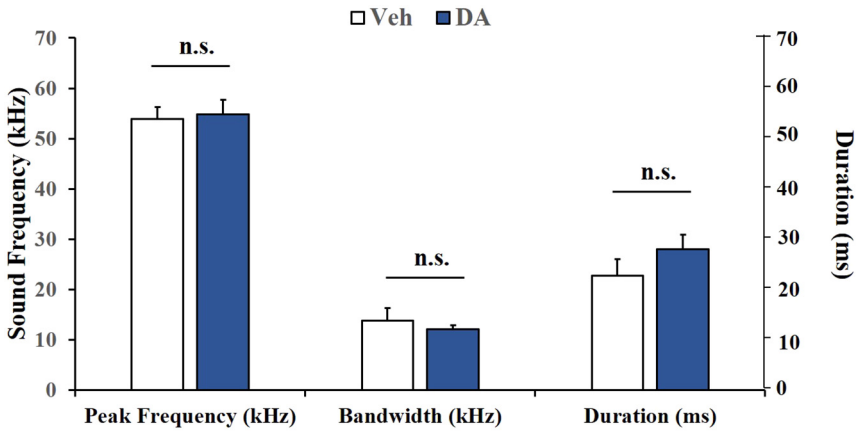

Fig. 3. Comparison of acoustic parameters of individual 50 kHz USVs between vehicle (Veh) and dopamine (DA) microinjections (n = 8) for experiment 1. Dopamine data represents averages collapsed across doses. Peak frequency in kHz, bandwidth in kHz, and duration in milliseconds were measured for individual single calls. Bandwidth is expressed on the same axis as peak frequency in kHz. No significant differences were found between injection conditions for all acoustic parameters measured. Results are represented as means ± SEM. Other abbreviations as in Fig. 1.

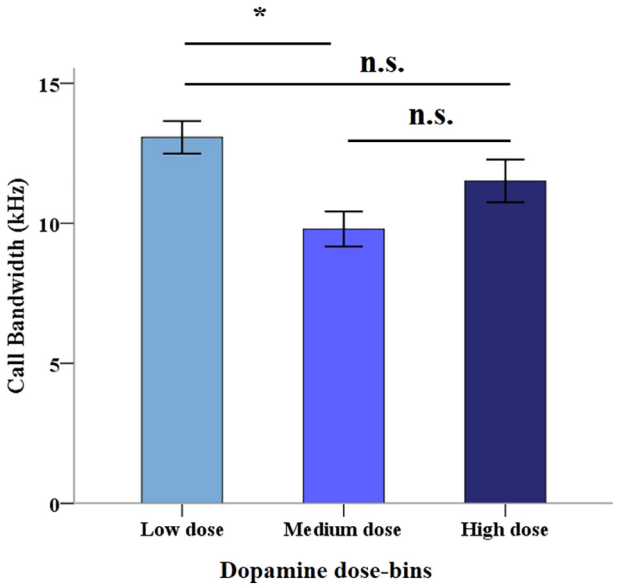

Fig. 4. Comparison of average bandwidth of individual 50 kHz USVs across dopamine dose-bins (n = 8) for experiment 1. Bandwidth (measured in kHz) was found to be significantly higher under low dopamine dose conditions when compared with medium doses at $p < .05$. Results are represented as means ± SEM.

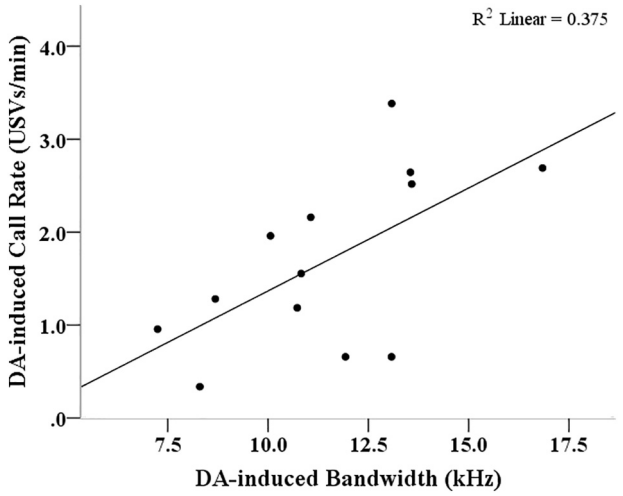

Fig. 5. Scatterplot illustrating the positive relationship between log-transformed 50 kHz USV call rate induced by dopamine and average bandwidth of individual 50 kHz calls induced by dopamine (n = 13) in experiment 1. Dopamine induced call rate represents average call rate collapsed across dose following logarithmic transformation using the natural base of *e*. Dopamine induced bandwidth represents average bandwidth collapsed across doses in kHz. Correlation coefficient was found to be significant at $p < .05$.

(see Fig. 8d).

3. Discussion

The purpose of this study was to explore the characteristics of 50 kHz USVs induced directly by DA microinjections into the shell of the NAc. The results support the hypothesis that DA microinjections (collapsed across doses ranging from 3.75 µg to 120 µg) can induce higher rates of 50 kHz USV emission with decreased latency as compared to calls after vehicle. However, we failed to find support for any differences in 50 kHz USV call rate induced across the individual doses used. Moreover, there was no evidence found that DA microinjections altered the acoustic parameters (duration, peak frequency, and bandwidth) of individual calls compared with those after vehicle. Unexpectedly, comparison among results of DA injections revealed differences in the average bandwidth of calls with the lowest doses producing the highest average bandwidth recorded. There was a significant positive relationship found between call rate induced by DA and the magnitude of average bandwidth of individual calls induced by DA. This finding was consistent with the hypothesis of a link between DA and frequency modulation.

Support for the hypothesis that direct intracerebral application of DA would affect the degree of frequency modulation observed among 50 kHz USVs was further found in comparisons of the ratio of FM to flat calls between recordings after DA or vehicle. Results found that calls recorded following DA injections had a higher ratio of FM calls relative to vehicle, which is in line with literature reports employing DA receptor agonists and antagonists (Wright et al., 2013).

In contrast with expectations, comparing results of DA and AMPH microinjections found that 7 µg AMPH was more effective at inducing 50 kHz USVs than DA and did appear to alter acoustic parameters of recorded calls. AMPH injections increased the average duration of individual calls compared with those after 15 µg DA though no difference was found for average sound frequency of calls. In contrast to several studies using psychostimulant USV induction (Thompson et al., 2006; Williams and Undieh, 2010; Wright et al., 2013), the hypothesis that raclopride antagonism of D_2 receptors prior to DA administration would affect the character of calling was not supported. Raclopride pretreatment was not found to alter the measured acoustic parameters of individual calls induced by DA when compared with vehicle pretreatment.

Employing a systemic application of the DAT antagonist (GBR-12909) prior to DA microinjections was found to attenuate the

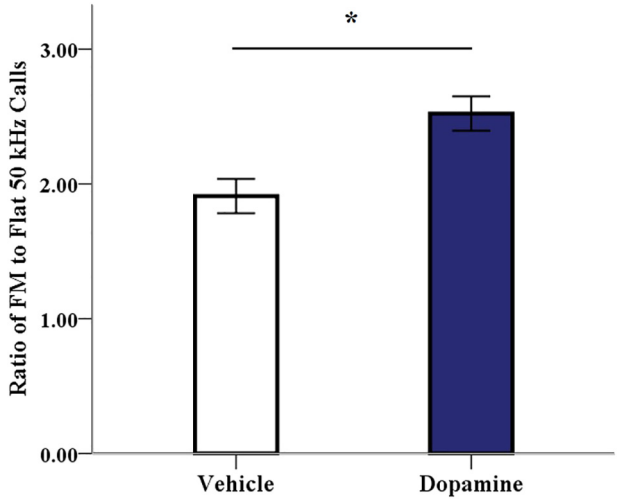

Fig. 6. Comparison of the ratio of frequency modulated (FM) to flat 50 kHz USVs recorded after vehicle and dopamine microinjections (n = 8) in experiment 1. Dopamine data represents average FM to flat ratio collapsed across doses. Dopamine microinjection recordings had a significantly higher ratio of FM to flat 50 kHz calls compared with vehicle. Results are represented as means ± SEM. * = $p < .05$.

differences in 50 kHz USV emission between DA and AMPH injections. Call rate induced by DA following GBR-12909 pretreatment was not significantly different from that after AMPH (though trended towards it). Given the lack of a control for the effect of GBR 12909 alone, we are unfortunately limited in our ability to comment on the specific contribution of dopamine within this comparison. The acoustic parameters of average duration and average sound frequency were also no longer different between DA and AMPH-induced 50 kHz USVs following GBR-12909 pretreatment. However, AMPH was found to induce a greater ratio of FM to flat calls compared with the ratio after DA following pretreatment with a transporter antagonist. Thus, the hypothesis that there would be no difference in the ratio of FM calls between AMPH and DA induced 50 kHz USVs was not supported. This dissimilar finding between ratio of frequency modulated calls and acoustic parameters may suggest differential sensitivity among these measures. AMPH has been found to preferentially increase the trill call subtype (Wright et al., 2010) and this appears consistent with our finding of increased FM calls relative to flat. One possible explanation as to why this increase in FM calls was not reflected in analysis of acoustic parameters may be due to the sonographic nature of the increased FM calls. Trill calls, which may have driven the difference, depend on peak-frequency modulation and our measure of average sound frequency may not have reflected this sonographic difference (Pereira et al., 2014).

In aggregate, the results of this study support the hypothesis that drugs capable of inducing 50 kHz USVs via increasing synaptic DA concentration (i.e. AMPH) reflect primarily the influence of DA within the NAcSh on 50 kHz call profile and acoustic parameters. Furthermore, the apparent relation of frequency modulation of 50 kHz USVs and DA found in studies employing invasive disruptions or exogenous ligands to alter the function of the mesolimbic DA system was supported using the native transmitter. To our knowledge, this study represents the first use of DA as a pharmacological agent to induce emission of 50 kHz USVs from the rat.

The lack of a difference in 50 kHz USV emission rate across DA doses found in the present study was unexpected. This finding likely informs that the doses and local microinjection procedure used in the current study were insufficient to overcome brain mechanisms of DA elimination. Prior work employing intracerebral DA microinjections to explore its behavioural effects in rats utilized a MAO inhibitor in order to prolong the synaptic action of DA (Pijnenburg and Van Rossum, 1973; Costall and Naylor, 1975; Jackson et al., 1975; Pijnenburg et al., 1975). In the current work we did not employ any such inhibitor as this would limit the value of comparing the effect of DA injections with that of AMPH injections, because as mentioned, one of the effects of AMPH is to inhibit the functional activity of MAO (Sulzer et al., 2005). Instead, we used an antagonist of the DA plasma membrane transporter (GBR-12909) to prolong the synaptic action of the injected DA. This pretreatment was found to show a trend of increasing the number of 50 kHz USVs induced by DA and abolished many of the differences that were found between DA and AMPH following only a vehicle pretreatment.

Even without MAO inhibition, Pijnenburg et al. (1976) found a stimulatory effect on locomotor activity with a dose of DA as low as 5 µg

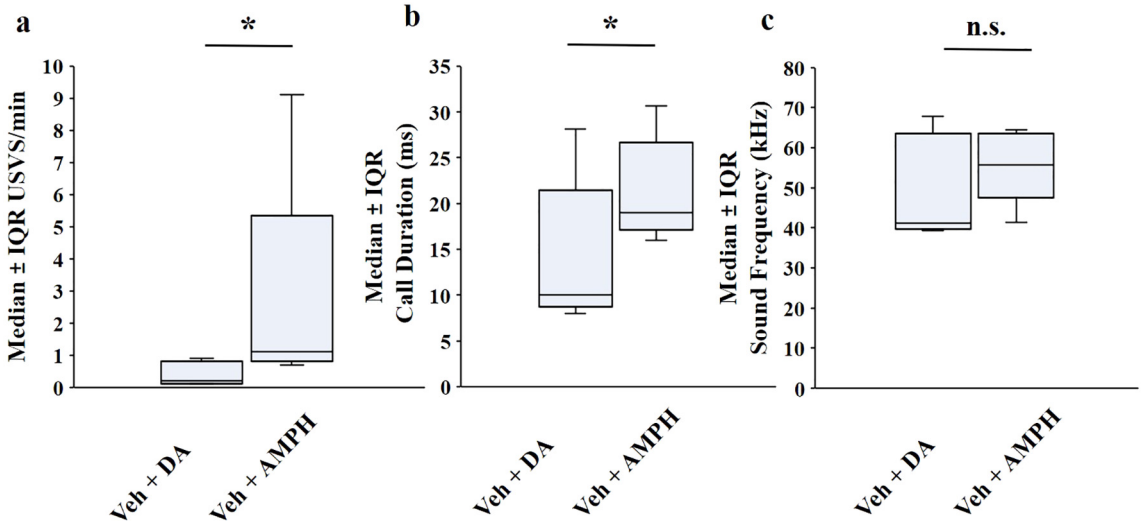

Fig. 7. (**a**) Comparison of recorded 50 kHz USV call rate measured as number of USVs per min after intracerebral vehicle pre-treatment with dopamine microinjections (Veh + DA) and intracerebral vehicle pre-treatment with amphetamine microinjections (Veh + AMPH) in experiment 2. (**b**) Comparison of average call duration (measured in ms) of individual 50 kHz USVs recorded after Veh + DA or Veh + AMPH microinjections in experiment 2. (**c**) Comparison of average sound frequency (measured in kHz) of individual 50 kHz USVs recorded after Veh + DA or Veh + AMPH microinjections in experiment 2. For all panels results are represented as medians ± the interquartile range (IQR) with n of 5. * - represents significant results of a related samples Wilcoxon signed-rank test at $p < .05$ level. Use of (**n.s.**) denotes no significant difference.

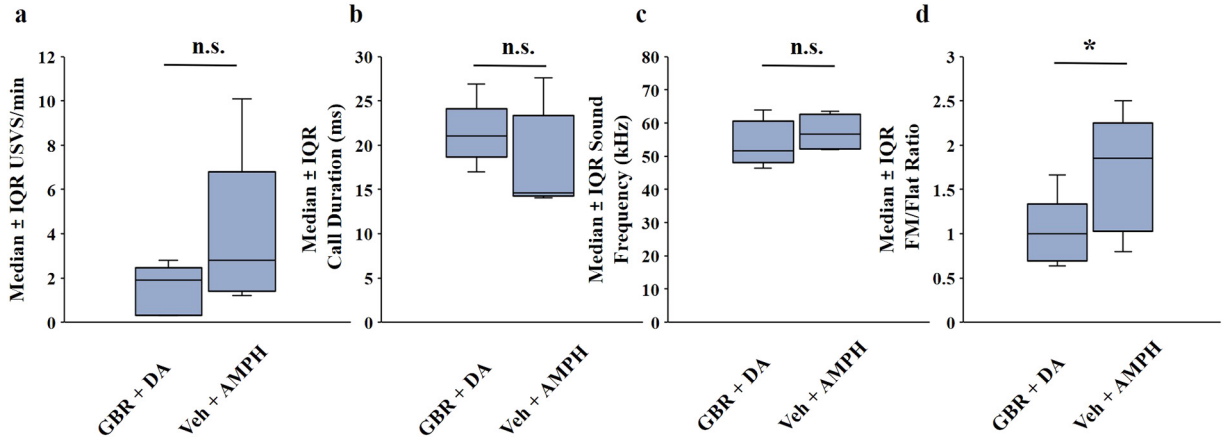

Fig. 8. (**a**) Comparison of recorded 50 kHz USV call rate measured as number of USVs per min after systemic GBR-12909 pre-treatment with dopamine microinjections (GBR + DA) and systemic vehicle pre-treatment with amphetamine microinjections (Veh + AMPH) in experiment 3. (**b**) Comparison of average call duration (measured in ms) of individual 50 kHz USVs recorded after GBR + DA and Veh + AMPH in experiment 3. (**c**) Comparison of average sound frequency (measured in kHz) of individual 50 kHz USVs recorded after GBR + DA and Veh + AMPH in experiment 3. (**d**) Comparison of the ratio of frequency modulated (FM) to flat 50 kHz USVs recorded after GBR + DA and Veh + AMPH in experiment 3. Results are represented as medians ± the interquartile range (IQR) with n of 5. * - represents significant results of a related samples Wilcoxon signed-rank test at $p < .05$ level. Use of (**n.s.**) denotes no significant difference.

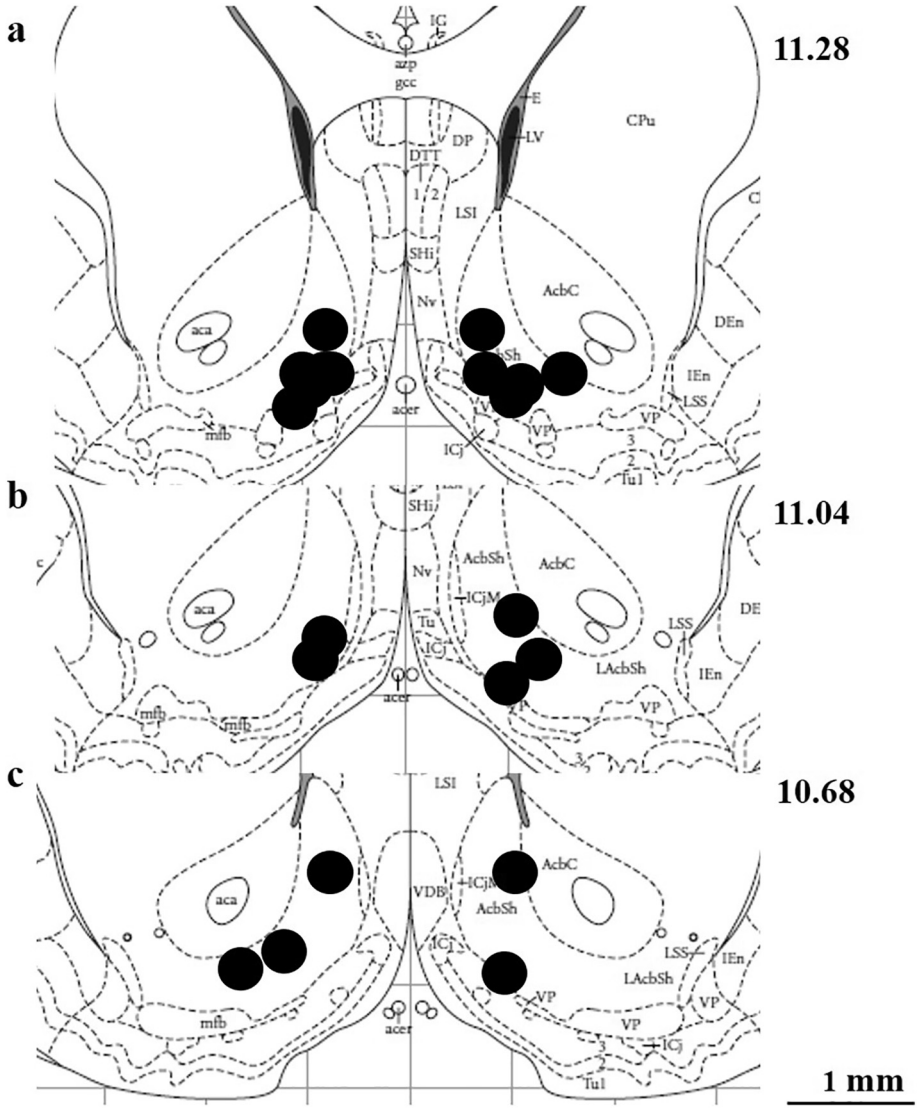

Fig. 9. Localization of valid injection sites utilized in all experimental groups on the coronal sections of the rat brain according to the stereotaxic atlas by Paxinos and Watson (1986). Panels **a**, **b**, and **c** represent movement along the anterior-posterior axis from 11.28 mm to 10.68 mm from the interaural zero plane (numbers on the top right corner of each panel). For sake of clearness any directly overlapping sites are not shown. Bar at bottom right corner represents 1 mm for scale. 11 valid subjects localized in left hemisphere, 14 localized in right hemisphere. Abbreviations: aca – anterior commissure, anterior part; AcbC – accumbens nucleus, core; AcbSh – accumbens nucleus, shell; CPu - caudate putamen (striatum); LAcbSh - lateral accumbens shell; LSI - lateral septal nucleus, intermediate part; mfb – medial forebrain bundle; VP – ventral pallidum.

injected directly into the NAc. In the present work, we were not able to obtain locomotor activity data coincident with USV recordings which would allow for a direct comparison. Also, the dose range for induction of USVs might be different from that for locomotor activity. Changes in rat behaviour after intraaccumbens injection of DA seem to be inducible by a wide range of doses and behavioural changes were reported after doses as high as 200 μg (Costall and Naylor, 1975).

Microinjections of AMPH into the NAcSh were found to be more efficacious than DA in inducing emission of 50 kHz USVs as well as altering the call profile in favour of frequency modulation. DA itself increased the ratio of FM to flat calls compared with vehicle, but AMPH appeared to increase this ratio when compared with DA following transporter inhibition. This effect of AMPH-increased frequency modulation is abundant in the literature, including evidence of sensitization of FM USVs which itself is different from locomotor activity sensitization (Wright et al., 2010; Taracha et al., 2014; Simola and Morelli, 2015). It is possible that this finding of AMPH-increased FM ratio compared to DA reflects a difference in the length of time for DA signaling in the synaptic cleft between the two conditions. In both conditions the clearance rate of transmitter is largely dependent on the enzymatic activity of catechol-*o*-methyltransferase (COMT). However, under AMPH conditions, the drug induces efflux of DA molecules from the cytoplasm and also interrupts the vesicular transporter cycle, thus, it may be capable of saturating this enzyme for longer periods of time (Fleckenstein et al., 2007; Sitte and Freissmuth, 2015). In accordance with this reasoning, the microinjections of dopamine used in this study (with their putatively fast transient duration) may have acted in a similar fashion as phasic dopamine release within the NAc. Such phasic dopamine release has been found to be associated with both the production and reception of 50 kHz USVs (Willuhn et al., 2014; Scardochio et al., 2015). The investigation of the role of this phasic dopamine release indicates that it may be capable of inducing 50 kHz calling but does not appear sufficient for maintenance of the behaviour (Scardochio et al., 2015). Delineating the association of local AMPH effects and tonic/phasic DA signaling in 50 kHz call induction may represent an exciting avenue for future research.

An alternative explanation for the difference in FM ratio induction between AMPH and DA observed in the present work relates to the general catecholaminergic effects of AMPH. In addition to its DAergic effects, the noradrenergic effects of the drug may explain its greater capacity to increase frequency modulation (for relevant review see Rippberger et al., 2015). Wright et al. (2012) found that systemic α_1 receptor antagonism (prazosin) or α_2 autoreceptor agonism (clonidine) dose-dependently reduced AMPH-induced 50 kHz USV emission. Importantly, the α_1 antagonism prevented the typical alteration in call profile of increased FM calls. Similar application of a β_1/β_2 receptor antagonist (propranolol) showed no effect on AMPH-induced 50 kHz USV call rate but did dose-dependently alter the call profile with an increased proportion of flat calls at the expense of the proportion of FM calls. These findings may be instructive as to how AMPH affects 50 kHz USVs, however, caution must be exerted in generalizing findings from systemic application of drugs to the current work involving local intracerebral NAc application.

There are several noted limitations specific to this study. Following exclusions due to cannulae localization, non-calling, or missing data across injection conditions, the effective sample sizes for any given statistical analysis were low. Thus, the findings of the present work should be considered exploratory in nature. Moreover, in part due to this low sample size, the comparisons between DA and AMPH microinjections required the use of non-parametric statistical tests which may have failed to detect differences between certain variables. The results also may not generalize beyond the doses used in the present study or subjects of different age, sex, or strain. Further research is required to perform an exhaustive comparison between DA- and psychostimulant-induced 50 kHz USV emission. Future studies may investigate the contributions of different locations within the brain to establishing the species-typical 50 kHz USV call profile inducible by microinjections of DA.

The two software programs used in the current study (Avisoft SASlab Pro and Sonotrack™ by Metris), although used for distinct purposes, were found to be generally complementary to each other. The automatic detection and screening for 50 kHz calls among spectrograms using Sonotrack enabled a much greater breadth of recordings to be analyzed. However, the manual precision afforded by Avisoft SASlab Pro was critical in determining the holistic sonographic architecture of recorded 50 kHz USVs (i.e. call subtypes).

4. Conclusion

Microinjections of DA into the NAc in the rat were demonstrated to increase call rate and decrease latency to call relative to vehicle. To our knowledge, this is the first study to employ intracerebral microinjections of DA into the brain to induce 50 kHz USVs. DA microinjections into the shell of the NAc were not found to differ from vehicle in any of the measured acoustic parameters for individual calls (peak sound frequency, duration, or bandwidth). However, calculated as a ratio of FM to flat calls, it was found that DA increased the ratio of FM calls compared with vehicle. Antagonism of the D_2 receptor using intracerebral raclopride was not found to inhibit DA-induced 50 kHz calling. Microinjections of AMPH into the NAc shell was found to be more effective than DA given alone (in the dose-range used) for increasing call rate and average duration of calls. Blocking the DA reuptake transporter successfully minimized these DA-AMPH differences in call rate and average duration of calls, however, AMPH was found to have increased the ratio of FM/flat calls. Together these results suggest that DA signaling even locally injected into the shell of the NAc is capable of inducing 50 kHz USV emission. Moreover, a number of psychostimulants may differ in the character of 50 kHz USV emission as a function of their pharmacological profile compared with the native transmitter acting in isolation. This consideration may be important when extracting meaningful contributions of singular brain neurochemical systems via application of pharmacological agents to induce organismal behaviour.

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Competing interests

None.

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