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Influence of dopamine transmission in the medial prefrontal cortex and dorsal striatum on the emission of 50-kHz ultrasonic vocalizations in rats treated with amphetamine: effects on drug-stimulated and conditioned calls

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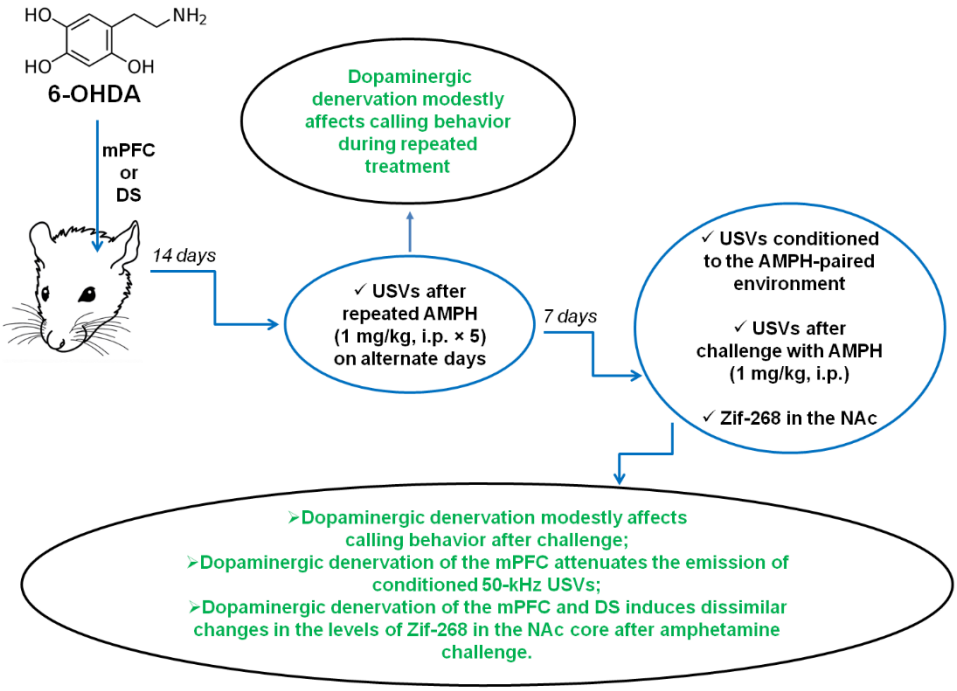

Graphical Abstract

Abstract

Rat ultrasonic vocalizations (USVs) of 50-kHz are increasingly being evaluated as a behavioral marker of the affective properties of drugs. Studies in amphetamine-treated rats have shown that activation of dopamine transmission in the nucleus accumbens (NAc) initiates the emission of 50-kHz USVs, but little is known on how dopamine transmission in other brain regions modulates the effects of drugs on calling behavior. To clarify this issue, we evaluated 50-kHz USV emissions in rats subjected to dopaminergic denervation of either the medial prefrontal cortex (mPFC) or the dorsal striatum (DS) and treated with amphetamine. Rats received amphetamine (1 mg/kg, i.p. × 5) on alternate days in a test cage; 7 days later, they were re-exposed to the test cage, to measure calling behavior that may reflect drug conditioning, and then challenged with amphetamine (1 mg/kg, i.p.). The numbers of total and categorized 50-kHz USVs emitted were evaluated, along with immunofluorescence for Zif-268 in the NAc. Dopamine-denervated and sham-operated rats displayed comparable patterns of calling behavior during amphetamine treatment and after amphetamine challenge. Conversely, rats that were dopamine-denervated in the mPFC, but not DS, emitted low numbers of 50-kHz USVs on test cage re-exposure. Finally, dopamine-denervated rats displayed a less marked increase in Zif-268-positive neurons in the NAc shell after amphetamine challenge, compared with sham-operated rats. These results may be relevant to identify the neuronal circuits that modulate 50-kHz USV emissions in rats treated with amphetamine, as well as the interplay between calling behavior and affective properties of drugs.

Keywords: conditioning; emotional state; reward; motivation; sensitization; Zif-268

1. Introduction

Rats emit the so-called 50-kHz ultrasonic vocalizations (USVs), which are contained within the 35-120 kHz frequency range, in response to a variety of stimuli that possess positive affective valence (Brudzynski, 2013; Schwarting et al., 2007). Notably, rats treated with psychoactive drugs may emit high numbers of 50-kHz USVs; accordingly, such calling behavior is considered a marker of the rewarding and motivational properties of drugs (Barker et al., 2014, 2015; Hamed and Kursa, 2018; Simola, 2015; Simola and Granon, 2019; Taracha et al. 2014).

Previous studies have demonstrated that the activation of dopamine receptors critically regulates the emission of 50-kHz USVs in rats treated with psychoactive drugs that may modify the emotional state, consistent with the bulk of evidence showing that dopamine plays a critical role in the processing of appetitive and aversive states (Bromberg-Martin et al., 2010; Lammel et al., 2011; Zweifel et al., 2011). Robust calling behavior has been demonstrated after the systemic administration of the dopaminomimetic drugs amphetamine, cocaine, methylphenidate, or of the mixed D_1/D_2 dopamine receptor agonist apomorphine (Kõiv et al., 2016; Mu et al., 2009; Pereira et al., 2014; Simola et al., 2012, 2014; Williams and Undieh, 2010; Wintink and Brudzynski, 2001). Emission of 50-kHz USVs has also been demonstrated after local injections into the nucleus accumbens (NAc) of either amphetamine or the mixed D_2/D_3 dopamine receptor agonist quinpirole (Brudzynski et al., 2012; Burgdorf et al., 2001; Thompson et al., 2006). Conversely, administration of dopamine receptor antagonists, either systemically or locally in the NAc, has been found to attenuate the emission of 50-kHz USVs elicited by amphetamine, cocaine or quinpirole (Brudzynski et al., 2012; Thompson et al., 2006; Williams and Undieh, 2010; Wright et al., 2013).

The results of studies that used local drug injections indicate that the activation of dopamine receptors in the NAc is a key event that underlies the emission of 50-kHz USVs stimulated by psychoactive drugs. Nevertheless, we and others have demonstrated that rats treated with amphetamine according to regimens that induced sensitized 50-kHz USV emission also showed increased levels of markers of neuronal activation (i.e., Zif-268, c-fos) in various brain regions that

receive dopaminergic innervation and regulate reward and motivation (Costa et al., 2015; Kaniuga et al., 2016; Lehner et al., 2017). Accordingly, it may be speculated that dopamine transmission in brain regions other than the NAc modulates the emission of 50-kHz USVs in rats treated with psychoactive drugs. Addressing this issue is relevant to describe the neuronal circuits that regulate the emission of 50-kHz USVs, and to further elucidate the interplay between calling behavior and alteration of the emotional state in rats treated with psychoactive drugs.

The present study investigated whether dopamine transmission in specific cortical and striatal regions modulates the emission of 50-kHz USVs in rats treated with amphetamine. To this end, rats were subjected to bilateral dopaminergic denervation with 6-hydroxydopamine (6-OHDA) in either the medial prefrontal cortex (mPFC) or the dorsal striatum (DS), two brain regions where we have previously demonstrated increased levels of Zif-268 in amphetamine-treated rats that developed sensitized emission of 50-kHz USVs (Costa et al., 2015). Thereafter, dopamine-denervated and sham-operated rats were repeatedly administered amphetamine according to a protocol that allowed to evaluate: i) sensitization of calling behavior, which is thought to reflect modifications in the motivational properties of drugs with repeated experience (Ahrens et al., 2009; Mu et al., 2009; Simola and Morelli, 2015), and ii) calling behavior on re-exposure to the drug-paired environment, which has been proposed to reflect drug conditioning (Knutson et al., 1999; Maier et al., 2010). The total numbers of 50-kHz USVs and the numbers of “flat”, “trill” and “frequency modulated” call subtypes emitted were evaluated, to clarify whether dopamine transmission in the mPFC and/or DS modulated general calling behavior as well as emission of specific call categories that are thought to possess dissimilar behavioral significance (Burgdorf et al., 2008; Wöhr et al., 2008). Finally, the present study evaluated the number of nuclei positive for Zif-268 in the NAc, to investigate whether any relationship between this structure and dopamine transmission in the mPFC or DS is involved in mediating the emission of 50-kHz USVs in rats treated with amphetamine.

2. Materials and methods

2.1. Subjects

A total of 67 male Sprague–Dawley rats (Harlan, Italy) were included in the study, whereas 3 rats were excluded from the study since they did not display signs of dopaminergic denervation. Rats weighed 275–300 g at the beginning of experiments and were housed in groups of 4-5 per cage under a 12-h light/dark cycle (lights on at 08:00 h). Standard laboratory chow and tap water were freely available except during USV recordings, which were performed between 10:00 and 16:00 h. All experiments were conducted in accordance with the guidelines for animal experimentation of the EU directives (2010/63/EU; L.276; 22/09/2010), and with the guidelines approved by the Ethical Committee of the University of Cagliari (opec271.20130626160529.28283.02.1.18, 26/06/2013). All efforts were made to minimize animal discomfort and numbers of animals used.

2.2. Drugs

D-Amphetamine (sulfate), desipramine (hydrochloride) and 6-OHDA (hydrochloride) were purchased from Sigma–Aldrich (Milan, Italy). Amphetamine and desipramine were dissolved in distilled water and administered intraperitoneally (i.p.) in a volume of 3 ml/kg, whereas 6-OHDA was dissolved in saline solution (0.9% NaCl) + 0.05% ascorbic acid and administered intracranially.

2.3. Lesion with 6-OHDA in the mPFC and DS

Rats were deeply anesthetized with Equithesin (pentobarbital 0.97 g, $MgSO_4$ 2.1 g, chloral hydrate 4.25 g, propylene glycol 42.8 mL, ethanol 90% 11.5 mL, to 100 mL with sterile distilled H_2O) (5 ml/kg, i.p.) and placed in a David–Kopf stereotaxic apparatus (Tujunga, CA, USA). 6-OHDA (4 μg/μl) was then infused bilaterally in either the mPFC or the DS by means of a stainless-steel cannula. For all surgeries, stereotaxic coordinates were defined as anterior-posterior (AP) from bregma, lateral (L) from midline and ventral (V) from skull, according to the rat brain atlas of Paxinos and Watson (1998). Dopaminergic denervation of the mPFC was performed according to the procedure described by Bjijou et al. (2002), which consisted in the infusion of 6-OHDA (0.5 μl/site) in 6 sites (3 sites per hemisphere), defined as follows: 1) AP = +3.8, L = ±0.5, V = −4.7; 2) AP = +3.5, L = ±0.5, V = −4.5

and 3) AP = +3.2, L = ±0.5, V= −4.3. Dopaminergic denervation of the DS was performed according to the procedure described by Coccurello et al. (2004), which consisted in the infusion of 6-OHDA (3 µl/site) in 2 sites (1 site per hemisphere) at the following coordinates: AP = +0.2, L = ±3.5, V = −4.8. A flow rate of 1 µl /min was always used and the cannula was left in place for an additional 2 min at each site after the completion of 6-OHDA infusions, in order to allow maximal diffusion of the toxin into the surrounding tissue. After completion of surgical procedures, rats were monitored until they regained the righting reflex and were then returned to their home cage pending further experiments. All rats used in the study were pretreated with desipramine-HCl (15 mg/kg i.p.) 30 min before surgeries, to prevent damage to noradrenergic neurons. In the case of dopamine-denervated rats, desipramine was followed by intracranial 6-OHDA, whereas in the case of sham-operated rats desipramine was followed by intracranial 0.9% NaCl + 0.05% ascorbic acid.

2.4. Experimental plan

Rats were randomly assigned to an experimental group and were handled daily (5 min) for 2 days before experiments, which began 14 days after surgeries. The experimental plan was designed based on previous studies of our group that evaluated USV emissions in rats repeatedly treated with psychoactive drugs (Costa et al., 2015, Simola et al., 2014). Experiments were structured in five phases: 1) habituation to the test cage (15 min), twice a day × 2 days; 2) acute administration of vehicle in the test cage, to evaluate basal calling behavior; 3) repeated administration of amphetamine (× 5) in the test cage every other day, to evaluate the induction of sensitized calling behavior; 4) amphetamine withdrawal (7 days) in the home cage; 5) re-exposure to the test cage in drug-free conditions (10 min), to evaluate calling behavior that may reflect conditioning to the amphetamine-paired environment (Knutson et al., 1999; Maier et al., 2010), immediately followed by drug challenge, to evaluate the expression of sensitized calling behavior. Distinct groups of sham-operated and dopamine-denervated rats received amphetamine at the dose of 1 mg/kg (i.p.). The dose of amphetamine was selected based on previous studies demonstrating that rats repeatedly treated with such a dose of amphetamine displayed sensitized emission of 50-kHz USVs, as well as sustained calling behavior upon re-exposure to the environment previously paired with amphetamine

administration (Ahrens et al., 2009; Costa et al., 2015; Simola and Morelli, 2015; Simola et al., 2016). Sham-operated and dopamine-denervated rats treated with vehicle according to the same protocol served as controls. Figure 1 demonstrates the experimental plan; please refer to Simola and Morelli (2015) for further details.

2.5. Recording of ultrasonic vocalizations

All experiments were performed in a quiet room. USVs were recorded from individual rats that were placed in Plexiglas cylinders (diameter, 25 cm; height, 30 cm) enclosed by four cardboard walls (height, 65 cm; distance from the cylinder, 15 cm). Cylinders had the bottom covered with sawdust, some of which was taken from the home cage of each specific rat evaluated, in order to reduce the negative influence that a brand novel environment may have on calling behavior (Natusch and Schwarting, 2010). Each cylinder was topped with a lid equipped with an ultrasonic microphone (CM16/CMPA, Avisoft, Berlin, Germany) connected to an ultrasound recording device (Ultrasound Gate 116 Hb, Avisoft, Berlin, Germany). Intensity gain was always kept at a constant level during recordings. USV emissions after the administration of amphetamine or vehicle were recorded for 30 min, starting immediately after injections. USV emissions on re-exposure to the test cage were recorded by placing rats in cylinders for 10 min immediately before challenge.

2.6. Evaluation of dopaminergic and noradrenergic denervation and of nuclei positive for Zif-268

At completion of the experiments, ninety minutes after amphetamine or vehicle challenge, rats were deeply anesthetized and transcardially perfused with 0.9% NaCl followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH=7.4). Afterwards, brains were removed, postfixed overnight in the same solution (4°C) and cut on a vibratome 2 days later to obtain sections (40 μm) suited for immunohistochemical processing. Three coronal sections were collected from each rat at the following stereotaxic coordinates: 1) 2.70 mm to 2.20 mm, mPFC; 2) 1.70 mm to 0.20 mm DS and NAc (shell and core). All coordinates were relative to bregma according to the rat brain atlas of Paxinos and Watson (1998). Sections from the mPFC and the DS were stored in a cryoprotective solution until they were processed to quantify the levels of tyrosine hydroxylase (TH), as a marker of

dopaminergic denervation, and of noradrenaline transporter (NET), as a marker of noradrenergic denervation. Sections from the NAc were processed to quantify the levels of Zif-268 as a marker of neuronal activation.

2.6.1. Reaction protocols

Free-floating sections were rinsed in 0.1 M PB, blocked in a solution containing 3% normal donkey or goat serum (Jackson ImmunoResearch Europe, UK; Vector, UK) and 0.3% Triton X-100 in 0.1 M PB at room temperature (2 h). The diaminobenzidine (DAB) technique was used for the visualization of TH and NET. Sections were incubated with the primary antibody, mouse monoclonal antibody anti-TH (1:1000, Sigma-Aldrich, Italy) or mouse monoclonal antibody anti-NET (1:1000, MAb Technologies, GA, USA). Thereafter, the biotinylated secondary antibody (goat anti-mouse IgG, Vector, UK) was added, followed by the avidin–biotin–peroxidase complex protocol (ABC, Vector, UK). Finally, sections were mounted onto glass slides coated with gelatin in Eukitt mounting medium for visualization (Costa et al., 2017). The immunofluorescence technique was used for the visualization of Zif-268. Sections were incubated with the primary antibody, rabbit polyclonal antibody anti-Zif-268 (1:1000, Santa Cruz Biotechnology, U.S.A.), rinsed three times in 0.1M PB, and then incubated with the secondary antibody, AlexaFluor® 594-labeled donkey anti-rabbit IgG, (1:400, Jackson ImmunoResearch Europe, UK) in 0.1M PB at room temperature (2 h). Afterwards, sections were rinsed and immediately mounted onto glass slides coated with gelatin in Mowiol mounting medium (Costa et al., 2017). In each of the reaction protocols, omission of either the primary or secondary antibodies served as negative control and yielded no labeling (data not shown).

2.6.2. Image acquisition and analysis

Images of single wavelength were obtained with an epifluorescence microscope (Axio Scope A1, Zeiss, Germany) connected to a digital camera (1.4 MPixels, Infinity 3-1, Lumenera, Canada) as previously described (Costa et al., 2017). Brain sections immunostained for TH, NET or Zif-268 were evaluated using a 20× objective in order to acquire: i) the whole prelimbic/infralimbic area of the mPFC; ii) two portions of the DS; iii) the shell and core subregions of the NAc. Sections

immunostained for TH or NET were captured in black and white 8-bit monochrome and the densities of immunoreactive fibers were determined in fixed regions by using a threshold level that was kept constant across all images (Costa et al., 2019). The Image J software (National Institutes of Health, USA) was used to quantify the densities of immunoreactive fibers positive for TH or NET. Densities were quantified by converting pixels into square micrometers using a suited calibration, in order to obtain the area occupied by a specific immunoreaction product. The final values of the densities of immunoreactive fibers counted in the mPFC and DS were expressed as percentages of the respective SHAM+VEH groups. For each level of every brain area analyzed, densities were first normalized with respect to SHAM+VEH then values from different levels were averaged. No significant differences in the densities of immunoreactive fibers were found among the three coronal sections of a given area from the same rat (data not shown). To allow visualization of cell nuclei (Maric et al., 2011) positive for Zif-268, slices were labeled with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI, 1:10.000, Sigma-Aldrich, Italy). Thereafter, the numbers of neurons labeled with DAPI were counted separately for each level of the NAc that was evaluated. All the sections were evaluated with the multi-points tool of the Image J software, which creates a point selection that enables counting the number of labeled cell bodies. The final values of Zif-268-positive nuclei counted in the NAc were expressed as percentages of the respective SHAM+VEH groups. For each level, the number of nuclei positive for Zif-268 was first normalized with respect to SHAM+VEH, then values from different levels were averaged. No significant differences in the number of nuclei positive for Zif-268 was found among the three coronal sections from the same rat (data not shown).

2.7. Data collection, analysis, and statistics

USV recordings were converted into spectrograms by means of the software SASLab Pro 4.52 (Avisoft, Berlin, Germany) with the following settings: 512 Fast Fourier Transform (FFT)-length, Hamming window and 75% overlap frame set-up. Spectrograms were manually processed to remove background noise and signals that could not be unambiguously classified as USVs (Simola et al., 2012). Thereafter, the total number of USVs isolated in each spectrogram was automatically counted by means of SASLab Pro 4.52. Furthermore, 50-kHz USVs were visually categorized into

flat, trill or FM call subtypes (Figure 2), according to the criteria proposed by Wright and coworkers (2010). Call categorization was performed by an experienced blind experimenter who visually evaluated each spectrogram every 7 days, for a total of three times (Simola et al., 2012). About the 1.6 % of categorized calls were not uniformly classified over the evaluations and were therefore excluded from statistical analysis. No emissions of aversive 22-kHz USVs were recorded in any of the experiments performed during the study.

Means ± S.E.M. were calculated for: 1) densities of TH-, and NET-positive fibers in the mPFC and DS; 2) numbers of total and categorized 50-kHz USVs emitted on days 1, 5 and 9 (repeated treatment) and 16 (test cage re-exposure and challenge) of the experimental protocol; and 3) ~~the~~ numbers of nuclei positive for Zif-268 in the NAc shell and core. Differences in the densities of TH- and NET-positive fibers were analyzed by two-way ANOVA (6-OHDA injection × subsequent pharmacological treatment). Differences in calling behavior during repeated treatment were first analyzed by three-way ANOVA (treatment × dopaminergic denervation × administration day). Considering the scarce calling behavior displayed by rats treated with vehicle, distinct two-way ANOVAs (dopaminergic denervation × administration day) were subsequently performed to selectively analyze the time course of calling behavior in amphetamine-treated and vehicle-treated rats. Differences in calling behavior recorded after challenge and on test cage re-exposure were analyzed by two-way ANOVA (treatment or previous treatment × dopaminergic denervation). Differences in nuclei positive for Zif-268 were analyzed by ~~means of~~ two-way ANOVA (treatment × dopaminergic denervation). ANOVAs were followed by Tukey's post-hoc test, when appropriate, and within-group post-hoc tests were applied to disclose the development of sensitized calling behavior during repeated treated with amphetamine.

All data were evaluated for normality and Levene's test was applied to check for homoscedasticity of datasets before ANOVA analysis. When appropriate, datasets were log transformed to preserve homoscedasticity and a constant (+1) was added to all datasets subjected to transformation, to

correct for null values. Figures report raw data for clarity. Statistical analysis was performed with Statistica (StatSoft, Tulsa, OK, USA), Prism (GraphPad, La Jolla, CA, USA), and QI Macros (KnowWare International, Denver, CO, USA) for Windows. Significance was set at $p<0.05$ for ANOVA analyses. For the sake of conciseness, the text reports only significant results.

3. Results

3.1. Dopaminergic denervation

Injection of 6-OHDA (4 µg/µl) in either the mPFC or the DS significantly reduced the density of TH-positive fibers, compared with sham surgery. Two-way ANOVA for rats operated in the mPFC showed a significant effect of 6-OHDA ($F_{1,30}$=60.93, p<0.01), but neither significant effect of subsequent pharmacological treatment nor significant treatment × 6-OHDA interaction. Tukey's test showed that injection of 6-OHDA in the mPFC decreased the levels of TH-positive fibers in rats that were later treated with either vehicle (p<0.01) or amphetamine (p<0.01) (Fig. 3A). Moreover, two-way ANOVA for rats operated in the DS showed a significant effect of 6-OHDA ($F_{1,29}$=483.11, p<0.01), but neither significant effect of subsequent pharmacological treatment nor significant treatment × 6-OHDA interaction. Tukey's test showed that injection of 6-OHDA in the DS reduced the levels of TH-positive fibers in rats that were later treated with either vehicle (p<0.01) or amphetamine (p<0.01) (Fig. 3B). Injection of 6-OHDA (4 µg/µl) in either the mPFC or the DS did not significantly modify the density of NET-positive fibers, compared with sham surgery (Fig. 3A-B). Taken together, these results indicate that in both the mPFC and the DS the toxic effects of 6-OHDA predominantly involved dopaminergic fibers.

3.2. Emission of 50-kHz USVs in rats bearing a dopaminergic denervation of the mPFC

3.2.1 General calling behavior during repeated treatment with amphetamine

Repeated treatment with amphetamine (1 mg/kg, i.p.) modified the total number of 50-kHz USVs emitted, compared with repeated treatment with vehicle. Three-way ANOVA revealed a significant effect of treatment ($F_{1,30}$=87.84, p<0.01) and time ($F_{2,60}$=12.27, p<0.01), as well as a significant treatment × time interaction ($F_{2,60}$=16.82, p<0.01). Tukey's test showed that amphetamine increased the emission of 50-kHz USVs at each time point evaluated in both sham-operated rats and rats bearing a dopaminergic denervation of the mPFC (p<0.01 for each time point) (Fig. 4A,B). Two-way ANOVA analysis of calling behavior narrowed to rats treated with amphetamine revealed a significant effect of time ($F_{2,32}$=14.37, p<0.01), but neither significant effect of denervation nor significant denervation × time interaction. Tukey's test revealed that sham-operated rats displayed a sensitized increase in calling behavior after the third (p<0.01) and fifth (p<0.01) amphetamine administration, whereas dopamine-denervated rats did so after the third (p=0.01) amphetamine administration only (Fig. 4A). Two-way ANOVA analysis of calling behavior narrowed to rats treated with vehicle revealed no significant effects (Fig. 4B).

3.2.2 Emission of categorized calls during repeated treatment with amphetamine

Repeated treatment with amphetamine (1 mg/kg, i.p.) modified the number of flat, trill and FM 50-kHz USVs emitted, compared with repeated treatment with vehicle. Three-way ANOVA for the emission of each category of calls evaluated revealed a significant effect of treatment (flat, $F_{1,30}$=33.96, p<0.01; trill, $F_{1,30}$=10.87, p<0.01; FM, $F_{1,30}$=24.89, p<0.01) and time (flat, $F_{2,60}$=15.09, p<0.01; trill, $F_{2,60}$=6.52, p<0.01; FM, $F_{2,60}$=10.49, p<0.01), as well as a significant treatment × time interaction (flat, $F_{2,60}$=21.48, p<0.01; trill, $F_{2,60}$=6.21, p<0.01; FM, $F_{2,60}$=10.51, p<0.01). Tukey's test showed that in both sham-operated and dopamine-denervated rats amphetamine increased the emission of flat, trill and FM calls at each time point evaluated (p<0.01 for each time point) (Fig. 4C-H). Two-way ANOVA analysis of calling behavior narrowed to rats treated with amphetamine revealed a significant effect of time for each category of calls evaluated (flat, $F_{2,32}$=8.11, p<0.01; trill, $F_{2,32}$=14.03, p<0.01; FM, $F_{2,32}$=10.18, p<0.01) and a significant denervation × time interaction for flat

calls ($F_{2,32}$=3.57, p<0.05). Tukey's test showed that in sham-operated rats amphetamine induced sensitized emissions of flat and FM calls after the third (p=0.01 for flat and FM) and fifth (p<0.01 for flat and FM) administration (Fig. 4C,G), whereas sensitized emission of trill calls occurred only after the fifth (p<0.01) administration (Fig. 4E). Dopamine-denervated rats displayed non-sensitized increases in the emissions of categorized calls during repeated treatment with amphetamine. Moreover, dopamine-denervated rats exhibited non-significant trends towards decreased emission of flat calls and increased emission of trill calls, compared with sham-operated rats. Two-way ANOVA analysis of the emission of categorized calls narrowed to rats treated with vehicle revealed no significant effects (Fig. 4D,F,H).

3.2.3. General calling behavior after amphetamine challenge

Challenge with amphetamine (1 mg/kg, i.p.) modified the total number of 50-kHz USVs emitted, compared with challenge with vehicle. Two-way ANOVA revealed a significant effect of treatment ($F_{1,30}$=78.50, p<0.01). Tukey's test showed that amphetamine challenge increased the emission of 50-kHz USVs in both sham-operated (p<0.01) and dopamine-denervated rats (p<0.01) (Fig. 5A). Calling behavior elicited by amphetamine appeared to be less marked in dopamine-denervated than sham-operated rats, although this effect did not reach statistical significance.

3.2.4 Emission of categorized calls after amphetamine challenge

Challenge with amphetamine (1 mg/kg, i.p.) modified the number of flat, trill and FM 50-kHz USVs emitted, compared with challenge with vehicle. Two-way ANOVA for the emission of each category of calls evaluated revealed a significant effect of treatment (flat, $F_{1,30}$=66.67, p<0.01; trill, $F_{1,30}$=12.30, p<0.01; FM, $F_{1,30}$=86.79, p<0.01). Tukey's test showed that amphetamine challenge increased the emission of flat, trill and FM calls in both sham-operated rats (p<0.01 for each category) and dopamine-denervated rats (p<0.01 for flat and FM calls, p=0.01 for trill calls) (Fig. 5A). Emission of flat and FM calls stimulated by amphetamine challenge appeared to be less marked in dopamine-denervated than sham-operated rats, although these effects did not reach statistical significance.

3.2.5. General calling behavior on re-exposure to the test cage

Previous treatment with amphetamine and dopaminergic denervation affected the total number of 50-kHz USVs that rats emitted when re-exposed to the test cage in treatment-free conditions. Two-way ANOVA revealed a significant effect of previous treatment ($F_{1,30}$=5.76, p<0.05) and of dopaminergic denervation ($F_{1,30}$=16.95, p<0.05), as well as a significant treatment × denervation interaction ($F_{1,30}$=5.09, p<0.05). Tukey's test showed that sham-operated rats previously treated with amphetamine emitted higher numbers of 50-kHz USVs compared with sham-operated rats previously treated with vehicle (p=0.01) (Fig. 5B). Conversely, dopamine-denervated rats previously treated with amphetamine displayed calling behavior comparable to that of dopamine-denervated rats previously treated with vehicle (Fig. 5B).

3.2.6 Emission of categorized calls on re-exposure to the test cage

Previous treatment with amphetamine and dopaminergic denervation affected the number of categorized 50-kHz USVs that rats emitted when re-exposed to the test cage in treatment-free conditions. Two-way ANOVA for FM calls revealed a significant effect of previous treatment ($F_{1,30}$=32.1; p<0.05) and of dopaminergic denervation ($F_{1,30}$=11.87; p<0.05), as well as a significant previous treatment × denervation interaction ($F_{1,30}$=13.21; p<0.05). Two-way ANOVA also revealed a significant effect of dopaminergic denervation for the emission of flat ($F_{1,30}$=34.68; p<0.05) and trill ($F_{1,30}$=6.29; p<0.05) calls. Tukey's test showed that sham-operated rats previously treated with amphetamine emitted higher numbers of FM calls compared with sham-operated rats previously treated with vehicle (p<0.01) (Fig. 5B). Conversely, dopamine-denervated rats previously treated with amphetamine emitted numbers of categorized calls comparable to those emitted by dopamine-denervated rats previously treated with vehicle (Fig. 5B).

3.3. Emission of 50-kHz USVs in rats bearing a dopaminergic denervation of the DS

3.3.1 General calling behavior during repeated treatment with amphetamine

Repeated treatment with amphetamine (1 mg/kg, i.p.) modified the total number of 50-kHz USVs emitted, compared with repeated treatment with vehicle. Three-way ANOVA revealed a significant

effect of treatment ($F_{1,30}$=108.95, p<0.01) and time ($F_{2,60}$=6.72, p<0.01), as well as a significant treatment × time interaction ($F_{2,60}$=5.15, p<0.01). Tukey's test showed that amphetamine increased the emission of 50-kHz USVs at each time point evaluated in both sham-operated rats and dopamine-denervated rats (p<0.01 for each time point) (Fig. 6A,B). Two-way ANOVA analysis of calling behavior narrowed to rats treated with amphetamine revealed a significant effect of time ($F_{2,30}$=15.73, p<0.01), but neither significant effect of denervation nor significant denervation × time interaction. Moreover, Tukey's test revealed that sham-operated rats displayed a sensitized increase in calling behavior after the third (p=0.01) and fifth (p<0.01) amphetamine administration, whereas dopamine-denervated rats did so after the third (p<0.01) amphetamine administration only (Fig. 6A). Two-way ANOVA analysis of calling behavior narrowed to rats treated with vehicle revealed no significant effects (Fig. 6B).

3.3.2 Emission of categorized calls during repeated treatment with amphetamine

Repeated treatment with amphetamine (1 mg/kg, i.p.) modified the number of flat, trill and FM 50-kHz USVs emitted, compared with repeated treatment with vehicle. Three-way ANOVA for the emission of each category of calls evaluated revealed a significant effect of treatment (flat, $F_{1,30}$=46.03, p<0.01; trill, $F_{1,30}$=18.49, p<0.01; FM, $F_{1,30}$=110.26, p<0.01) and time (flat, $F_{2,60}$=6.74; p<0.01; trill, $F_{2,60}$=5.52; p<0.01; FM, $F_{2,60}$=6.27; p<0.01). A significant treatment × time interaction was found for the emission of flat ($F_{2,60}$=7.63; p<0.01) and trill ($F_{2,60}$=5.73; p<0.01) calls. Tukey's test showed that in both sham-operated and dopamine-denervated rats amphetamine increased the emission of flat and FM calls at each time point evaluated (p<0.01 for each time point) (Fig. 6C,D,G,H), whilst it increased the emission of trill calls after the third (p<0.01) and fifth administration (p<0.01) only (Fig. 6E,F). Two-way ANOVA analysis of calling behavior narrowed to rats treated with amphetamine revealed a significant effect of time for all the categories of calls emitted (flat, $F_{2,30}$=5.82, p<0.01; trill, $F_{2,30}$=8.62, p<0.01; FM, $F_{2,30}$=8.06, p<0.01) and a significant denervation × time interaction for trill calls ($F_{2,32}$=4.66; p<0.01). Tukey's test showed that in sham-operated rats amphetamine induced sensitized increases in the emission of trill and FM calls after the fifth administration (p<0.01 for trill calls; p=0.01 for FM calls) (Fig. 6C,E,G). Dopamine-denervated rats

displayed non-sensitized increases in the emission of categorized calls during repeated treatment with amphetamine (Fig. 6,E,G). Moreover, dopamine-denervated rats exhibited a non-significant trend towards an increased emission of flat calls, compared with sham-operated rats. Two-way ANOVA analysis of the emission of categorized 50-kHz USVs narrowed to rats treated with vehicle revealed no significant effects (Fig. 6D,F,H).

3.3.3 General calling behavior after amphetamine challenge

Challenge with amphetamine (1 mg/kg, i.p.) modified the total number of 50-kHz USVs emitted, compared with challenge with vehicle. Two-way ANOVA revealed a significant effect of treatment ($F_{1,30}$=152.57, p<0.01). Tukey's test showed that amphetamine challenge increased the emission of 50-kHz USVs in both sham-operated (p<0.01) and dopamine-denervated (p<0.01) rats (Fig. 7A). Calling behavior elicited by amphetamine appeared to be less marked in dopamine-denervated than in sham-operated rats, although this effect did not reach statistical significance.

3.3.4 Emission of categorized calls after amphetamine challenge

Challenge with amphetamine (1 mg/kg, i.p.) modified the number of flat, trill and FM 50-kHz USVs emitted, compared with challenge with vehicle. Two-way ANOVA for the emission of each category of calls evaluated revealed a significant effect of treatment (flat, $F_{1,30}$=66.84, p<0.01; trill, $F_{1,30}$=82.22, p<0.01; FM, $F_{1,30}$=155.57, p<0.01). Tukey's test showed that amphetamine challenge increased the emission of flat, trill and FM calls in both sham-operated (p<0.01 for each call category) and dopamine-denervated (p<0.01 for each call category) rats (Fig. 7A). Moreover, dopamine-denervated rats exhibited a non-significant trend towards an increased emission of flat calls, and towards a decreased emission of trill and FM calls, all compared with sham-operated rats.

3.3.5. General calling behavior on re-exposure to the test cage

Two-way ANOVA revealed a significant effect of previous treatment with amphetamine ($F_{1,30}$=25.71, p<0.01) on the total number of 50-kHz USVs emitted on re-exposure to the test cage in treatment-free conditions. Tukey's test showed that sham-operated (p<0.01) and dopamine-denervated

(p=0.01) rats previously treated with amphetamine emitted higher numbers of 50-kHz USVs compared with sham-operated rats or dopamine-denervated rats previously treated with vehicle, respectively (Fig. 7B).

3.3.6 Emission of categorized calls on re-exposure to the test cage

Two-way ANOVA revealed a significant effect of previous treatment with amphetamine ($F_{1,30}$=63.01; p<0.01) on the number of FM 50-kHz USVs that rats emitted when re-exposed to the test cage in treatment-free conditions. Tukey's test showed that sham-operated (p<0.01) and dopamine-denervated (p<0.01) rats previously treated with amphetamine emitted higher numbers of FM calls, compared with sham-operated rats or dopamine-denervated rats previously treated with vehicle, respectively (Fig. 7B).

3.4 Number of Zif-268-positive nuclei in the NAc after amphetamine challenge

Challenge with amphetamine (1 mg/kg, i.p.) modified the number of nuclei positive for Zif-268 in the NAc shell and core of sham-operated and dopamine-denervated rats, compared with challenge with vehicle. Two-way ANOVA for rats operated in the mPFC revealed a significant effect of treatment for shell ($F_{1,30}$=9.20, p<0.01) and core ($F_{1,30}$=8.59, p<0.01). Tukey's test showed that amphetamine challenge significantly increased the number of nuclei positive for Zif-268 in the NAc shell and core of sham-operated rats (p<0.05 for each region) (Fig. 8A-A'). A trend towards an increase in the numbers of nuclei positive for Zif-268 was detected in the NAc shell and core of dopamine-denervated rats, but this effect did not reach statistical significance. Two-way ANOVA for rats operated in the DS revealed a significant effect of treatment for shell ($F_{1,30}$=23.47, p<0.01) and core ($F_{1,30}$=14.43, p<0.01). Tukey's test showed that amphetamine challenge significantly increased the number of nuclei positive for Zif-268 in the NAc shell (p<0.05) and core (p<0.05) of sham-operated rats and did so also in the NAc core (p<0.05) of dopamine-denervated rats (Fig. 8B-B').

4. Discussion

Emission of 50-kHz USVs is being increasingly evaluated in studies of psychopharmacology as a marker of the effects that drugs may elicit on the emotional state (Simola and Brudzynski, 2018). However, limited information is currently available on the neuronal circuits that modulate calling behavior in rats treated with psychoactive drugs. The present study provides new insights in this regard, by demonstrating that dopamine transmission in the mPFC and DS may influence certain aspects of 50-kHz USV emissions in rats treated with the psychostimulant drug of abuse amphetamine.

Dopamine-denervated rats emitted high numbers of 50-kHz USVs after each amphetamine administration, which indicates that activation of dopamine transmission in the mPFC or DS is not necessary for the initiation of calling behavior stimulated by amphetamine. Since emission of 50-kHz USVs elicited by acute amphetamine administration is considered a marker of the positive emotional state induced by the drug (Burgdorf et al., 2001; Wintink and Brudzynski, 2001), the sustained calling behavior displayed by dopamine-denervated rats on the first day of repeated treatment with amphetamine may indicate that the activation of dopamine transmission in the mPFC or DS is not required for the manifestation of the acute rewarding effects of that drug. Yet, rats bearing a dopaminergic denervation of either the mPFC or the DS displayed less evident sensitization of 50-kHz USV emissions during repeated amphetamine administration, as well as a trend towards less marked calling behavior after amphetamine challenge, compared with sham-operated rats. In this regard, it is noteworthy that dopamine transmission in the mPFC and DS modulates the modifications in the motivational properties of psychoactive drugs that occur with repeated drug experience (Koob and Volkow, 2010). Therefore, our findings further underline the important role of dopamine in calling behavior, and may support the usefulness of measuring 50-kHz USV emissions to investigate the modifications in the affective state of rats repeatedly treated with psychoactive drugs (Ahrens et al., 2009; Mu et al., 2009; Simola and Morelli, 2015; Taracha et al., 2014).

Rats bearing a dopaminergic denervation of the mPFC and previously treated with amphetamine emitted very low numbers of 50-kHz USVs upon re-exposure to the drug-paired environment. Conversely, the same situation elicited significant 50-kHz USV emissions in amphetamine-treated rats that were sham-operated in the mPFC or DS as well as in rats that were dopamine-denervated in the DS, albeit a trend towards a less marked calling behavior was observed in the latter animals. Earlier investigations have suggested that emission of 50-kHz USVs elicited by the presentation of environmental cues previously paired with drug administration may reflect the presence of drug conditioning (Knutson et al., 1999; Hamed et al., 2012; Maier et al., 2010; Simola and Morelli, 2015). Besides, it has been demonstrated that dopaminergic denervation of the mPFC may induce memory deficits in rats (Bubser and Schmidt, 1990; Clinton et al., 2006; Kadowaki Horita et al., 2013). On these bases, we may hypothesize that dopaminergic denervation of the mPFC impaired the generation of memories for the context of amphetamine administration, and that such an impairment eventually led to scarce calling behavior on re-exposure to the drug-paired environment. This hypothesis may be consistent with the evidence showing that the mPFC tightly interacts with the cornu Ammonis (CA) CA1 and CA3 regions of the hippocampus (de Souza et al., 2016; Thierry et al., 2000), which are critically involved in the generation of memories (Hunsaker and Kesner, 2008). Interestingly, a recent study has demonstrated that the functional interaction between the mPFC and the CA1 is crucial for the processing of episodic memory, as well as for the integration of spatial memory components (Chao et al., 2017). Moreover, additional evidence demonstrates that dopamine release and intact function of D_1 receptors is necessary for the generation of spatial memories in CA1 (Retailleau and Morris, 2018). Based on this evidence, we may speculate that dopaminergic denervation altered the connection between the mPFC and the hippocampus, and that this mechanism could underlie the scarce vocalization displayed by rats lesioned in the mPFC upon their re-exposure to the environment previously paired with amphetamine administration. The results of the present study may also suggest that dopamine transmission in the mPFC and DS have a dissimilar influence on the vocal expression of positive emotional states that are triggered by environmental cues previously associated with the administration of psychoactive drugs. Notably, the latter hypothesis may be substantiated by the finding that amphetamine challenge induced

dissimilar modifications in the levels of Zif-268 in the NAc core of rats bearing a dopaminergic denervation of the mPFC or DS, since the NAc core is critically involved in the generation of drug-associated memories (see below).

Analysis of the categorized 50-kHz USVs emitted during repeated treatment and after challenge with amphetamine revealed non-significant trends towards increased or decreased emission of definite call categories in rats bearing a dopaminergic denervation of either the mPFC or DS. Conversely, analysis of categorized calls emitted on test cage re-exposure showed that rats bearing a dopaminergic denervation of the mPFC and previously treated with amphetamine emitted very low numbers of FM calls on re-exposure to the environment paired with drug administration. Earlier investigations have suggested that modifications in the emission of categorized 50-kHz USVs may provide information about the effects that psychoactive drugs elicit on the emotional state of rats. For example, it has been reported that amphetamine robustly stimulates the emission of trill and other FM calls, and may also increase the ratio between the trill and flat calls emitted (Simola et al., 2010; Wright et al., 2010). These effects have been proposed to selectively mark the induction of positive affect by amphetamine, based on the evidence that rats emit high numbers of trills and other FM calls and in response to social stimuli with positive emotional valence (Burgdorf et al., 2008; Wöhr et al., 2008). Nevertheless, the behavioral significance of the categorized 50-kHz USVs emitted by rats treated with psychoactive drugs is still disputed. In fact, amphetamine may also stimulate a sustained emission of flat calls (Simola et al., 2012; Wright et al., 2010). Moreover, a recent investigation by our group in rats treated with amphetamine has demonstrated the existence of positive correlations between the numbers of various categories of 50-kHz USVs and the total number of calls (i.e., the sum of all call categories) that were emitted (Simola and Costa, 2018). The latter finding could suggest the hypothesis that it is the total number of 50-kHz USVs emitted, rather than the numbers of categorized calls, that communicates the modifications in the emotional state of rats treated with psychoactive drugs. On the one hand, the results obtained here may lend support to this view, since dopaminergic denervation of the mPFC also reduced the total number of 50-kHz USVs that rats previously treated with amphetamine emitted upon re-exposure to the environment

paired with drug administration. On the other hand, it is noteworthy that sham-operated and dopamine-denervated rats used in the present study displayed interindividual variability in the emission of 50-kHz USVs, in agreement with previous investigations (Schwarting et al., 2007; Simola et al., 2018; Wöhr et al., 2008). Moreover, the modifications in the emissions of categorized calls observed during repeated treatment and after challenge with amphetamine did not always parallel those involving the total number of calls emitted. Hence, the present findings claim for more exhaustive investigations aimed at elucidating whether specific brain regions regulate the emission of definite categories of 50-kHz USVs, which may help to clarify the significance of categorized calls in terms of affective properties of drugs.

As mentioned in the Introduction, the emission of amphetamine-stimulated 50-kHz USVs is initiated by the activation of dopamine receptors in the NAc (Burgdorf et al., 2001; Thompson et al., 2006). Moreover, the NAc is interconnected with the mPFC and the DS (Bimpisidis et al., 2012; Ikeda et al., 2013). On these bases, we speculated that dopaminergic denervation of the mPFC or the DS could alter the connectivity of either region with the NAc, and in turn affect calling behavior. Accordingly, we evaluated the numbers of nuclei positive for Zif-268 in the NAc shell and core as a measure of neuronal activation. Challenge with amphetamine increased the numbers of nuclei positive for Zif-268 in the NAc shell and core of rats that were sham-operated or dopamine-denervated in either the mPFC or DS, although this increase was less marked in the latter rats. In detail, rats that were dopamine-denervated in the mPFC displayed an attenuated increase in Zif-268 in both accumbal subregions, whereas rats that were dopamine-denervated in the DS showed an attenuated increase in Zif-268 in the NAc shell only. Attenuated increase in the immunoreactivity for Zif-268 in the NAc shell may reflect a reduced responsiveness of accumbal neurons to amphetamine, which could be consistent with the finding that dopamine-denervated rats showed a trend towards decreased emission of 50-kHz USVs after amphetamine challenge, compared with sham-operated rats. In addition, the present results suggest that the NAc core may modulate the conditioned calling behavior that is displayed by rats previously treated with amphetamine on re-exposure to the environment where they receive drug treatment. In fact, rats bearing a dopaminergic denervation of

the DS showed significantly increased immunoreactivity for Zif-268 in the NAc core after amphetamine challenge and displayed a sustained emission of 50-kHz USVs on re-exposure to the environment previously paired with amphetamine administration. Conversely, the same situation elicited scarce calling behavior in rats bearing a dopaminergic denervation of the mPFC, which also showed an attenuated increase in the immunoreactivity for Zif-268 in the NAc core after amphetamine challenge. Notably, the possible involvement of the NAc in calling behavior elicited by the exposure to environmental cues previously paired with the administration of psychoactive drugs has also been suggested by a recent study in rats treated with morphine (Hamed and Kursa, 2018). In fact, that study demonstrated that re-exposure of rats to the morphine-paired context after drug withdrawal elicited the emission of 50-kHz USVs and increased the glutamatergic activity in the NAc, although the latter effect was measured in the whole nucleus and no distinctions were made between the shell and core subregions (Hamed and Kursa, 2018). Nevertheless, the possibility that the NAc core modulates calling behavior in response to environmental cues previously paired with drug administration may be consistent with the evidence that the NAc core is a region critically involved in the retention of drug-associated memories (Li et al., 2011; Crespo et al., 2012; Ding et al., 2013).

It must be acknowledged that the present study has some potential limitations. Thus, a single dose of amphetamine was used here, and previous investigations have reported that amphetamine may elicit contrasting behavioral effects in rats with cortical or striatal dopaminergic lesions that depend on the dose administered. For example, in rats bearing a dopaminergic denervation of the mPFC and treated with acute amphetamine increased stereotyped activity has been demonstrated after moderate-to-high doses (2.5 mg/kg or higher) (Carter and Pyckock, 1980; Sokolowski and Salamone, 1994) whereas no increase in locomotor activity has been reported after low-to-moderate doses (0.5-1.5 mg/kg) (Banks and Gratton, 1995; King and Finlay, 1995; Bjijou et al., 2002). Although further dose-response studies may be warranted to exhaustively describe how dopamine transmission in the mPFC and DS modulates 50-kHz USV emissions in rats treated with amphetamine, it should be emphasized that ~~the~~ amphetamine elicits effects on motor activity that do not always overlap with those it exerts on calling behavior (Simola and Morelli, 2015; Taracha et al.,

2014). Moreover, the present study employed lesion procedures that did not discriminate among the subregions of the mPFC (i.e., infralimbic/prelimbic cortex) or DS (i.e., dorsomedial/dorsolateral striatum), and it is known that different cortical or striatal subregions may have dissimilar influences on several aspects of rats' behavior (Jinks and McGregor, 1997; Oualian et al., 2010; Pelloux et al., 2013; Yin et al., 2004, 2005). Accordingly, we cannot rule out the possibility that dopamine depletion in specific subregions of the mPFC or DS may influence calling behavior of amphetamine-treated rats in a fashion different from that observed here. Finally, independent studies have demonstrated that non-dopaminergic transmitters may modulate the emission of 50-kHz USVs in rats treated with amphetamine (de Oliveira Guaita et al., 2018; Hamed et al., 2016; Simola and Brudzynski, 2018; Wöhr et al., 2015; Wright et al., 2012). In addition, it is well known that amphetamine may significantly impact the GABAergic, glutamatergic noradrenergic, and serotonergic systems (Sulzer et al., 2005; Jiao et al., 2015). Although a previous study that applied the surgical procedures used here to the mPFC demonstrated only partial damage in the noradrenergic and serotonergic pathways (Bjijou et al., 2002), based on the above considerations we cannot exclude that such a partial damage may have contributed, at least in part, to the alterations in calling behavior observed here in dopamine-denervated rats treated with amphetamine.

5. Conclusions

In conclusion, the present study demonstrates that dopamine transmission in the mPFC and DS modulates certain aspects of 50-kHz USV emissions in rats treated with amphetamine. These findings are of interest to identify the neuronal circuits that regulate the effects of psychoactive drugs on calling behavior. Indeed, measuring calling behavior may represent a useful ethological methodology able to clarify the mechanisms of drug-induced effects on emotional and motivational states.

Contributors

NS designed the study. GC, MS, JM, and NS performed the experiments. GC and NS analyzed the data and wrote the manuscript. MM contributed to the interpretation of data and critically revised the manuscript. All authors have approved the final version of the manuscript.

Declaration of interest

The authors declare no conflict of interest.

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Figure Legends

Figure 1. Experimental plan. Experiments began 14 days after surgeries. Rats were habituated to the test cage for 2 consecutive days and then treated with acute vehicle (i.p.), to evaluate basal calling behavior. Starting from the day after, rats were repeatedly treated with amphetamine (1 mg/kg, i.p.) in the test cage (×5) on alternate days, followed by drug withdrawal in the home cage (7 days), re-exposure to the test cage, and drug challenge. Amph=amphetamine; Chall=challenge; DS=dorsal striatum; Hab=habituation to the test cage; mPFC=medial prefrontal cortex; Re-exp=re-exposure to the test cage.

Figure 2. Example of sonograms of categorized 50-kHz ultrasonic vocalizations isolated in the present study. Vocalizations demonstrated are examples of independent calls emitted by different rats. FM=frequency modulated.

Figure 3. Densities of fibers positive to tyrosine hydroxylase or noradrenaline transporter in the medial prefrontal cortex (A) and dorsal striatum (B) of rats that received either injections of 6-OHDA or sham surgery at the same sites. * indicates $p<0.01$ vs. SHAM mPFC + VEH; # indicates $p<0.01$ vs. SHAM mPFC + AMPH 1; § indicates $p<0.01$ vs. SHAM DS + VEH; ^ indicates $p<0.01$ vs SHAM DS + AMPH 1. N=8-10 rats for each group. 6-OHDA=6-hydroxydopamine; AMPH=amphetamine; DS=dorsal striatum; mPFC=medial prefrontal cortex; NET=noradrenaline transporter; TH= tyrosine hydroxylase

Figure 4. Effects of repeated treatment with amphetamine (1 mg/kg i.p.) (A,C,E,G) or vehicle (B,D,F,H) on the numbers of total and categorized 50-kHz ultrasonic vocalizations emitted in rats subjected to dopaminergic denervation of the medial prefrontal cortex or sham-surgery at the same site. Filled black symbols indicate $p<0.01$ vs. the respective group of vehicle-treated rats, as revealed by three-way ANOVA. * indicates $p\leq0.01$ vs. the first administration within each experimental group, as revealed by two-way ANOVA. Emission of 50-kHz ultrasonic vocalizations was recorded for 30 minutes. N=8-10 rats for each experimental group. 6-OHDA=6-

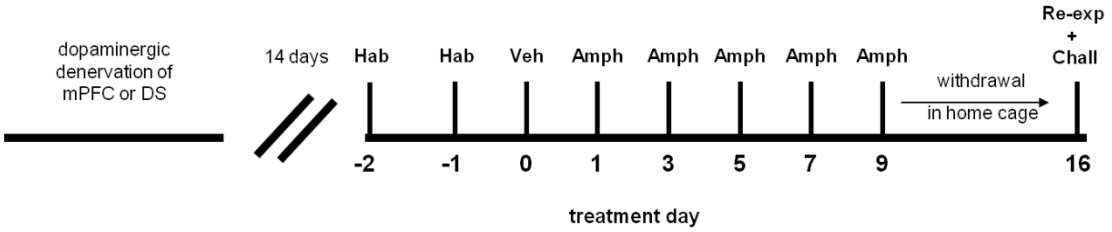
hydroxydopamine; AMPH=amphetamine; mPFC=medial prefrontal cortex; USVs=ultrasonic vocalizations; VEH=vehicle.

Figure 5. Effects of challenge with amphetamine (1 mg/kg i.p.) or vehicle (A) and of re-exposure to the test cage (B) on calling behavior in rats subjected to either dopaminergic denervation of the medial prefrontal cortex or sham-surgery at the same site. The figure demonstrates the numbers of total and categorized 50-kHz ultrasonic vocalizations emitted. * indicates $p \leq 0.01$ vs. SHAM mPFC + VEH; # indicates $p < 0.01$ vs. 6-OHDA mPFC + VEH. Emission of 50-kHz ultrasonic vocalizations was recorded for 30 minutes after challenge or for 10 minutes on test cage re-exposure. N=8-10 rats for each experimental group. 6-OHDA=6-hydroxydopamine; AMPH=amphetamine; FM=frequency modulated; mPFC=medial prefrontal cortex; USVs=ultrasonic vocalizations; VEH=vehicle.

Figure 6. Effects of repeated treatment with amphetamine (1 mg/kg i.p.) (A,C,E,G) or vehicle (B,D,F,H) on the numbers of total and categorized 50-kHz ultrasonic vocalizations emitted in rats subjected to dopaminergic denervation of the dorsal striatum or sham-surgery at the same site. Filled black symbols indicate $p < 0.01$ vs. the respective group of vehicle-treated rats, as revealed by three-way ANOVA. * indicates $p \leq 0.01$ vs. the first administration within each experimental group, as revealed by two-way ANOVA. Emission of 50-kHz ultrasonic vocalizations was recorded for 30 minutes. N=8-10 rats for each experimental group. 6-OHDA=6-hydroxydopamine; AMPH=amphetamine; DS=dorsal striatum; USVs=ultrasonic vocalizations; VEH=vehicle.

Figure 7. Effects of challenge with amphetamine (1 mg/kg i.p.) or vehicle (A) and of re-exposure to the test cage (B) on calling behavior in rats subjected to either dopaminergic denervation of the dorsal striatum or sham-surgery at the same site. The figure demonstrates the numbers of total and categorized 50-kHz ultrasonic vocalizations emitted. § indicates $p < 0.01$ vs. SHAM DS + VEH; ^ indicates $p \leq 0.01$ vs. 6-OHDA DS + VEH. Emission of 50-kHz ultrasonic vocalizations was recorded for 30 minutes after challenge or for 10 minutes on test cage re-exposure. N=8-10 rats for each experimental group. 6-OHDA=6-hydroxydopamine; AMPH=amphetamine; DS=dorsal striatum; FM=frequency modulated; USVs=ultrasonic vocalizations; VEH=vehicle.

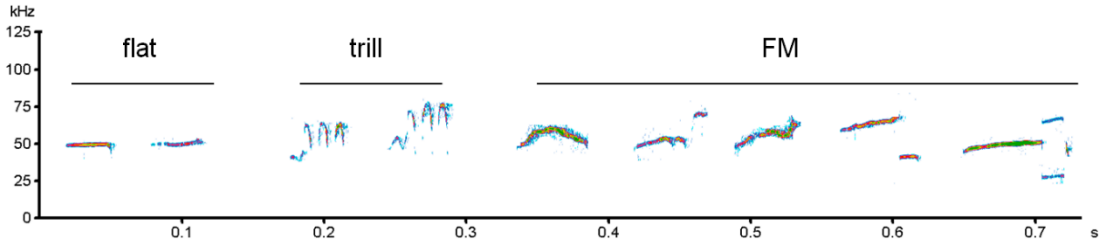
Figure 8. Effects of challenge with amphetamine (1 mg/kg, i.p.) or vehicle on the levels of Zif-268 in the nucleus accumbens shell and core. The figure demonstrates representative high-resolution images (×20) immunostained for Zif-268 and histograms for rats subjected to either dopaminergic denervation or sham-surgery in the medial prefrontal cortex (A) or dorsal striatum (B). Each graph reports the percentage of Zif-268-positive nuclei compared with the respective group of sham-operated rats challenged with vehicle (SHAM mPFC + VEH or SHAM DS + VEH). * indicates $p<0.05$ vs. SHAM mPFC + VEH; § indicates $p<0.05$ vs. 6-SHAM DS + VEH; ^ indicates $p<0.05$ vs. 6-OHDA DS + VEH. N=8-10 rats for each experimental group. aca=anterior commissure, anterior part; AcbSh=nucleus accumbens shell; AcbC=nucleus accumbens core; 6-OHDA=6-hydroxydopamine; AMPH=amphetamine; DS=dorsal striatum; mPFC=medial prefrontal cortex; VEH=vehicle. Scale bar, 50 μm.


dopaminergic
denervation of
mPFC or DS
14 days
Hab
Hab
Veh
Amph
Amph
Amph
Amph
Amph
withdrawal
in home cage
Re-exp
+
Chall
-2
-1
0
1
3
5
7
9
16
treatment day

1

2 Figure 1

3



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2 Figure 2

3

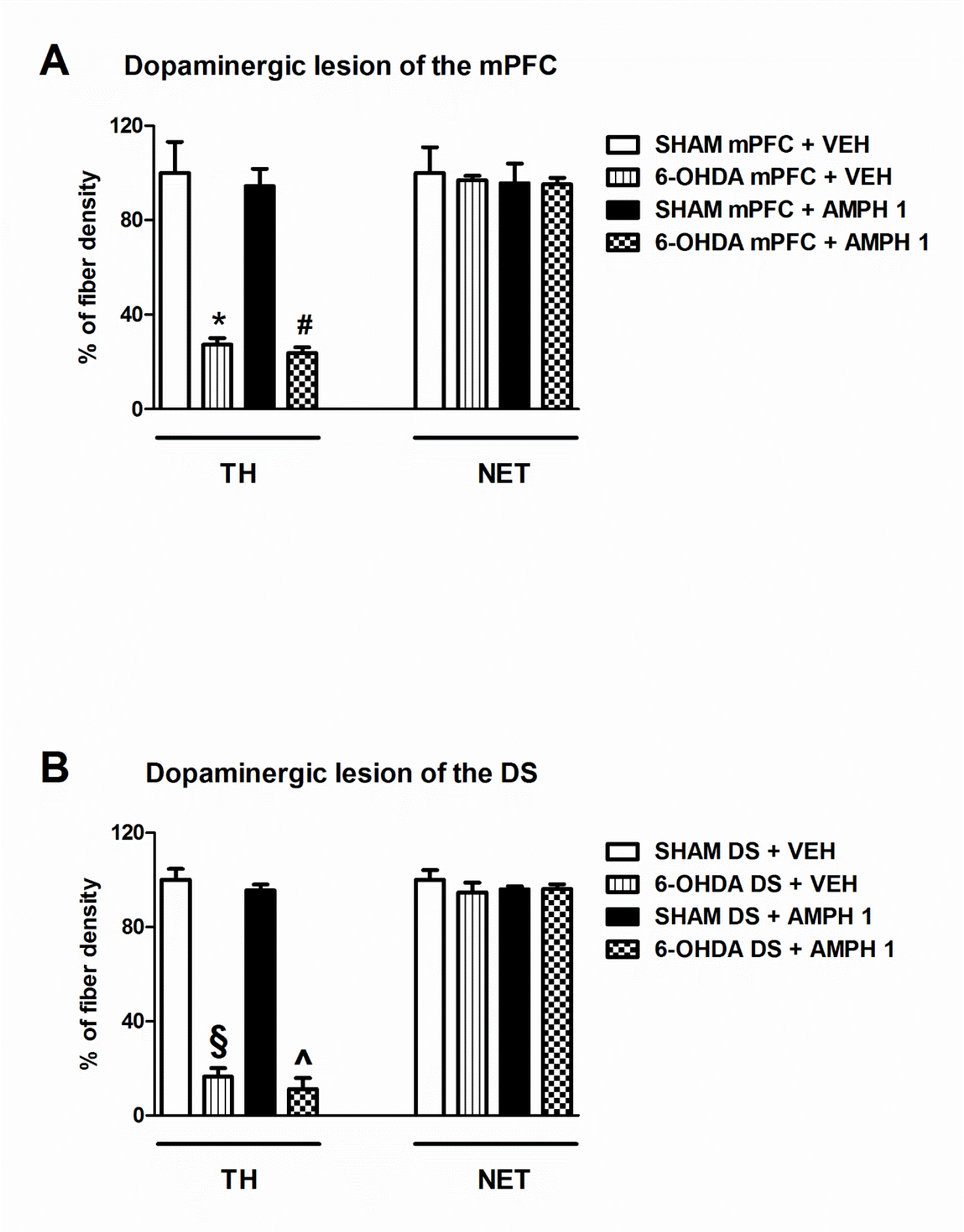
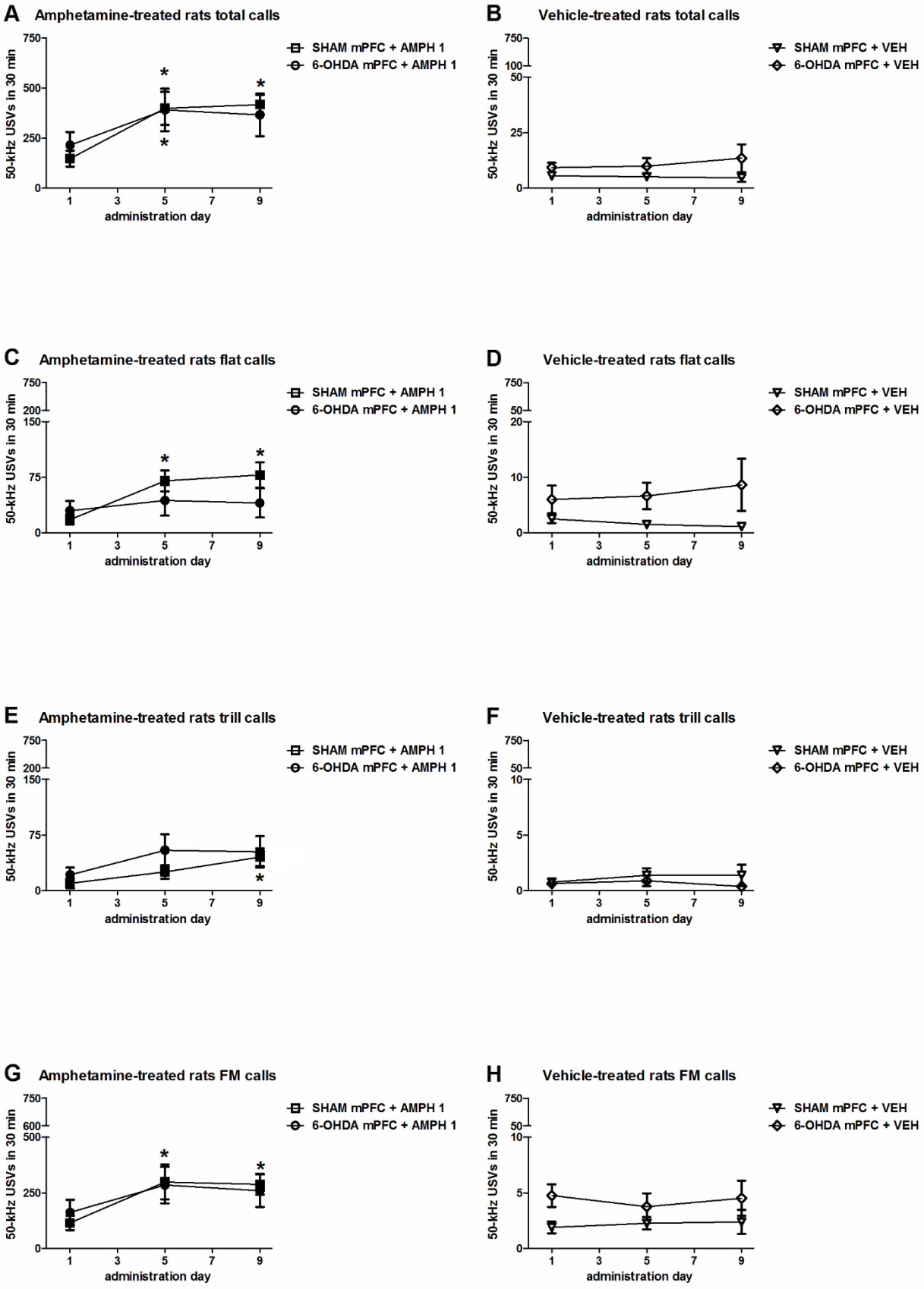

A
Dopaminergic lesion of the mPFC
% of fiber density
120
80
40
0
*
#
TH
NET
SHAM mPFC + VEH
6-OHDA mPFC + VEH
SHAM mPFC + AMPH 1
6-OHDA mPFC + AMPH 1
B
Dopaminergic lesion of the DS
% of fiber density
120
80
40
0
§
^
TH
NET
SHAM DS + VEH
6-OHDA DS + VEH
SHAM DS + AMPH 1
6-OHDA DS + AMPH 1

Figure 3


A
Amphetamine-treated rats total calls
50-kHz USVs in 30 min
administration day
SHAM mPFC + AMPH 1
6-OHDA mPFC + AMPH 1
B
Vehicle-treated rats total calls
SHAM mPFC + VEH
6-OHDA mPFC + VEH
C
Amphetamine-treated rats flat calls
D
Vehicle-treated rats flat calls
E
Amphetamine-treated rats trill calls
F
Vehicle-treated rats trill calls
G
Amphetamine-treated rats FM calls
H
Vehicle-treated rats FM calls

1

Figure 4

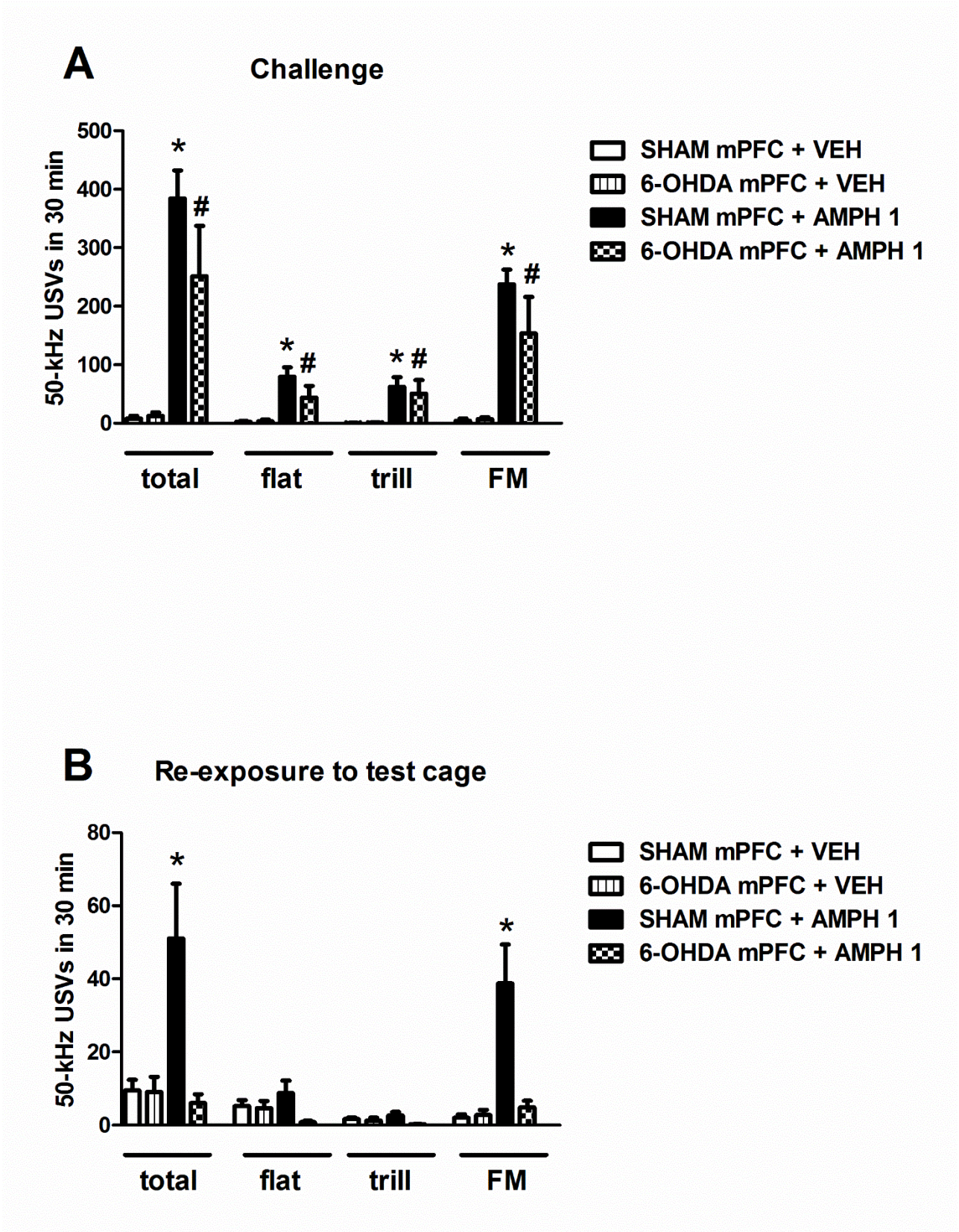


Figure 5

1

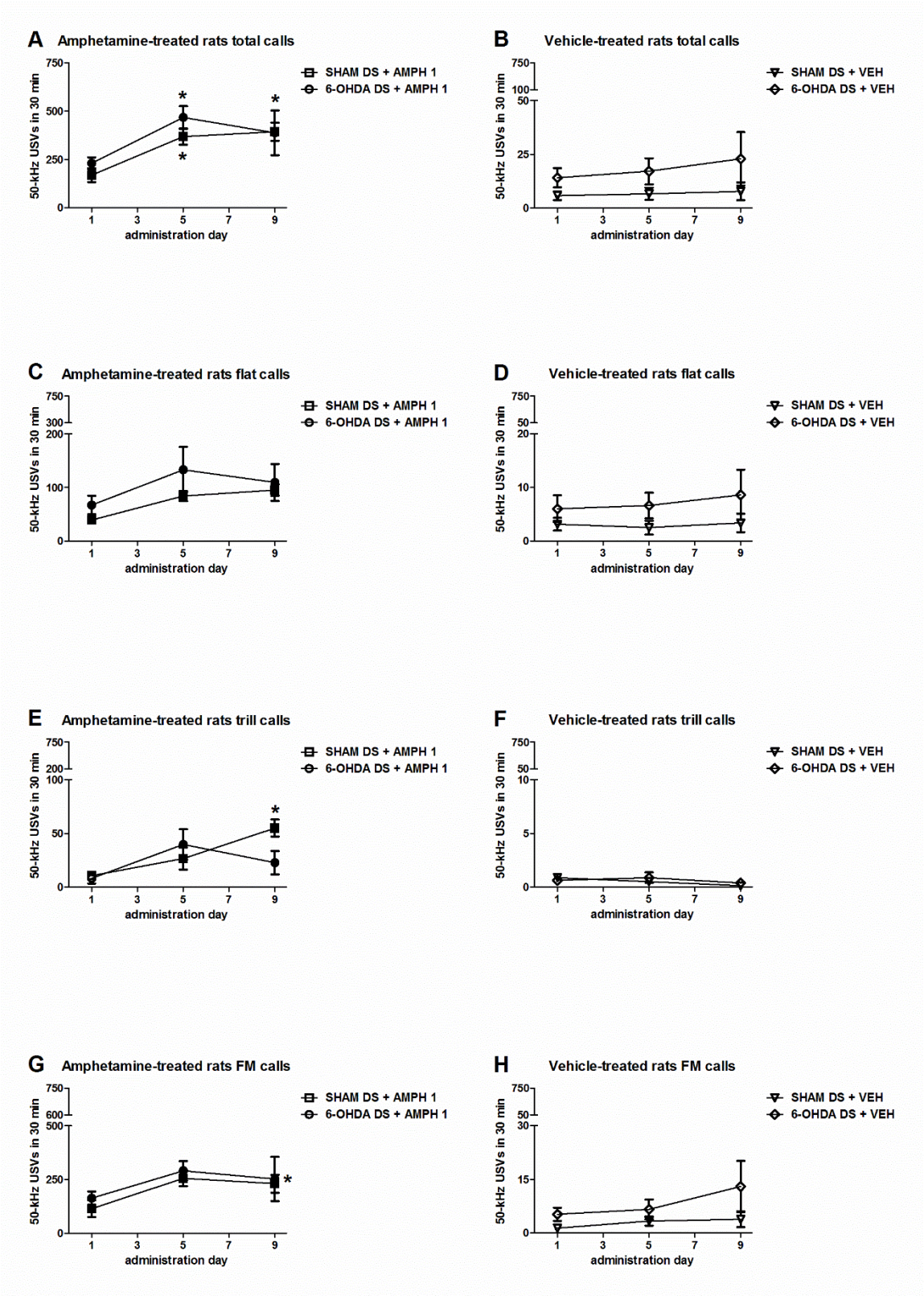
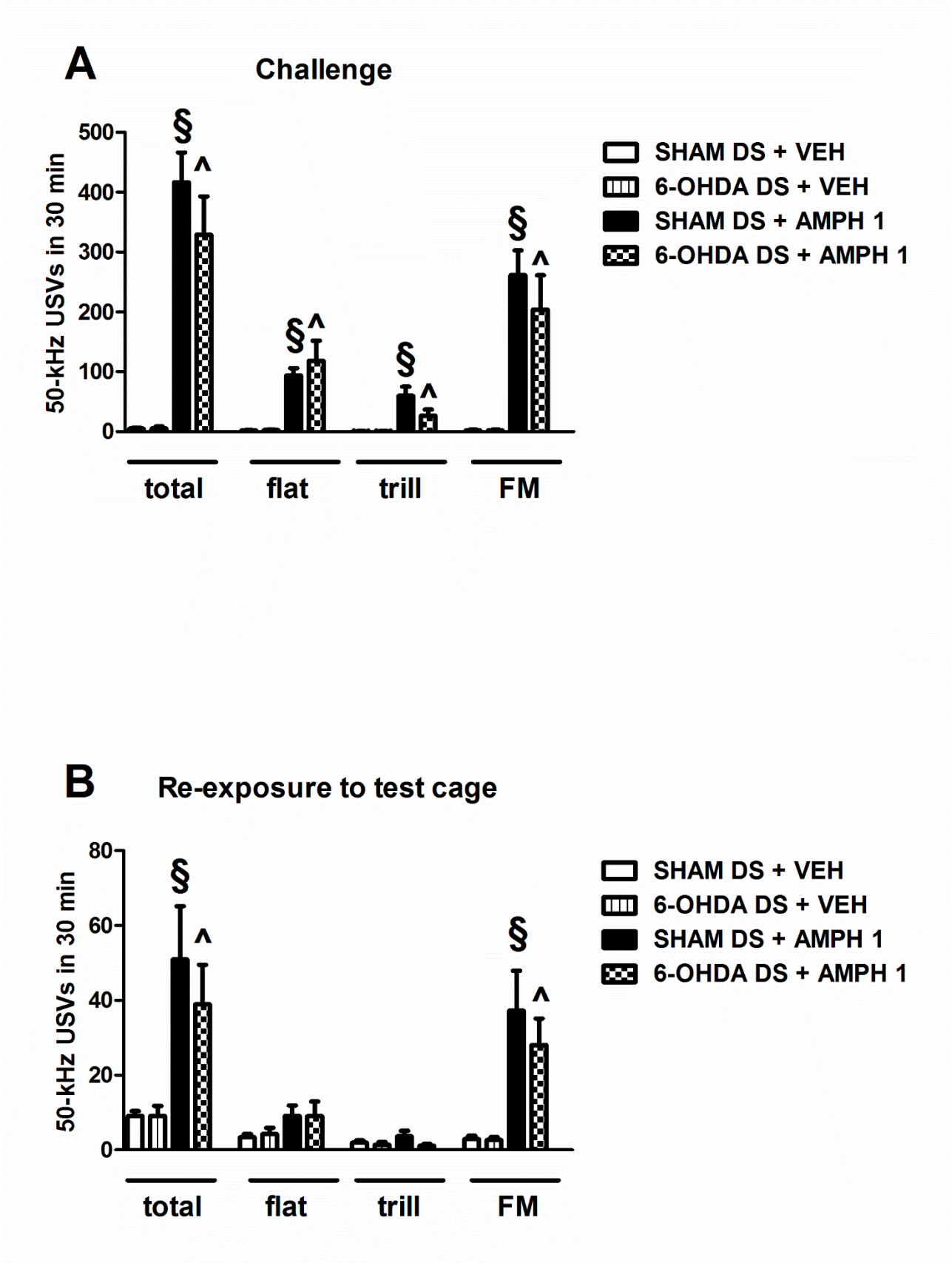

A
Amphetamine-treated rats total calls
50-kHz USVs in 30 min
administration day
SHAM DS + AMPH 1
6-OHDA DS + AMPH 1
B
Vehicle-treated rats total calls
SHAM DS + VEH
6-OHDA DS + VEH
C
Amphetamine-treated rats flat calls
D
Vehicle-treated rats flat calls
E
Amphetamine-treated rats trill calls
F
Vehicle-treated rats trill calls
G
Amphetamine-treated rats FM calls
H
Vehicle-treated rats FM calls

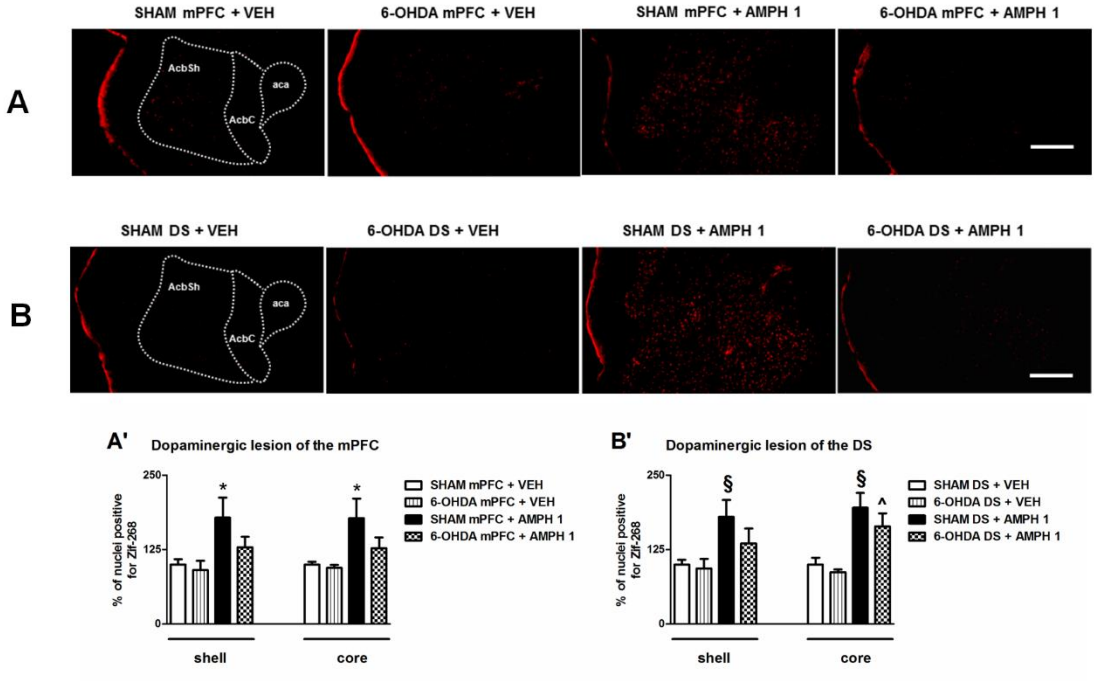
Figure 6


A
Challenge
50-kHz USVs in 30 min
500
400
300
200
100
0
§
^
total
flat
trill
FM
SHAM DS + VEH
6-OHDA DS + VEH
SHAM DS + AMPH 1
6-OHDA DS + AMPH 1
B
Re-exposure to test cage
50-kHz USVs in 30 min
80
60
40
20
0
§
^
total
flat
trill
FM
SHAM DS + VEH
6-OHDA DS + VEH
SHAM DS + AMPH 1
6-OHDA DS + AMPH 1

1

2 Figure 7

1


SHAM mPFC + VEH
6-OHDA mPFC + VEH
SHAM mPFC + AMPH 1
6-OHDA mPFC + AMPH 1
A
AcbSh
aca
AcbC
SHAM DS + VEH
6-OHDA DS + VEH
SHAM DS + AMPH 1
6-OHDA DS + AMPH 1
B
AcbSh
aca
AcbC
A'
Dopaminergic lesion of the mPFC
% of nuclei positive for Zif-268
250
125
0
*
*
SHAM mPFC + VEH
6-OHDA mPFC + VEH
SHAM mPFC + AMPH 1
6-OHDA mPFC + AMPH 1
shell
core
B'
Dopaminergic lesion of the DS
% of nuclei positive for Zif-268
250
125
0
§
§
^
SHAM DS + VEH
6-OHDA DS + VEH
SHAM DS + AMPH 1
6-OHDA DS + AMPH 1
shell
core

1

2 Figure 8