

## RESEARCH ARTICLE

# Involvement of Glutamate NMDA Receptors in the Acute, Long-Term, and Conditioned Effects of Amphetamine on Rat 50 kHz Ultrasonic Vocalizations

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## Abstract

**Background:** Rats emit 50 kHz ultrasonic vocalizations (USVs) in response to either natural or pharmacological pleasurable stimuli, and these USVs have emerged as a new behavioral measure for investigating the motivational properties of drugs. Earlier studies have indicated that activation of the dopaminergic system is critically involved in 50 kHz USV emissions. However, evidence also exists that non-dopaminergic neurotransmitters participate in this behavioral response.

**Methods:** To ascertain whether glutamate transmission plays a role in 50 kHz USV emissions stimulated by amphetamine, rats received five amphetamine (1–2 mg/kg, i.p.) administrations on alternate days in a test cage, either alone or combined with the glutamate N-methyl-D-aspartate receptor antagonist MK-801 (0.1–0.5 mg/kg, i.p.). Seven days after treatment discontinuation, rats were re-exposed to the test cage to assess drug conditioning, and afterwards received a drug challenge. USVs and locomotor activity were evaluated, along with immunofluorescence for Zif-268 in various brain regions and spontaneous alternation in a Y maze.

**Results:** Amphetamine-treated rats displayed higher 50 kHz USV emissions and locomotor activity than vehicle-treated rats, and emitted conditioned vocalizations on test cage re-exposure. Rats co-administered amphetamine and MK-801 displayed lower and dose-dependent 50 kHz USV emissions, but not lower locomotor activity, during repeated treatment and challenge, and scarce conditioned vocalization compared with amphetamine-treated rats. These effects were associated with lower levels of Zif-268 after amphetamine challenge and spontaneous alternation deficits.

**Conclusions:** These results indicate that glutamate transmission participates in the acute, long-term, and conditioned effects of amphetamine on 50 kHz USVs, possibly by influencing amphetamine-induced long-term neuronal changes and/or amphetamine-associated memories.

**Keywords:** MK-801, psychostimulant, reward, sensitization, spontaneous alternation, Zif-268

## Introduction

Solid experimental evidence indicates that the emission of ultrasonic vocalizations (USVs) may reflect changes in the emotional state of rats (Panksepp, 2005; Schwarting et al., 2007; Brudzynski, 2013; Simola, 2015). In particular, the so-called

50 kHz USVs, which are contained within the 35–80 kHz frequency range (Brudzynski, 2013), are thought to indicate positive affective states, since rats emit these vocalizations in response to or anticipation of pleasurable stimuli, such as

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mating, heterospecific play with familiar humans (or “tickling”), and non-aggressive homospecific social encounters (Burgdorf et al., 2008, 2011). Interestingly, drugs of abuse can affect 50kHz USV emissions (Wintink and Brudzynski, 2001; Mu et al., 2009; Simola et al., 2014); therefore, 50kHz USVs are increasingly being used to study the motivational properties of drugs (Wright et al., 2012a; Mahler et al., 2013; Barker et al., 2014; Taracha et al., 2014).

Earlier studies have demonstrated that activation of dopamine receptors, particularly in the shell of the nucleus accumbens (NAc), is critically involved in the emission of 50kHz USVs by rats (Thompson et al., 2006; Burgdorf et al., 2011). This finding agrees with the results showing that drugs with marked dopaminomimetic properties robustly stimulate 50kHz USV emissions (Wintink and Brudzynski, 2001; Mu et al., 2009), while drugs devoid of these properties do not (Simola et al., 2010, 2014). However, evidence also exists showing that neurotransmitters other than dopamine influence 50kHz USVs. Emission of 50kHz USVs by naïve rats has been observed after the intracerebral infusion of glutamate, as well as following the antagonism of either the  $\kappa$ -opioid or the serotonin 5HT<sub>2c</sub> receptors (Fu and Brudzynski, 1994; Hamed et al., 2015; Wöhr et al., 2015). Moreover, activation of either the  $\alpha_1$  or  $\alpha_2$  noradrenaline receptors, or of the 5HT<sub>2c</sub> serotonin receptors attenuates the emission of 50kHz USVs stimulated by amphetamine (Wright et al., 2012b; Wöhr et al., 2015). These latter results are particularly relevant, since amphetamine is the prototype of drugs that stimulate 50kHz USV emissions (Wright et al., 2012b). Moreover, recent studies have suggested that long-term changes in amphetamine’s effects on 50kHz USVs may reflect modifications in the motivational properties of this drug with repeated experience (Ahrens et al., 2009; Simola and Morelli, 2015). Therefore, elucidating the role of different neurotransmitters in the effects of amphetamine on 50kHz USVs may help to develop new experimental models for studying the addictive properties of this drug. Amphetamine acts by stimulating the release of monoamines and glutamate in various brain regions (Shoblock et al., 2003; Iversen, 2008), and glutamate is involved in amphetamine’s addictive properties (Kalivas, 2007). However, while the role of monoamines in amphetamine’s effects on 50kHz USVs has been described (Wright et al., 2012b, 2013; Wöhr et al., 2015), the role of glutamate is unknown.

To address this issue, this study evaluated the influence of N-methyl-D-aspartate (NMDA) glutamate receptor antagonism on the acute, long-term, and conditioned effects of amphetamine on 50kHz USVs. To further clarify the importance of the results of this study, development of locomotor sensitization was also evaluated, to obtain an additional behavioral correlate of changes in amphetamine motivational properties with repeated experience (see Robinson and Berridge, 2001). This study specifically evaluated the involvement of NMDA receptors in the effects of amphetamine on 50kHz USVs, since these receptors are those most involved in the modulatory effects of glutamate transmission on amphetamine-induced behavioral sensitization (Vanderschuren and Kalivas, 2000). Moreover, MK-801 was used to antagonize NMDA receptors, since this drug counteracts amphetamine-induced sensitization (Karler et al., 1989; Wolf and Khansa, 1991). Finally, to investigate the mechanisms by which NMDA receptor antagonism influences amphetamine-stimulated 50kHz USVs, this study evaluated the expression of the protein Zif-268 (an index of neuronal activation) in the NAc shell and core, dorsolateral striatum (DLS), and medial prefrontal cortex (mPFC), as well as rats’ spontaneous alternation in a Y maze as a measure of cognitive performance.

## Methods

### Subjects

Male Sprague–Dawley rats (Harlan) weighing 275–300 g were used. Rats (4 or 5 per cage) were housed in standard polycarbonate cages with sawdust bedding, and maintained on a 12-h light/dark cycle (lights on at 08:00). Food and water were freely available, except during the experiments, which were performed from 10:00 to 16:00.

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. Efforts were made to minimize animal discomfort and reduce the number of animals used.

### Drugs

D-Amphetamine (sulfate) and MK-801 {dizocilpine, (5S,10R)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine apomorphine hydrogen maleate}, both obtained from Sigma–Aldrich, were dissolved in distilled water and administered intraperitoneally (i.p.), in a 3 ml/kg (amphetamine) or 1 ml/kg (MK-801) volume. Drug doses were based on our previous studies on amphetamine-stimulated 50kHz USVs (Simola et al., 2014; Simola and Morelli, 2015), and on our preliminary experiments on the effects of MK-801 on 50kHz USV emissions during non-aggressive social interactions (data not shown). Doses of MK-801 refer to the maleate salt. MK-801 was administered 15 min before behavioral evaluation at the doses of 0.1, 0.2, or 0.5 mg/kg (i.p.). Amphetamine was administered immediately before behavioral evaluation at the doses of 1 or 2 mg/kg (i.p.).

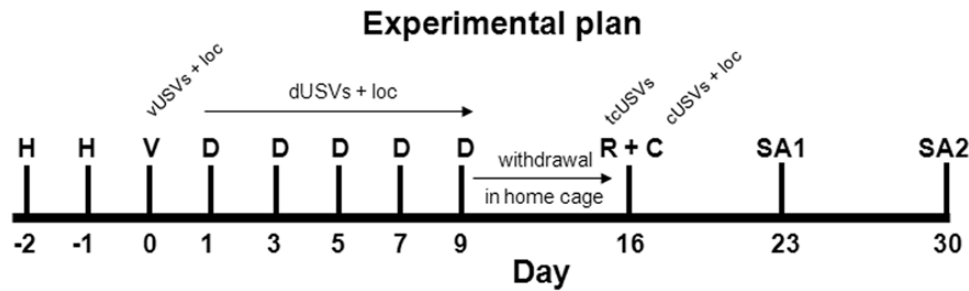
### Experimental Procedure

Experiments were designed according to Simola et al. (2014), and consisted of six phases: (1) test cage habituation; (2) acute vehicle administration in the test cage, to quantify basal USVs; (3) repeated drug administration ( $\times 5$ ) in the test cage every other day; (4) drug withdrawal in the home-cage; (5) re-exposure to the test cage, immediately followed by drug challenge; and (6) evaluation of rats’ performance in a Y maze.

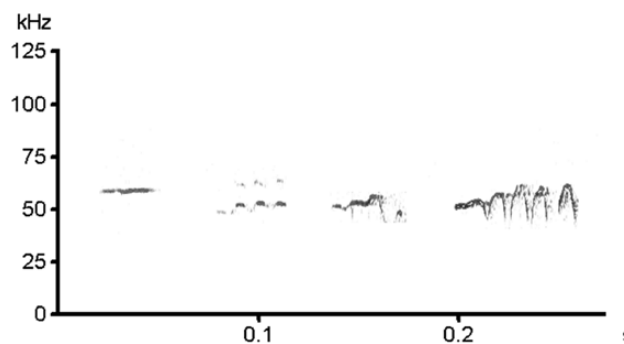
USVs and locomotor activity were evaluated throughout phases 1–5. Rats were randomly assigned to their experimental group and were used only once. Rats treated with distilled water only (vehicle, i.p.) served as controls. Each experimental group included at least 7 rats (see figure legends for exact numbers). Figure 1 reports the detailed experimental plan.

### Recording of USVs and Locomotor Activity

Experiments were performed in a quiet room. USV recordings were performed according to Simola et al. (2012). Briefly, each rat was placed alone in a Plexiglas cylinder (diameter, 25 cm; height, 30 cm) topped with a lid equipped with an ultrasonic microphone (CM16/CMPA, Avisoft) and connected to an ultrasound-recording device (UltraSoundGate 116 Hb, Avisoft). During recordings, constant gain was always maintained. Figure 2 shows examples of 50kHz USVs recorded during this study. USVs were recorded after drug or vehicle administration for 30 min, starting immediately after rats’ placement in the test cage. USVs emitted on test cage re-exposure were recorded for 10 min, starting immediately after re-exposure (Figure 1). The recording times were selected



**Figure 1.** Experimental plan. Rats were habituated (15 min, twice a day) to the test cage for 2 days (H), then received an acute administration of vehicle (V) to evaluate basal emission of 50 kHz ultrasonic vocalizations (USVs). Starting from the day after vehicle administration, rats underwent repeated drug treatment on alternate days in the test cage (D). Recording of 50 kHz USVs was performed after each vehicle (vUSVs) or drug (dUSVs) administration in the test cage, re-exposure to the test cage (tcUSVs), or drug challenge administered immediately after test cage re-exposure (cUSVs), at the time points indicated. Rats' locomotor activity (loc) was also recorded at the time points indicated. Finally, rats' spontaneous alternation in a Y maze was evaluated after the completion of repeated drug treatment at the time points indicated. C, drug challenge in the test cage; R, re-exposure to the test cage; SA1, spontaneous alternation evaluated in drug-free conditions; SA2, spontaneous alternation evaluated after acute pharmacological treatment.



**Figure 2.** Example of sonograms of 50 kHz ultrasonic vocalizations (USVs). USVs reported are examples of independent vocalizations emitted by different rats.

as reported in [Simola and Morelli \(2015\)](#). Locomotor activity was recorded simultaneously with USVs by means of automated counters (Opto-Varimex; Columbus Instruments) equipped with a horizontal infrared beam emitter-detector system. The system was placed outside the cylinder, and oriented in order to project the infrared beams parallel to the floor of the cylinder.

### Spontaneous Alternation

Spontaneous alternation behavior in a Y maze is widely used as a tool to evaluate the sensory/attentional and cognitive functions (e.g. spatial working memory) of rodents and the amnesic effect of drugs (reviewed in [Hughes, 2004](#)). In the present study, spontaneous alternation was evaluated after completion of repeated drug treatment at the time points reported in [Figure 1](#). To minimize the influence of previous drug treatment, spontaneous alternation was firstly evaluated in drug-free conditions (day 23) to obtain a baseline value. Seven days later (day 30), spontaneous alternation was evaluated after acute pharmacological treatment which consisted of either: (i) MK-801 (0.1, 0.2, 0.5 mg/kg, i.p.); (ii) amphetamine (1 mg/kg, i.p.); or (iii) amphetamine (1 mg/kg, i.p.) in combination with MK-801 (0.1, 0.2 mg/kg, i.p.). On day 30, each rat received a pharmacological treatment different from the one it received in days 1–16.

The Y maze used was made of black PVC and had three equal-sized symmetrical arms (named A, B, and C; length 50 cm, width 20 cm, height 35 cm) converging onto a central triangular area. The maze was thoroughly cleaned with odorless soapy water and dried between each trial to remove olfactory cues. The test was performed by individually placing each rat in the central area and leaving it free to explore the maze for 8 min. Rats were

videotaped, and alternation was subsequently calculated based on the sequence of arm entries. An arm entry was scored when a rat had all its four paws inside a specific arm. Alternation is defined as successive entries to the three arms on overlapping triplet sets in which three different arms are entered. For example, a sequence of arm entries such as ABCBAB corresponds to two alternations: ABC and CBA. Spontaneous alternation was then expressed as a percentage obtained by dividing the number of alternations by the total number of triplets, which corresponds to the number of arm entries minus two (see [Hughes, 2004](#) for further details).

### Immunofluorescence

Immunofluorescence experiments were performed to investigate the modifications in the levels of the protein Zif-268 in brain areas related to the emission of 50 kHz USVs and the motivational properties of drugs of abuse. In the brain, the mRNA encoding for the early gene *zif-268* is constitutively expressed in areas such as the striatum and the cortex ([Schlingensiepen et al., 1991](#)). Moreover, *zif-268* can be rapidly and transiently induced by a variety of pharmacological and physiological stimuli ([Beckmann and Wilce, 1997](#)), making this gene and its protein useful and sensitive markers in the evaluation of neuronal activity and its modifications.

Seven days after repeated treatment discontinuation, a subset of rats were firstly re-exposed to the test cage in drug-free conditions for 10 min, and then challenged with either one of: (i) vehicle (i.p.); (ii) amphetamine (1 mg/kg, i.p.); or (iii) MK-801 (0.2 mg/kg, i.p.). Experimental groups were composed as: (1) rats pretreated with vehicle and challenged with vehicle (VEH/VEH

group); (2) rats pretreated with MK-801 (0.2 mg/kg, i.p.) and challenged with MK-801 (MK-801/MK-801 group); (3) rats pretreated with amphetamine (1 mg/kg, i.p.) and challenged with amphetamine (AMPH/AMPH group); and (4) rats pretreated with amphetamine (1 mg/kg, i.p.) in combination with MK-801 (0.2 mg/kg, i.p.) and challenged with amphetamine (AMPH + MK-801/AMPH group).

Each experimental group included 6 rats.

Ninety minutes after the challenge, rats were anesthetized with chloral hydrate and transcardially perfused with 0.9% NaCl followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH = 7.4). Brains were postfixed overnight in the same solution (4°C) and coronally cut on a vibratome (40 µm) 2 days later. For each rat, four coronal sections were collected from 2.70 mm to 2.20 mm (mPFC) and from 1.70 mm to 0.20 mm (DLS and NAC shell and core) relative to bregma, according to the rat brain atlas of Paxinos and Watson (1998).

Free-floating sections were rinsed in 0.1 M PB, blocked in a solution containing 3% normal goat serum and 0.3% Triton X-100 in 0.1 M PB at room temperature for 1 h, and incubated at 4°C in the same solution with the primary antibody for 2 nights. A purified rabbit polyclonal antibody against Zif-268 (1:1000, Santa Cruz Biotechnology) was used. AlexaFluor 594-labeled goat anti-rabbit immunoglobulin G (IgG) (1:400, Jackson ImmunoResearch) was used as secondary antibody. The Alexa Fluor 594 secondary antibody has a maximal light absorption around 591 nm, which allows its use for immunofluorescence detection in the deep-red region of the visible spectrum. After incubation, sections were rinsed and mounted immediately onto glass slides coated with gelatin in Mowiol mounting medium.

### Statistical Analysis

USV recordings were converted into spectrograms using the software SASLab Pro 4.52 (Avisoft) with the following settings: 512 FFT-length, Hamming window, and 75% overlap frame setup. After visual inspection and manual cleaning of all signals that could not be univocally classified as vocalizations (see Simola et al., 2012), the number of 50 kHz USVs was automatically calculated by SASLab Pro 4.52.

USV data collected in this study showed non-parametric distribution, and were square-root transformed before analyses. Figures report raw data for clarity. Means ± standard errors of the mean of the number of 50 kHz USVs and locomotor activity counts were calculated, and only data collected on days 0, 1, 5, 9, and 16 were analyzed by means of t-tests or analyses of variance (ANOVA). Baseline emissions of USVs and locomotor activity recorded after acute vehicle (day 0) revealed no significant group differences (data not shown). Three rats emitted 22 kHz USVs, which indicate negative affective states (Brudzynski, 2013), following pharmacological treatment, and were excluded from the analyses to avoid any possible influence of aversive effects of drugs on the results observed.

For repeated drug treatment, a two-factor ANOVA (treatment × administration day) was used. For test cage re-exposure in the drug-free conditions and drug challenge, a one-factor ANOVA (treatment) was used. A t-test was employed to verify the presence of significant differences between challenge with MK-801 and first administration of MK-801, as well as between challenge with amphetamine and first administration of amphetamine. Spontaneous alternation performed on days 23 and 30 was analyzed by means of a one-factor ANOVA (condition).

Stained brain sections were analyzed at ×20 magnification using an Axio Scope A1 light microscope (Zeiss) connected with a digital camera (1.4 MPixels, Infinity 3-1, Lumenera). The images obtained were processed using the Image J analysis software (US National Institutes of Health). Nuclei positive for Zif-268 were then identified for each slice and counted with the manual particle counting option of Image J. For each level of each brain area considered, the obtained value was first normalized with respect to the VEH/VEH group, then values from different levels were averaged. Data were analyzed by means of a one-factor ANOVA (treatment).

Significance was set at  $p < 0.05$  for each analysis, and ANOVA analyses were followed by Tukey's post-hoc test, when appropriate. Statistical analysis was performed with Statistica (StatSoft) and with Prism (GraphPad) for Windows.

## Results

### Emission of 50 kHz USVs and Locomotor Activity During Repeated Drug Treatment and Challenge

#### MK-801

Emission of 50 kHz USVs by rats repeatedly treated with MK-801 significantly differed from that of vehicle-treated rats. Two-way ANOVA revealed effects of treatment ( $F_{3,36} = 7.12, p = 0.0007$ ) and treatment × time interaction ( $F_{6,72} = 2.20, p = 0.0006$ ). Tukey's test showed that rats treated with each MK-801 dose vocalized less than vehicle-treated rats, although rats treated with MK-801 (0.1 mg/kg) developed tolerance to this effect (Figure 3A, Supplementary Table S1).

Locomotor activity of rats repeatedly treated with MK-801 significantly differed from that of vehicle-treated rats. Two-way ANOVA revealed effects of treatment ( $F_{3,36} = 80.33, p = 0.0001$ ), time ( $F_{2,72} = 5.76, p = 0.005$ ), and treatment × time interaction ( $F_{6,72} = 5.96, p = 0.0004$ ). Tukey's test showed that rats treated with MK-801 (0.5 mg/kg) exhibited higher locomotor activity than vehicle-treated rats at every time point considered (Figure 3B, Supplementary Table S1).

The MK-801 challenge influenced vocalization in a fashion significantly different from the vehicle challenge. One-way ANOVA revealed effect of treatment ( $F_{3,36} = 14.27, p = 0.0001$ ), and Tukey's test showed that rats challenged with MK-801 (0.1–0.5 mg/kg) vocalized less than vehicle-challenged rats. Moreover, t-test comparisons revealed that vocalization stimulated by MK-801 challenge was lower than that elicited by the first MK-801 administration for every dose tested (0.1 mg/kg:  $t = 4.510, df = 16, p = 0.0004$ ; 0.2 mg/kg:  $t = 3.42, df = 20, p = 0.003$ ; 0.5 mg/kg:  $t = 2.94, df = 14, p = 0.03$ ; Figure 3A, Supplementary Table S1).

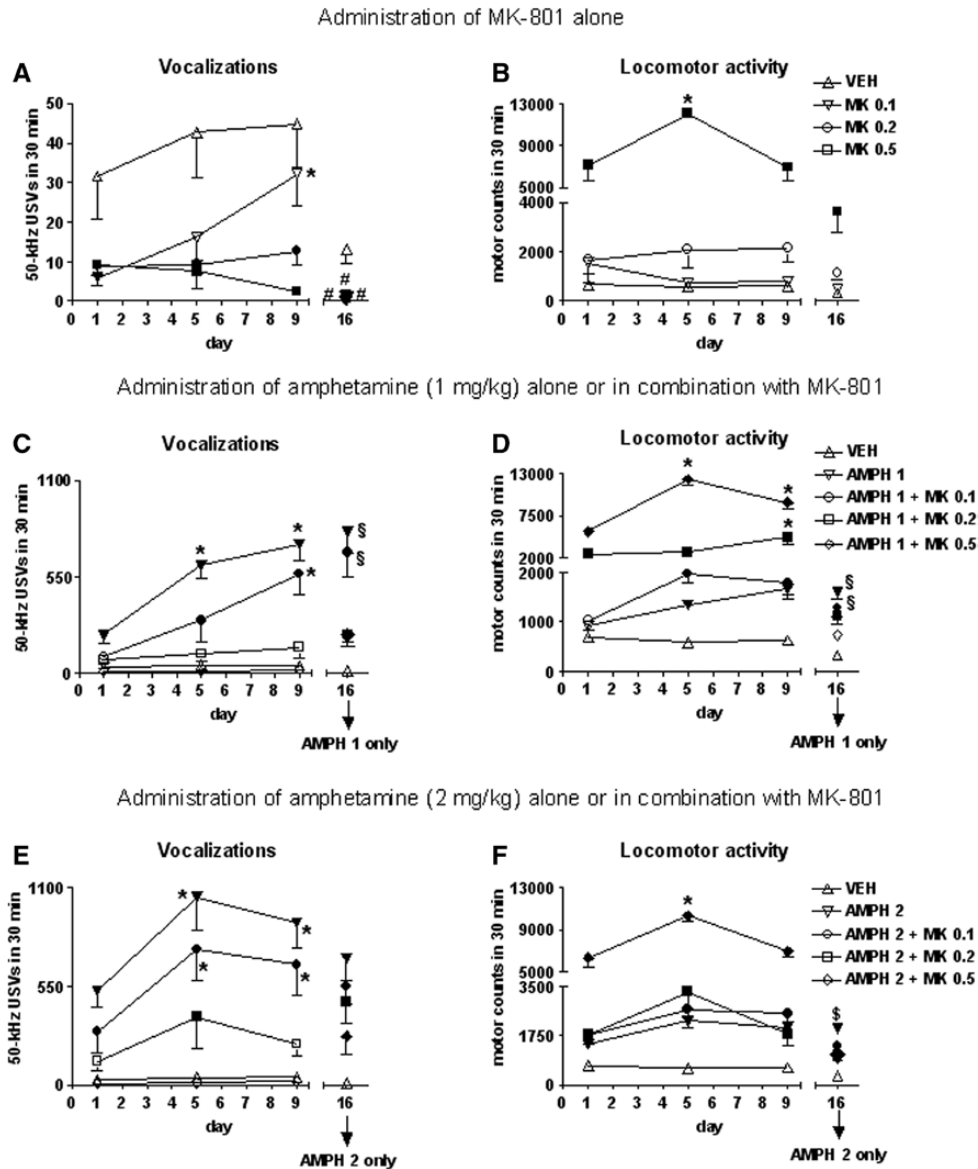
The MK-801 challenge stimulated locomotor activity in a fashion significantly different from the vehicle challenge, as shown by one-way ANOVA ( $F_{3,36} = 14.78, p = 0.0001$ ). Tukey's test showed higher locomotor activity in rats challenged with MK-801 (0.5 mg/kg) than in vehicle-challenged rats (Figure 3B, Table 1).

### Amphetamine and Amphetamine + MK-801

#### Amphetamine (1 mg/kg)

Emission of 50 kHz USVs by rats repeatedly treated with amphetamine (1 mg/kg) significantly differed from that of vehicle-treated rats, and MK-801 coadministration influenced amphetamine effects. Two-way ANOVA revealed effects





**Figure 3.** Changes in the emission of 50 kHz ultrasonic vocalizations (USVs) and in locomotor activity induced by repeated treatment with MK-801 (MK, 0.1–0.5 mg/kg, i.p.), or amphetamine (AMPH, 1 or 2 mg/kg, i.p.), administered alone or in combination with MK-801, and by subsequent challenge with either MK-801 or amphetamine. Upper panels: (A) emission of 50 kHz USVs and (B) locomotor activity in rats repeatedly treated and challenged with MK-801 (0.1–0.5 mg/kg). Middle panels: (C) emission of 50 kHz USVs and (D) locomotor activity in rats repeatedly treated with amphetamine (1 mg/kg, i.p.), alone or in combination with MK-801 (0.1–0.5 mg/kg), and challenged with amphetamine (1 mg/kg, i.p.). Lower panels: (E) emission of 50 kHz USVs and (F) locomotor activity in rats repeatedly treated with amphetamine (2 mg/kg, i.p.), alone or in combination with MK-801 (0.1–0.5 mg/kg), and challenged with amphetamine (2 mg/kg, i.p.). Recording of USVs and locomotor activity lasted 30 min. Filled black symbols indicate  $p < 0.05$  compared with vehicle (VEH)-treated rats. \* $p < 0.05$  compared with the first drug administration within each experimental group. # $p < 0.05$  compared with the first administration of the corresponding dose of MK-801. \$ $p < 0.05$  compared with the first administration of amphetamine (1 mg/kg, i.p.). \$ $p < 0.05$  compared with the first administration of amphetamine (2 mg/kg, i.p.). VEH,  $n = 12$ ; MK 0.1,  $n = 9$ ; MK 0.2,  $n = 11$ ; MK 0.5,  $n = 8$ ; AMPH 1,  $n = 22$ ; AMPH 1 + MK 0.1,  $n = 7$ ; AMPH 1 + MK 0.2,  $n = 12$ ; AMPH 1 + MK 0.5,  $n = 8$ ; AMPH 2,  $n = 15$ ; AMPH 1 + MK (0.1–0.5),  $n = 8$ .

of treatment ( $F_{4,56} = 22.09$ ,  $p = 0.0001$ ), time ( $F_{2,112} = 14.43$ ,  $p = 0.0001$ ), and treatment  $\times$  time interaction ( $F_{8,112} = 5.25$ ,  $p = 0.0001$ ). Tukey's test showed that amphetamine-stimulated vocalizations developed sensitization with repeated treatment. Coadministration of MK-801 (0.1 mg/kg) suppressed amphetamine-stimulated vocalizations only on the first administration day, while coadministration of MK-801 (0.2 or 0.5 mg/kg) elicited this effect at every time point considered (Figure 3C, Supplementary Table S2).

The locomotor activity of rats repeatedly treated with amphetamine (1 mg/kg) significantly differed from that of

vehicle-treated rats, and MK-801 coadministration influenced amphetamine effects. Two-way ANOVA revealed effects of treatment ( $F_{4,56} = 140.16$ ,  $p = 0.0001$ ), time ( $F_{2,112} = 33.78$ ,  $p = 0.0001$ ), and treatment  $\times$  time interaction ( $F_{8,112} = 16.08$ ,  $p = 0.0001$ ). Tukey's test showed a further increase in locomotor activity in rats coadministered MK-801 (0.2 or 0.5 mg/kg). This effect was present at every time point considered, and developed sensitization (Figure 3D, Supplementary Table S2).

The challenge with amphetamine (1 mg/kg) stimulated 50 kHz USV emissions significantly different from the vehicle challenge, and previous MK-801 coadministration influenced

this effect (one-way ANOVA,  $F_{4,56} = 21.39$ ,  $p = 0.0001$ ). Tukey's test revealed that the amphetamine challenge stimulated higher vocalization than the vehicle challenge in rats pretreated with either amphetamine or amphetamine + MK-801 (0.1–0.5 mg/kg). Moreover, t-test comparisons revealed that the amphetamine challenge elicited higher vocalization compared with the first amphetamine administration in rats pretreated with either amphetamine ( $t = 5.27$ ,  $df = 42$ ,  $p = 0.0001$ ) or amphetamine + MK-801 (0.1 mg/kg;  $t = 3.59$ ,  $df = 27$ ,  $p = 0.001$ ; [Figure 3C](#), [Supplementary Table S2](#)).

The challenge with amphetamine (1 mg/kg) stimulated locomotor activity in a fashion significantly different from the vehicle challenge, and previous MK-801 coadministration influenced this effect (one-way ANOVA,  $F_{4,56} = 14.02$ ,  $p = 0.0001$ ). Tukey's test showed that the amphetamine challenge stimulated higher locomotor activity than the vehicle challenge in rats pretreated with either amphetamine or amphetamine + MK-801 (0.1 or 0.2 mg/kg). Moreover, t-test comparisons revealed that the amphetamine challenge elicited higher locomotor activity compared with the first amphetamine administration in rats pretreated with either amphetamine ( $t = 4.57$ ,  $df = 42$ ,  $p = 0.0001$ ) or amphetamine + MK-801 (0.1 mg/kg) ( $t = 2.19$ ,  $df = 27$ ,  $p = 0.04$ ; [Figure 3D](#), [Supplementary Table S2](#)).

#### Amphetamine (2 mg/kg)

Emissions of 50 kHz USVs by rats repeatedly treated with amphetamine (2 mg/kg) significantly differed from those displayed by vehicle-treated rats, and MK-801 coadministration influenced amphetamine effects. Two-way ANOVA revealed effects of treatment ( $F_{4,46} = 28.49$ ,  $p = 0.0001$ ), time ( $F_{2,92} = 21.05$ ,  $p = 0.0001$ ), and treatment  $\times$  time interaction ( $F_{8,92} = 3.34$ ,  $p = 0.002$ ). Tukey's test showed that amphetamine-stimulated vocalizations developed sensitization with repeated treatment. Coadministration of MK-801 (0.2 mg/kg) suppressed amphetamine-stimulated vocalization on the first and fifth administration days, while coadministration of MK-801 (0.5 mg/kg) elicited this effect at every time point considered ([Figure 3E](#), [Supplementary Table S3](#)).

The locomotor activity of rats repeatedly treated with amphetamine (2 mg/kg) significantly differed from that of vehicle-treated rats, and MK-801 coadministration influenced amphetamine effects. Two-way ANOVA revealed effects of treatment ( $F_{4,46} = 143.95$ ,  $p = 0.0001$ ), time ( $F_{2,92} = 32.76$ ,  $p = 0.0001$ ), and treatment  $\times$  time interaction ( $F_{8,92} = 8.76$ ,  $p = 0.0001$ ). Tukey's test showed that coadministration of MK-801 (0.5 mg/kg) potentiated amphetamine-stimulated locomotion at every time point considered ([Figure 3F](#), [Supplementary Table S3](#)).

The challenge with amphetamine (2 mg/kg) stimulated 50 kHz USV emissions significantly different from the vehicle challenge, and previous MK-801 coadministration influenced this effect (one-way ANOVA,  $F_{4,46} = 18.80$ ,  $p = 0.0001$ ). Tukey's test showed that the amphetamine challenge stimulated higher vocalization than the vehicle challenge in rats pretreated with either amphetamine or amphetamine + MK-801 (0.1–0.5 mg/kg; [Figure 3E](#), [Supplementary Table S3](#)).

The challenge with amphetamine (2 mg/kg) stimulated locomotor activity in a fashion significantly different from the vehicle challenge, and previous MK-801 coadministration influenced this effect (one-way ANOVA,  $F_{4,46} = 31.75$ ,  $p = 0.0001$ ). Tukey's test revealed that the amphetamine challenge induced higher locomotor activity than the vehicle challenge in rats pretreated with either amphetamine or amphetamine + MK-801 (0.1–0.5 mg/kg). Moreover, t-test comparisons revealed that the amphetamine

challenge stimulated higher locomotor activity compared with the first amphetamine administration in amphetamine-pretreated rats ( $t = 3.23$ ,  $df = 28$ ,  $p = 0.004$ ; [Figure 3F](#), [Supplementary Table S3](#)).

### Emission of Conditioned 50 kHz USVs During Test-Cage Re-Exposure Under Drug-Free Conditions

#### MK-801

When re-exposed to the test cage, rats previously treated with MK-801 (0.1–0.5 mg/kg) displayed similar 50 kHz USV emissions compared with vehicle-pretreated rats ([Figure 4A](#), [Supplementary Table S1](#)).

#### Amphetamine and Amphetamine + MK-801

##### Amphetamine (1 mg/kg)

When re-exposed to the test cage, rats previously treated with amphetamine (1 mg/kg) displayed 50 kHz USV emissions significantly different from those in vehicle-pretreated rats, and previous MK-801 coadministration influenced this effect (one-way ANOVA,  $F_{4,56} = 14.27$ ,  $p = 0.0002$ ). Tukey's test showed that amphetamine-pretreated rats vocalized more than vehicle-pretreated rats, while rats pretreated with amphetamine + MK-801 (0.1–0.5 mg/kg) did not ([Figure 4B](#), [Supplementary Table S2](#)).

##### Amphetamine (2 mg/kg)

When re-exposed to the test cage, rats previously treated with amphetamine (2 mg/kg) exhibited 50 kHz USV emissions significantly different from those in vehicle-pretreated rats, and previous MK-801 coadministration influenced this effect (one-way ANOVA,  $F_{4,46} = 6.78$ ,  $p = 0.0002$ ). Tukey's test showed that amphetamine-pretreated rats vocalized more than vehicle-pretreated rats, while rats pretreated with amphetamine + MK-801 (0.1–0.5 mg/kg) did not ([Figure 4C](#), [Supplementary Table S3](#)).

### Spontaneous Alternation

Acute pharmacological treatment significantly modified spontaneous alternation compared with drug-free conditions (one-way ANOVA,  $F_{10,66} = 3.92$ ,  $p = 0.0001$ ). Tukey's test showed reduced spontaneous alternation in rats treated with MK-801 (0.5 mg/kg) and amphetamine (1 mg/kg) + MK-801 (0.2 mg/kg; [Figure 5](#)).

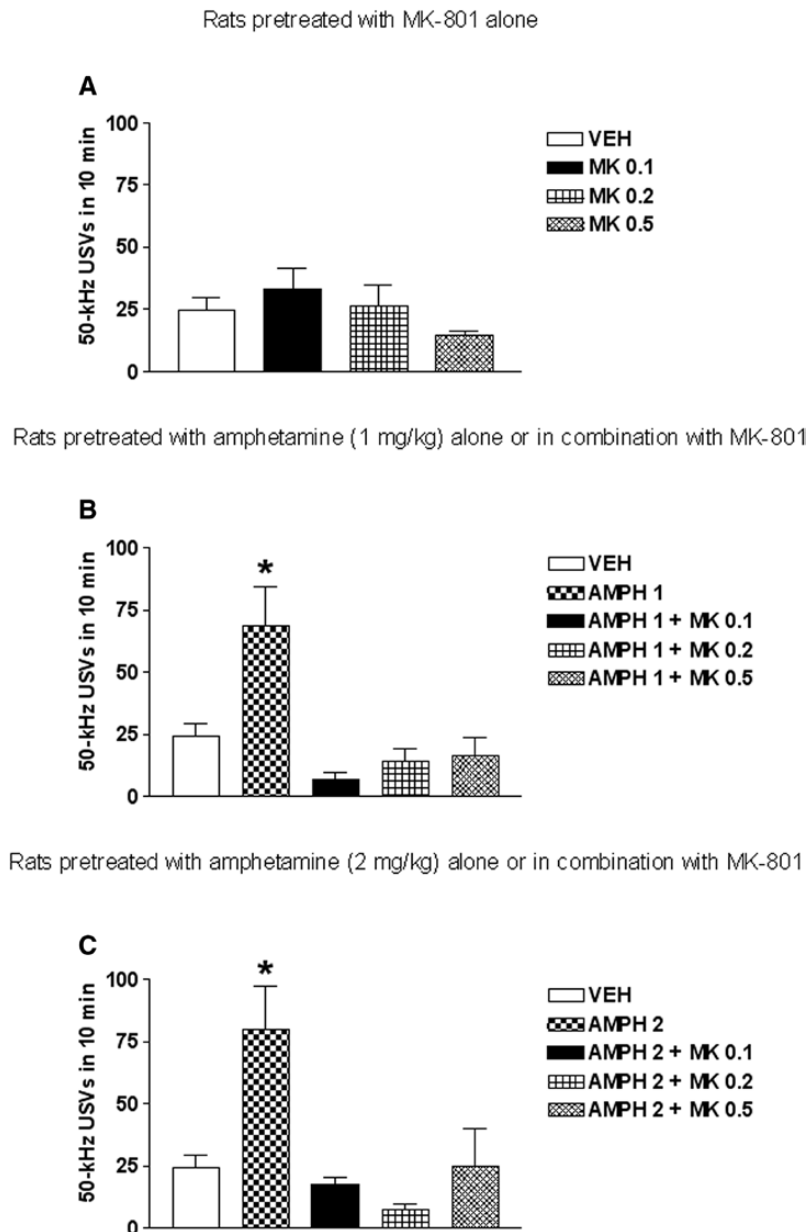
### Immunofluorescence

The drug challenge modified the levels of Zif-268 in a fashion significantly different from the vehicle challenge. One-way ANOVA revealed effect of treatment in the NAc shell ( $F_{3,19} = 19.26$ ,  $p = 0.0001$ ), NAc core ( $F_{3,19} = 5.74$ ,  $p = 0.006$ ), mPFC ( $F_{3,19} = 8.17$ ,  $p = 0.001$ ), and DLS ( $F_{3,19} = 9.64$ ,  $p = 0.0004$ ). Tukey's test showed higher levels of Zif-268 in the NAc shell and core, mPFC, and DLS of the AMPH/AMPH group, compared with the VEH/VEH and the MK-801/MK-801 groups. Higher levels of Zif-268 were also found in the NAc shell, mPFC, and DLS of the AMPH/AMPH group compared with the AMPH + MK-801/AMPH group ([Figure 6A–D](#)).

### Summary of Major Findings

The major findings of this study can be summarized as follows.

Coadministration of MK-801 suppressed the induction and expression of 50 kHz USV sensitization in rats repeatedly



**Figure 4.** Emission of conditioned 50kHz ultrasonic vocalizations (USVs) by rats previously treated in the test cage with MK-801 (MK, 0.1–0.5 mg/kg, i.p.), amphetamine (AMPH, 1 or 2 mg/kg, i.p.), or their combination. (A) Emission of conditioned 50kHz USVs by rats pretreated with MK-801 (0.1–0.5 mg/kg, i.p.) alone. (B) Emission of conditioned 50kHz USVs by rats pretreated with amphetamine (1 mg/kg, i.p.), alone or in combination with MK-801 (0.1–0.5 mg/kg). (C) Emission of conditioned 50kHz USVs by rats pretreated with amphetamine (2 mg/kg, i.p.), alone or in combination with MK-801 (0.1–0.5 mg/kg, i.p.). USV recordings were performed in drug-free conditions immediately after the re-exposure of rats to the test cage and before the drug challenge, and lasted 10 min. \* $p < 0.05$  compared with vehicle (VEH)-pretreated rats and rats pretreated with amphetamine in combination with MK-801 (AMPH 1 or 2 mg/kg, i.p. + MK, 0.1–0.5 mg/kg, i.p.). VEH,  $n = 12$ ; MK 0.1,  $n = 9$ ; MK 0.2,  $n = 11$ ; MK 0.5  $n = 8$ ; AMPH 1,  $n = 22$ ; AMPH 1 + MK 0.1,  $n = 7$ ; AMPH 1 + MK 0.2,  $n = 12$ ; AMPH 1 + MK 0.5,  $n = 8$ ; AMPH 2,  $n = 15$ ; AMPH 1 + MK (0.1–0.5),  $n = 8$ .

treated with amphetamine. However, this effect could be observed only at doses of MK-801 that themselves suppressed vocalization.

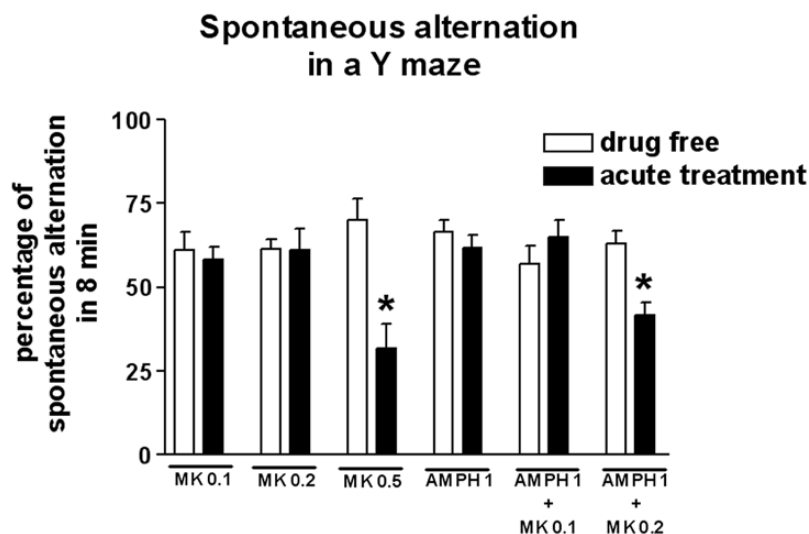
Previous coadministration of MK-801 suppressed the emission of conditioned 50kHz USVs by amphetamine-treated rats re-exposed to the previously drug-paired environment. This effect occurred even at a low dose of MK-801 that did not alter the vocalization recorded immediately after amphetamine administration.

MK-801 elicited divergent effects on USVs and locomotor activity stimulated by amphetamine.

The effects of MK-801 on amphetamine-stimulated 50kHz USVs were paired with modifications in spontaneous alternation and Zif-268 expression in different brain regions.

## Discussion

Previous studies have demonstrated that the activation of glutamate transmission has a permissive influence on rat 50kHz USVs. Intracerebral administration of glutamate stimulates 50kHz USVs (Fu and Brudzynski, 1994), and the NMDA receptor partial agonist D-cycloserine promotes vocalization in



**Figure 5.** Effects of acute treatment with MK-801 (MK, 0.1–0.5 mg/kg, i.p.), amphetamine (AMPH, 1 mg/kg, i.p.), and amphetamine (1 mg/kg, i.p.) in combination with MK-801 (0.1 or 0.2 mg/kg, i.p.) on rats' spontaneous alternation in a Y maze. Spontaneous alternation is reported as a percentage of alternation triplets calculated over the total number of triplets, the latter corresponding to the number of arm entries minus two. \* $p < 0.05$  compared with spontaneous alternation evaluated in drug-free conditions. Drug free,  $n = 7$ ; acute treatment,  $n = 7$ .

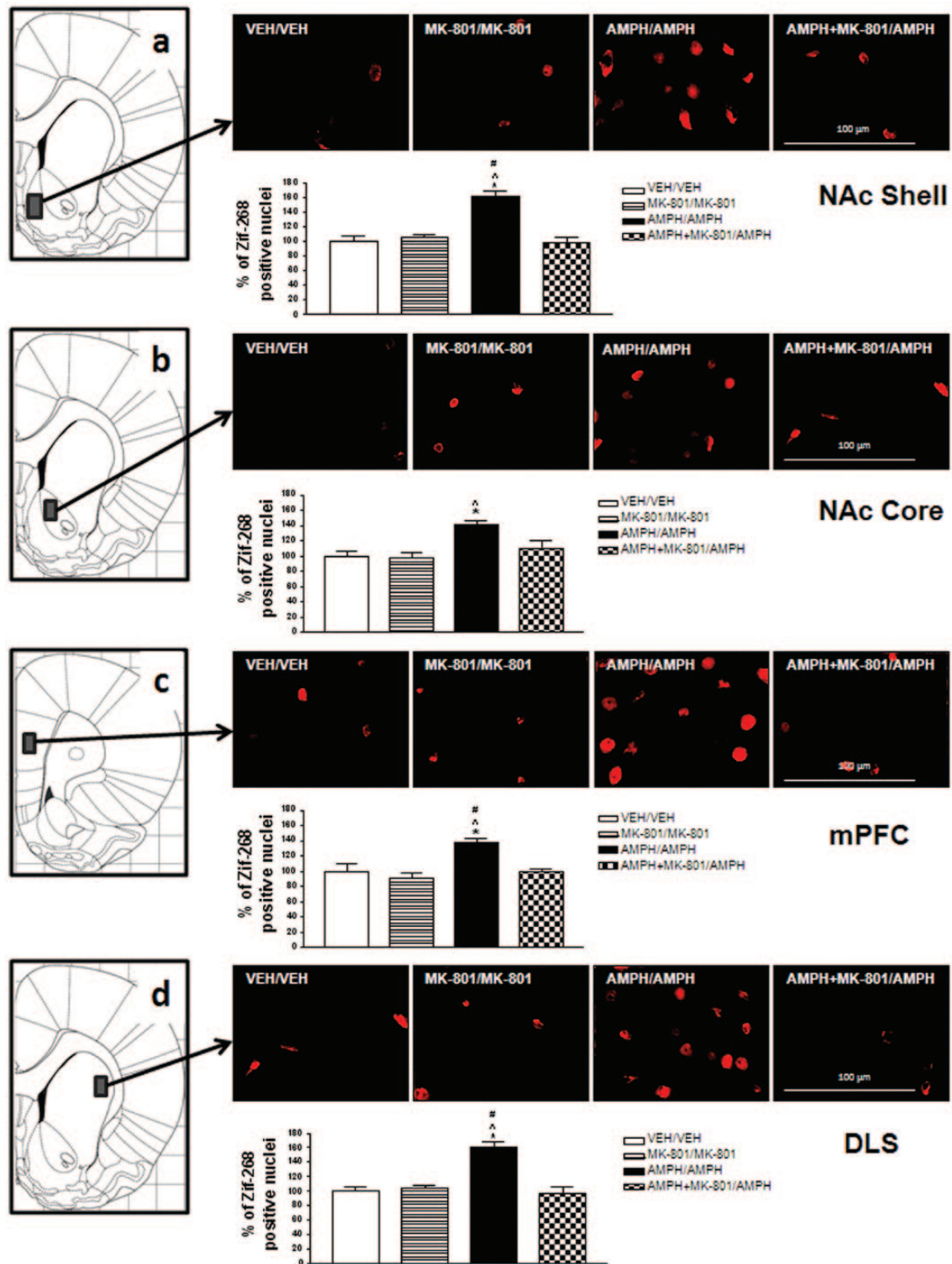
rats selected for low levels of tickling-stimulated 50 kHz USVs (Moskal et al., 2011). Conversely, the NMDA receptor antagonists MK-801 and phencyclidine suppress tickling-stimulated 50 kHz USVs (Panksepp and Burgdorf, 2000; Boulay et al., 2013). The present study demonstrates, for the first time, that the permissive influence of glutamate transmission extends to the vocalizations stimulated by a drug with abuse properties such as amphetamine.

Besides the findings on 50 kHz USVs, this study confirmed that MK-801 coadministration suppresses locomotor sensitization in rodents repeatedly treated with amphetamine (reviewed in Wolf, 1998). Locomotor sensitization is considered to be a correlate of the changes in the motivational properties of addictive drugs with repeated administration (Robinson and Berridge, 2001), and recent studies have suggested that sensitization in drug-stimulated 50 kHz USVs could have similar significance (Ahrens et al., 2009; Mu et al., 2009; Simola and Morelli, 2015). In this regard, it is noteworthy that neuroplastic changes in brain regions involved in motivational processes (e.g. NAc, mPFC) are thought to underlie drug-induced sensitization (Vanderschuren and Kalivas, 2000), and that NMDA receptors are critically involved in neuroplasticity (Lau and Zukin, 2007). However, this study found that NMDA receptor antagonism elicited opposite effects on amphetamine-stimulated locomotor activity and 50 kHz USV emissions. Moreover, this study found that previous MK-801 coadministration prevented the emission of conditioned 50 kHz USVs by amphetamine-treated rats upon re-exposure to the drug-paired environment. Interestingly, this effect could be observed even in rats administered MK-801 at a dose that was ineffective in sensitization of amphetamine-stimulated 50 kHz USVs, suggesting that the NMDA receptor antagonism is more effective on conditioned vocalizations.

Previous investigations have demonstrated that concomitant NMDA receptor antagonism prevents the molecular changes that accompany the behavioral sensitization produced by amphetamine (Wolf et al., 1994; Wolf, 1998). These findings could explain why MK-801 suppressed both the induction and expression of 50 kHz USV sensitization in amphetamine-treated rats. Moreover, these earlier findings

would agree with the immunofluorescence results of this study, showing that rats previously coadministered MK-801 at a dose (0.2 mg/kg) that prevented 50 kHz USV sensitization displayed reduced levels of Zif-268 following amphetamine challenge. Notably, this effect was observed in the NAc shell, which plays a crucial role in 50 kHz USV emissions. This would suggest that previous NMDA receptor antagonism may cause a persistent reduced sensitivity of NAc shell neurons to the effects of amphetamine, which could explain the lower effects of the amphetamine challenge on 50 kHz USV emissions in rats previously administered MK-801. However, lower levels of Zif-268 were also observed in the DLS and mPFC, which are major targets of the neuroplastic changes induced by repeated treatment with addictive substances (Vanderschuren and Kalivas, 2000; Robbins et al., 2008). This, in turn, would suggest that regions other than the NAc shell might participate in the effects of NMDA receptor antagonism on amphetamine-stimulated 50 kHz USVs. Alternatively, it is possible that changes in neuronal activity in the DLS and mPFC may influence the long-term changes in the responsiveness of NAc shell neurons to amphetamine (see King et al., 1997). However, the proposed influence of NMDA receptor antagonism on amphetamine-induced neuroplasticity seems in apparent contrast with the scarce emission of conditioned 50 kHz USVs by the rats pretreated with amphetamine and MK-801 (0.1 mg/kg), a dose that did not attenuate sensitization in amphetamine-stimulated 50 kHz USVs. One hypothesis that could explain these discrepant results is that NMDA receptor antagonism might modulate amphetamine-induced neuroplasticity in a way that selectively affects conditioned vocalizations. It is noteworthy that the 0.1 mg/kg dose of MK-801 has been reported to suppress the behavioral responses mediated by the activation of  $D_1$  receptors (Morelli and Di Chiara, 1990; Kreipke and Walker, 2004), and that these receptors are critically involved in drug conditioning (Di Chiara et al., 2004). However, other mechanisms besides the opposite NMDA- $D_1$  receptor interactions could underlie the effects of MK-801 on conditioned 50 kHz USV emissions by amphetamine-treated rats.





**Figure 6.** Effects of challenge with MK-801 (0.2 mg/kg, i.p.) or amphetamine (1 mg/kg, i.p.) administered 7 days after repeated treatment discontinuation on the levels of Zif-268 in the nucleus accumbens (NAc) shell and core, medial prefrontal cortex (mPFC), and dorsolateral striatum (DLS). The figure reports representative high-resolution images ( $\times 100$ ) immunostained for Zif-268 and histograms for NAc (A) shell and (B) core, (C) mPFC, and (D) DLS. Rats challenged with MK-801 were pretreated with MK-801 (0.2 mg/kg, i.p.). Rats challenged with amphetamine (AMPH) were pretreated with either amphetamine (1 mg/kg, i.p.) alone (AMPH/AMPH) or amphetamine (1 mg/kg, i.p.) in combination with MK-801 (0.2 mg/kg, i.p.; AMPH+MK-801/AMPH). The graphs show the percentage of Zif-268-positive nuclei compared with rats pretreated with vehicle (VEH) and challenged with vehicle (VEH/VEH). <sup>^</sup> $p < 0.05$  compared with VEH/VEH rats. <sup>#</sup> $p < 0.05$  compared with AMPH+MK-801/AMPH rats. Scale bar, 100  $\mu$ m. VEH/VEH, MK-801/MK-801, AMPH/AMPH, AMPH+MK-801/AMPH,  $n = 6$ .

Antagonism of NMDA receptors induces amnesia in rats (Zhang et al., 2001; Tronel and Sara, 2003) and, in line with this, MK-801 was here found to impair rats' spontaneous alternation. This finding appears relevant to amphetamine-stimulated 50 kHz USVs, since NMDA receptor antagonism can interfere with Pavlovian conditioning and long-lasting memories associated with the administration of addictive drugs (Sadler et al., 2007; Milton et al., 2012). However, rats coadministered amphetamine with the lower dose of MK-801 (0.1 mg/kg) in this study, while displaying negligible conditioned vocalization, exhibited no significant changes in spontaneous alternation. In this regard, it could be hypothesized that the emission of conditioned vocalizations is sensitive to submaximal amnesic deficits induced by NMDA receptor antagonism. Interestingly, this view would agree with earlier evidence showing that spontaneous alternation in a Y maze is relatively unaffected by subtle amnesic deficits (Simola et al., 2008). Alternatively, it is possible that NMDA receptor antagonism might have impaired reconsolidation of memory for environmental cues previously associated with amphetamine, an effect that has been reported after the administration of 0.1 mg/kg MK-801 (Sadler et al., 2007). Therefore, the modifications involving 50 kHz USVs in amphetamine-treated rats observed here could depend on a dual modulatory effect of NMDA receptor blockade on neuroplastic changes and long-term memories associated with amphetamine administration.

An additional issue to consider regarding the involvement of learning in the present results is that MK-801 has been suggested to promote state-dependent learning (defined in Sripada et al., 2001) when coadministered with psychostimulants (Carlezon et al., 1995; Ranaldi et al., 2000). Accordingly, state-dependent learning has been proposed to underlie the lack of behavioral sensitization in rats coadministered amphetamine and MK-801. However, others have challenged this hypothesis (Li and Wolf, 1999; Sripada et al., 2001), and similar considerations seem to apply to the present results. Regarding the vocalizations emitted immediately after amphetamine administration, it is noteworthy that while rats coadministered amphetamine (1 mg/kg) and MK-801 (0.1 mg/kg) developed 50 kHz USV sensitization during repeated treatment, they also displayed sensitized vocalization following the amphetamine challenge in the absence of MK-801. Nevertheless, it could be hypothesized that state-dependent learning may underlie the scarce conditioned vocalization by rats pretreated with amphetamine and MK-801. However, the present study always evaluated conditioned 50 kHz USVs in drug-free conditions, and rats treated with amphetamine alone exhibited a strong conditioned vocalization. Together, these considerations suggest that state-dependent learning is not involved in the results of this study.

In addition to the mechanisms discussed above, it is possible that other effects of MK-801 on rats' behavior could have contributed to the present results. This study observed a significant attenuation of amphetamine-stimulated 50 kHz USVs during repeated treatment only in rats coadministered doses of MK-801 that suppressed vocalization. Therefore, the 50 kHz USV emissions during repeated treatment by rats coadministered amphetamine and MK-801 could simply represent the net effect of these drugs on vocalization. Moreover, the present study has found that MK-801 elicits dose-dependent opposite effects on locomotor activity and 50 kHz USV sensitization induced by amphetamine. Interestingly, these effects were not observed following the amphetamine challenge (in the absence of MK-801), which stimulated both locomotor activity and 50 kHz USV emissions, though neither one sensitized in the same rats. These findings would suggest that other effects of MK-801 on

rats' behavior (e.g. suppression of vocalization, excessive motor stimulation) could interfere with the vocalization evaluated immediately after amphetamine and MK-801 coadministration. Therefore, this study suggests that pharmacological investigations of the role of non-dopaminergic transmitters in 50 kHz USV emissions should cautiously consider the behavioral effects of drugs targeting these transmitters that could interfere with the results observed.

In conclusion, this study provides the first demonstration that glutamate transmission mediated by NMDA receptors participates in the effects of amphetamine on 50 kHz USVs. In light of the crucial role of NMDA receptors in drug-induced behavioral sensitization, these findings further validate the use of 50 kHz USVs in the study of the motivational properties of amphetamine. Moreover, the present results agree with earlier data on the significance of 50 kHz USVs in the study of addictive drugs. First, this study provides further evidence that the acute and long-term effects of drugs on 50 kHz USVs may differ (Simola et al., 2014), and that vocalizations emitted immediately after drug administration and conditioned vocalizations may have a different significance with regard to the motivational properties of drugs (Simola and Morelli, 2015). Second, this study strengthens the idea that the significance of locomotor activity may not completely overlap that of 50 kHz USV emissions, with regard to the changes in the motivational properties of amphetamine after repeated experience (Taracha et al., 2014; Simola and Morelli, 2015).

## Supplementary Material

For supplementary material accompanying this paper, visit <http://www.ijnp.oxfordjournals.org>

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## Statement of Interest

None.

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