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Dopamine-sensitive signaling mediators modulate psychostimulant-induced ultrasonic vocalization behavior in rats

Stacey N. Williams and

Department of Pharmaceutical Sciences, Notre Dame of Maryland University, School of Pharmacy, Baltimore, MD 21210

Ashiwel S. Undieh

School of Medicine, City University of New York, City College, 160 Convent Avenue, New York, NY 10031

Stacey N. Williams: swilliams@ndm.edu; Ashiwel S. Undieh: aundieh@ccny.cuny.edu

Abstract

The mesolimbic dopamine system plays a major role in psychostimulant-induced ultrasonic vocalization (USV) behavior in rodents. Within this system, psychostimulants elevate synaptic concentrations of dopamine thereby leading to exaggerated activation of postsynaptic dopamine receptors within the D1-like and D2-like subfamilies. Dopamine receptor stimulation activate several transmembrane signaling systems and cognate intracellular mediators; downstream activation of transcription factors then conveys the information from receptor activation to appropriate modulation of cellular and physiologic functions. We previously showed that cocaine-induced USV behavior was associated with enhanced expression of the neurotrophin BDNF. Like cocaine, amphetamine also increases synaptic dopamine levels, albeit primarily through facilitating dopamine release. Therefore, in the present study we investigated whether amphetamine and cocaine similarly activate dopamine-linked signaling cascades to regulate intracellular mediators leading to induction of USV behavior. The results show that amphetamine increased the emission of 50 kHz USVs and this effect was blocked by SCH23390, a D1 receptor antagonist. Similar to cocaine, amphetamine increased BDNF protein expression in discrete brain regions, while pretreatment with K252a, a trkB neurotrophin receptor inhibitor, significantly reduced amphetamine-induced USV behavior. Inhibition of cyclic-AMP/PKA signaling with H89 or inhibition of PLC signaling with U73122 significantly blocked both the acute and subchronic amphetamine-induced USV behavior. In contrast, pharmacologic inhibition of either pathway enhanced cocaine-induced USV behavior. Although cocaine and amphetamine similarly modulate neurotrophin expression and USV, the molecular mechanisms by which these psychostimulants differentially activate dopamine receptor subtypes or other monoaminergic systems may be responsible for the distinct aspects of behavioral responses.

Correspondence to: Stacey N. Williams, swilliams@ndm.edu.

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Keywords

psychostimulant; ultrasonic vocalization; brain-derived neurotrophic factor; D1-like receptor; dopamine; trkB receptor

1. INTRODUCTION

Spontaneous emission of ultrasonic vocalizations (USVs) at 50 kHz frequencies is thought to reflect a positive affective state in rats based on studies showing the emission of these calls during appetitive behaviors such as anticipation of copulation [1] and rough and tumble play [2]. Furthermore, the mesolimbic dopamine system, which is the brain circuitry directly implicated in reward behaviors [3, 4], is the major system responsible for the production of these calls [5, 6]. Specifically, rats will emit increased rates of 50 kHz USVs upon delivery of electrical brain stimulation to several reward-associated brain regions [7, 8]. Additionally, psychostimulant agents known to stimulate the mesolimbic system also elicit spontaneous emission of 50 kHz USVs in rats [5, 9–14]. These findings support the notion that elevated dopamine levels within the mesolimbic region may unconditionally elicit a state of reward anticipation which, in rats, may be expressed by the emission of 50 kHz USV calls. Psychostimulant agents elevate synaptic concentrations of dopamine thereby leading to enhanced activation of postsynaptic G protein-coupled dopamine receptors. There are currently five well-characterized dopamine receptor subtypes that are categorized into D1-like (D1 and D5) and D2-like (D2, D3 and D4) subfamilies. Stimulation of both D1-like and D2-like receptors is necessary to produce full psychostimulant-induced increases in 50 kHz USV although blockage of either receptor is sufficient to inhibit USV production [5, 13]. Biochemically, the two receptor classes exert opposing effects on intracellular effector signaling whereby D1-like receptors couple positively to adenylate cyclase (AC) via Gs activation and D2-like receptors couple negatively to adenylate cyclase via Gi activation [15]. Adenylate cyclase activity ultimately increases cyclic AMP formation which then activates protein kinase A (PKA). Activation of dopamine D1-like receptors also enhances phosphatidylinositol synthesis and phospholipase C activity, thus stimulating phosphoinositol hydrolysis and mobilization of intracellular calcium stores [16]. Specifically, PLC cleaves phosphatidylinositol 4,5-bisphosphate (PIP₂) to form diacylglycerol which activates protein kinase C (PKC) [17] and D-myo-inositol-1,4,5-trisphosphate (IP₃) [16]. IP₃ regulates intracellular Ca²⁺ by binding to the IP₃ receptor on the endoplasmic reticulum (ER) and stimulating Ca²⁺ release from the ER. D1-like receptor-associated increase in PI synthesis also implies potential enhancement of the PI-3-kinase signaling and modulation of protein kinase B (Akt) and its downstream systems [18]. Activation of these signaling pathways plays a critical role in mediating the behavioral responses of the drugs [19]. Following transmembrane G-protein stimulation and release of cognate second messengers, intracellular activation of the PKA or PKC signaling pathways leads to an increase in the activity of the transcription factor CREB, which serves as a possible downstream mediator of responses or adaptations to psychostimulant exposure [19]. BDNF is a CREB-regulated gene that is implicated in the actions of psychostimulants [13, 20, 21]. Psychostimulant agents also induce increased expression of BDNF in various brain

regions [13, 22–25], while heterozygous BDNF knockout mice are significantly less responsive to cocaine-induced sensitization and motor activation [26].

Although there are various chemical entities that are highly selective for dopamine D1-like receptors, there remains a lack of agonists or antagonists that sufficiently discriminate between the D1 and D5 subtypes since typical agonists of D1-like receptors generally activate both the D1 and D5 subtypes and elicit multiple signaling responses in mammalian tissues [27]. Interestingly, however, some studies have excluded a role for the cloned D1 receptor in mediating phospholipase C (PLC) signaling [17, 28] while another study showed that mice lacking D5 dopamine receptors lost the ability to produce inositol phosphate or diacylglycerol messengers after stimulation with dopamine or several selective D1-like receptor agonists [29]. These results suggest that the dopamine D5 receptor may be the D1-like receptor subtype that mediates the PLC response. Due to the differential involvement of dopamine receptors in downstream signaling cascades, and the possibility that these signaling systems may differentially contribute to various dopaminergic functions, the present work sought to determine the possible contributions of dopamine D1-like receptor-linked signaling mediators in psychostimulant-induced 50kHz ultrasonic vocalization behavior in the rat.

2. MATERIALS AND METHODS

2.1 Animals

Male Sprague–Dawley rats weighing 175–200g were caged in groups of three and housed in climate-controlled facilities with a 12-h light/dark cycle and free access to food and water. Protocols for the care and use of the experimental animals were approved by the Institutional Animal Care and Use Committee and conformed to the NIH *Guide for the Care and Use of Laboratory Animals*.

2.2 Drugs

D-Amphetamine-sulfate and SCH23390 (Sigma-Aldrich, St. Louis, MO) were dissolved in saline and administered subcutaneously (s.c). SCH23390 was administered 20 minutes prior to amphetamine administration. The Protein kinase A (PKA) inhibitor, H89 (Sigma Aldrich, St. Louis, MO), was dissolved in DMSO and diluted in saline to a final concentration of 8 nmol/μL. The Phospholipase C-β (PLCβ) inhibitor U73122 (Sigma Aldrich, St. Louis, MO), was dissolved in DMSO and diluted with saline to a final concentration of 9 nmol/μL. The trkB receptor antagonist K-252a (Sigma Aldrich, St. Louis, MO) was dissolved in 25% DMSO/saline and diluted down to a final concentration of 25 ng/μl [30, 31]. Sterile saline (0.9% sodium chloride) was used for vehicle control injections.

2.3 Intracranial drug administration

Animals were surgically implanted with a stainless steel bilateral guide cannula (Plastics One, Roanoke VA) having an external diameter of 0.5 mm in order to facilitate the subsequent acute administration of drugs. Control rats also underwent sham surgery. Cannulas were aseptically implanted bilaterally into the cerebral ventricles under ketamine/xylazine (80%/12%) anesthesia. The intracerebroventricular (i.c.v.) coordinates used were

AP -0.8 mm, ML ± 1.5 mm, and DV -2.5 mm based on the atlas of Paxinos and Watson. Following the surgical procedure, animals were allowed at least one week to recover before being used in experiments. The drug to be administered was dissolved and diluted to appropriate concentration as described above and loaded into a Hamilton syringe connected through a 21-gauge silane tubing to the injection cannula; care was taken to expel air from the system. Prior to each injection, rats were briefly anesthetized in a chamber of isoflurane delivered from a controlled vaporizer. The injection cannula was carefully inserted into the guide cannula and 1–2 μ L of drug material was slowly infused into each of the lateral ventricles at the rate of 2 μ L/min. The injection cannula was left in place for an extra minute and then was slowly withdrawn and replaced with a dummy cannula to prevent loss of the drug or cerebrospinal fluid.

2.4 Measurement of USV behavior

Animals were tested following an acute injection of saline or amphetamine to naïve rats or rats pretreated with SCH23390 or signaling pathway modulators. For each experiment, parallel sets of animals were administered saline to serve as controls. On the day of testing, animals were first acclimated to the test apparatus which consisted of modified Med-Associates Activity Chambers each over-fitted with a pair of high-sensitivity SONOTRACK ultrasonic microphones (Metris B.V., KA Hoofddorp, The Netherlands). The insides of the chambers were shielded by lining the floor and side walls with techboard material. The main Sonotrack unit was connected to a Dell Computer workstation running the Sonotrack software in a Windows XP environment. After 15 min in the chamber, the apparatus was activated for 10 min in order to record any baseline USV or detect potential interfering noise. The animals were then carefully injected with saline or drug and placed back into the chamber. Both the Med-Associates activity monitoring system (see above) and the Sonotrack ultrasonic system were then simultaneously activated and monitored concurrently. Drug-induced USV behavior was continuously monitored for up to 90 min. The Sonotrack system captures multiple parameters for each emission, including the time (from start of experiment), the actual frequency of the emission, and its amplitude and duration. The combination of vocalization frequency and duration characterized two modes of emissions – a long-duration (>500 ms) 22 ± 4 KHz mode that is associated with aversive responses, and a short-duration (<300 ms) 50 ± 6 KHz mode that is associated with reward or appetitive behaviors in rodents [9, 32]. Since this system is capable of detecting ultrasound vocalizations of animals generated at frequencies ranging from 15 kHz to 100 kHz, it can be used with rats to concurrently monitor positive (50 kHz) and aversive (22 kHz) calls. Ultrasonic vocalizations were first identified and collated by the Sonograph system based on the established spectral characteristics for 22 KHz and 50 kHz emissions. The raw data were exported into Microsoft Excel and the data re-examined and validated using an in-house Excel algorithm. There was consistently strong correlation ($r>0.99$) between the algorithm-derived counts and the manual determinations, thus validating our use of the computerized algorithm which greatly increased analysis throughput. We report here on the 50 kHz responses that characteristically lasted less than 300 ms and showed a strong dose correlation for amphetamine and other rewarding drugs as previously reported [5, 6, 33]. No other form of vocalization between 15–100 KHz and lasting from 1–1000 ms was emitted to any appreciable degree by the animals throughout the course of these experiments. In no

instance did rats administered amphetamine or cocaine emit aversive 22 kHz USVs. Administered alone, amphetamine produced an inverted U-shaped dose response curve for the emission of ultrasonic vocalizations (data not shown) and showed 2 mg/kg to be an optimal dose for amphetamine induction of 50 kHz USV behavior as previously determined [34, 35]. Additionally, animals generally began emitting calls within five min after drug administration and continued to emit calls up to approximately 90 minutes (data not shown).

2.5 Measurement of in vivo BDNF protein expression

Animals were rapidly decapitated 24 h after the last injection of drug. The whole brain was removed and the striatum, hippocampus and prefrontal cortex was quickly dissected out anatomically intact, transferred to a polypropylene tube containing ice-cold homogenization buffer [137 mM NaCl, 20 mM Tris (pH 8.0), 1% Triton X-100, 10% glycerol, 1 mM protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO)] and homogenized for 30 s pulsing using an ultrasonic homogenizer. Tubes containing the homogenates were incubated for 20 min at 4 °C on a rocking platform and then centrifuged at 17000 g and 4 °C for 15 min. The supernatant was transferred to another tube and aliquots of the protein extract were assayed for BDNF protein using the Promega BDNF ELISA immunoassay kit (Promega, Madison WI) according to the manufacturer's protocol. Briefly, 96-well plates pre-coated with anti-BDNF monoclonal antibody were incubated with blocking buffer to saturate nonspecific binding. The immobilized anti-BDNF monoclonal antibody was incubated with BDNF standards or test samples followed by incubation with anti-human BDNF polyclonal antibody. After incubation with anti-IgY horse radish peroxidase conjugate, 3,3',5,5'-tetramethylbenzidine (TMB) One solution was added and color detection was accomplished at 450 nm using a Spectramax Pro plate reader (Molecular Devices, Sunnyvale CA).

2.6 Data Analysis

Each experiment was performed on multiple occasions for the molecular studies or in multiple animals for the behavioral studies so that sample sizes of 6–9 were accumulated. In general, the data were analyzed by one-way analysis of variance (ANOVA) using GraphPad Prism software (GraphPad Software, Inc, San Diego, CA), followed post-hoc either by the Dunnett test to determine which of the tested treatments differed significantly from the respective control group, or by the Tukey test to determine which among the various pairs of treatment or control groups were significantly different. Statistical comparisons were considered significant at $p < 0.05$ or better.

3. RESULTS

3.1 Single injection of amphetamine alters BDNF protein expression

Rats injected with a single amphetamine dose of 2 mg/kg and examined 24 h afterwards showed significant increases in BDNF protein expression in the striatum, hippocampus and prefrontal cortex (Figure 4) ($p < 0.05$ in each tissue). While the results are presented as % of control, it should be noted that basal BDNF protein expression differed among the tested brain regions, with the hippocampus having the highest basal concentrations of the expressed protein.

3.2 BDNF signaling mediates amphetamine-induced 50 kHz USV behavior

This experiment tested the role of intact BDNF signaling via the TrkB receptor in the acute effects of amphetamine. Groups of rats pretreated with either saline or 25 µg of K252a per brain hemisphere were administered a fixed dose (2 mg/kg) of amphetamine and USV behavior was immediately assessed for up to 90 min. Amphetamine increased USV responses (Figure 4). Rats pretreated with K252a prior to administration of amphetamine showed decreased responses to amphetamine-induced USVs as compared to animals receiving saline before amphetamine (Figure 4). K252a alone had no effect on USV emissions. The inhibitory effect of K252a on amphetamine's actions was evident in naïve animals and in animals receiving daily amphetamine administration for three or five days.

3.3 Inhibitors of different dopamine-sensitive signaling pathways differentially modulate amphetamine-induced USV behavior

These experiments tested the effects of known inhibitors of signaling via adenylyl cyclase (H89) or phospholipase C (U73122) cascades. As shown in Figure 5, mean USV calls significantly increased with repeated exposure of the animals to amphetamine ($p < 0.001$), indicating the occurrence of conventional psychostimulant sensitization of behavior with regard to the induction of USV. Compared to the effects of amphetamine alone, H89 significantly inhibited amphetamine-induced USV calls on the first day of exposure ($p < 0.05$); however, the ability of H89 to block the acute effects of amphetamine diminished with repeated exposures to amphetamine. U73122 had no significant effects on amphetamine-induced USV calls in naïve animals; however, with repeated amphetamine administration, U73122 gained inhibitory efficacy toward amphetamine-induced USVs.

3.4 Signaling pathway inhibitors differentially modulate cocaine-induced USV behavior

Given the differences in the mechanism of synaptic dopamine elevation by amphetamine and cocaine, we also tested the effects of cyclic-AMP-related and phosphoinositide-related signaling pathway modulators on cocaine-induced USV behavior. Cocaine significantly induced USV calls in naïve rats as previously reported [13]. Following repeated cocaine administration, cocaine-induced USV responses underwent significant sensitization from the third to fifth day of treatment ($p < 0.05$). Unlike the amphetamine results, both H89 and U73122 significantly enhanced the acute effects of cocaine in naïve rats ($p < 0.01$ for each drug). The enhancing effects of H89 or U73122 on cocaine-induced USV behavior were not lost or reversed following repeated treatment for five days.

4. DISCUSSION

The present data show that amphetamine induces the emission of 50 kHz ultrasonic vocalizations, while D1-like receptor blockade with SCH23390 significantly blocked the amphetamine effect. Similar to our previous findings with cocaine [13], amphetamine induced BDNF protein expression in various brain regions that receive dopaminergic input, and pretreatment with a trkB neurotrophin receptor inhibitor attenuated amphetamine-induced USV behavior. While this observation suggests that cocaine and amphetamine similarly induce USV behavior by utilizing D1-like receptors and BDNF/TrkB signaling, the

rest of the data suggest some differences in how these psychostimulants could utilize various signaling pathways upstream of BDNF/TrkB signaling to induce USV behavior.

The production of high numbers of 50 kHz calls by rats has been associated with a positive appetitive state [2, 36–38]. Consistent with previous results [10, 14], our present study showed that amphetamine increased flat 50 kHz USV calls and can be attributed to effects in regions of the mesolimbic dopaminergic system [5, 6]. Additionally, our results corroborate with previous studies [13, 39] by showing that selective antagonism of D1-like dopamine receptors by use of SCH23390 significantly prevented amphetamine-induced USV (data not shown).

We previously reported that cocaine increases BDNF protein expression in various brain regions that receive dopaminergic input [13], while pretreatment with a trkB inhibitor attenuated cocaine-induced USV behaviors [13, 20, 26, 40, 41]. The present results showed that another psychostimulant, amphetamine, significantly increased BDNF protein expression in various brain regions (Figure 2) while animals pretreated with the trkB receptor inhibitor K252a showed attenuated amphetamine-induced USV behaviors following single and repeated daily treatment. Taken together, these findings suggest that BDNF/TrkB signaling mediates psychostimulant-induced USV behavior, an inference that is consistent with previous findings [13, 20, 26, 40, 41].

In order to gain some insights on the contributions of upstream dopamine-sensitive (though not necessarily dopamine-exclusive) signaling cascades on the actions of amphetamine or cocaine, we challenged the psychostimulant drug effect by pretreatment with various signaling pathway modulators. H89 acutely inhibited amphetamine-induced ultrasonic vocalization behavior, but failed to maintain this behavioral inhibition of amphetamine-induced USV behavior with repeated exposure. On the other hand, pretreatment with the PLC inhibitor U73122 produced no effect on acute amphetamine-induced USV behavior, but inhibited amphetamine-induced USV behavior following repeated drug exposure. These results suggest that both adenylyl cyclase and PLC signaling are involved in mediating amphetamine-induced USV behavior, but the signaling pathways exert opposite or reciprocal effects on behavior mediation. A possible explanation for these results may lie in the differential coupling mechanisms for D1-like receptors such that stimulation of adenylyl cyclase/PKA elicits an inhibitory crosstalk with dopaminergic stimulation of PLC signaling [42]. Alternatively, adenylyl cyclase and phosphoinositide-dependent signaling systems may be working in tandem to regulate amphetamine-induced USV behavior. Evidence favors the distinct and separable coupling of D1/D1A receptors to AC signaling whereas the D5/D1B receptor subtype accounts for D1-like receptor coupling to phosphoinositide signaling in native brain tissues [18]. Although the manifestation of sensitization to the effects of amphetamine on 50 kHz ultrasonic vocalizations has already been demonstrated by others [12, 43], the current data suggests that phosphoinositide-linked signaling may play a significant role in the mediation of this behavior.

Similar to the findings with amphetamine, modulation of either cyclic AMP/PKA or PLC signaling altered the behavioral effects of cocaine. However, pretreatment with either the protein kinase A inhibitor H89 or the PLC inhibitor U73122 enhanced cocaine-induced USV

behavior after acute administration of cocaine in naïve or cocaine-sensitized rats. This suggests that activation of either the adenylyl cyclase or phospholipase C signaling pathways could exert a braking effect on cocaine-induced USV behavior, and this interaction exists on first exposure to cocaine and following repeated or sub-chronic exposure. The ability of dopamine-sensitive signaling pathways to differentially modulate cocaine and amphetamine-induced behavior was an unexpected result considering that these drugs are similarly capable of mediating USV behavior, as well as BDNF expression. It is well known that the dopamine transporter (DAT) serves as the target for all psychostimulants and is central to psychostimulant mediated increases in synaptic dopamine levels. However, amphetamine is also able to stimulate the release of neurotransmitter from the synaptic vesicle and prevents the breakdown of neurotransmitter through inhibition of the monoamine oxidase enzyme. Additionally, amphetamine and cocaine have different relative affinities for the DA, 5-HT and NE transporters [44]. In fact, an involvement of serotonin in the development of amphetamine-induced USV behavior has been established [35], although such involvement has not been established with cocaine. Thus, the molecular mechanisms by which amphetamine and cocaine increase synaptic dopamine level may be responsible for the distinct aspects of behavioral responses observed after both acute and repeated administration. Specifically, it is possible that the signaling pathway modulators exert additional effects on the mechanisms underlying transmitter release and or monoamine reuptake. Indeed, phosphorylation reactions mediated by either PKA or PKC have been shown to modulate transmitter release and/or reuptake [44–48]. Hence, further studies will be needed in order to better clarify the signaling pathway effects on behavioral modulation.

In conclusion, the results of this study indicate that both amphetamine and cocaine induce USV behavior via dopamine-dependent mechanisms, that these mechanisms involve intracellular signaling via protein kinase A and phosphoinositide-linked cascades, and that BDNF may serve as a converging intracellular nexus for the molecular mechanisms that mediate psychostimulant-induced USV behavior. The BDNF link is crucially important as it suggest a plausible path toward better understanding of the molecular mechanisms underlying the neuroplasticity and behavioral modulations that accompany repeated drug exposure. Further studies are needed in order to better understand the complex signaling cascade regulation and interactions that underlie dopamine-related phenomena such as positive-affective ultrasonic vocalization and addiction.

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Highlights

- Similar to cocaine, amphetamine increased BDNF protein expression in discrete brain regions, while pretreatment with a trkB neurotrophin receptor inhibitor, significantly reduced amphetamine-induced USV behavior.
- Inhibition of cyclic- AMP/PKA signaling with H89 or inhibition of PLC signaling with U73122 significantly blocked both the acute and subchronic amphetamine-induced USV behavior.
- In contrast to amphetamine, inhibition of cyclic- AMP/PKA signaling with H89 or inhibition of PLC signaling with U73122 further enhanced cocaine-induced USV behavior.

Figure 1. Effect of a single injection of amphetamine on BDNF protein expression

Male Sprague Dawley rats were given an i.p. injection of amphetamine 2 mg/kg. Animals were killed 24 h after drug treatment and BDNF levels in dissected striatal, hippocampal and prefrontal cortical tissues were measured by ELISA method. BDNF levels were computed as percentages relative to basal BDNF levels. Each bar is the mean $\pm$ SEM (n=6–9 animals).

**p<0.01, compared to the control group.

Figure 2. Effects of TrkB receptor inhibition on amphetamine-induced USV and locomotor behaviors

Groups of male Sprague Dawley rats were injected with 2 mg/kg amphetamine after pretreatment with an i.c.v. injection of saline or the TrkB receptor antagonist K252a (50 μ g). Following each drug treatment, USV behavior was measured for up to 90 min. Each bar is the mean $\pm$ SEM (N=6 animals). * p <0.05, compared to the control group as determined by ANOVA.

Figure 3. Effects of signaling pathway modulators on amphetamine-induced USV behavior
Groups of male Sprague Dawley rats were injected with 2 mg/kg amphetamine after a 20-min pretreatment with an i.c.v. injection of saline or the PKA inhibitor H89 (8nmol) or the PLCB inhibitor U73122 (9nmol). Immediately following amphetamine administration, USV behavior was measured for each animal for up to 90 min. Each bar is the mean $\pm$ SEM (N=6 animals). * p <0.05, compared to the control group as determined by posthoc Dunnett tests.

Figure 4. Effects of signaling pathway modulators on cocaine-induced USV behavior

Groups of male Sprague Dawley rats were injected with 20 mg/kg cocaine after pretreatment with an i.c.v. injection of the PKA inhibitor H89 (8nmol) or the PLCB inhibitor U73122 (9nmol). Immediately following cocaine administration, USV behavior was measured for each animal for up to 90 minutes. Each bar is the mean $\pm$ SEM (N=6 animals). * $p < 0.05$, compared to control group by posthoc Dunnett test