Effect of Anxiolytic Drugs on Air-Puff-Elicited Ultrasonic Vocalization in Adult Rats

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Abstract: Ultrasonic vocalization (USV) responses elicited by air-puff stimuli were compared in regard to both quality and quantity with those elicited by electric foot-shock(s) in adult rats. Frequency pattern, duration, repetition rate and interpulse interval of air-puff-elicited USV were comparable to those observed on foot-shock-elicited USV. Diazepam (0.25–1.0 mg/kg, s.c.) and buspirone (0.1–1.0 mg/kg, s.c.) attenuated equally and dose-dependently the USV responses elicited by both aversive stimuli. Air-puff-elicited USV was specifically attenuated in a dose-dependent manner by the anxiolytic properties of several psychotropic agents: diazepam (1.0–10.0 mg/kg, p.o.), buspirone (10.0–100.0 mg/kg, p.o.), 8-OH-DPAT (0.01–0.5 mg/kg, s.c.). Haloperidol (0.2–1.0 mg/kg, s.c.) weakly attenuated the USV response. Imipramine (0.2–1.0 mg/kg, s.c.) which has no anxiolytic property had no effect. Consequently, air-puff-elicited USV as well as foot-shock-elicited USV may provide a reliable tool for the study of anxiety.

Key words: ultrasonic vocalization, air-puff, anxiety, benzodiazepine, 5-HT₁A receptor ligand

Introduction

Ultrasonic vocalizations (USV), i.e., ultrasonic distress calls, in the 20–30 kHz range have been elicited in adult rats under stressful and painful situations such as electric shocks applied to the feet [1, 14, 16] or the tail [18] and acoustic startle [5, 6, 9, 19]. In view of the behavioral contexts in which USV are emitted, they are often interpreted as expressions of “fear” or “anxiety” [1, 8]. Many attempts have been made to develop animal models to test the anxiolytic effects of drugs [2, 6, 10, 16]. Suppression by anxiolytics of electric shock-elicited or acoustic startle-elicited USV in adult rats have been proposed as animal models of psychopharmacology of anxiolytic action which are behaviorally compatible with human anxiolytic function. However, they require pretreatments of rats by repeated aversive stimuli.

A new method recently developed by Knapp and Pohorecky [7], employing an air-puff as a stimulus, was found to efficiently induce USV responses in adult rats without requiring any pretreatments and painful stimuli. The present study was undertaken in order to examine whether 1) air-puff-elicited USV are expressions of anxiety by adult rats; and whether 2) many anxiolytics also attenuate air-puff-elicited USV.

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Materials and Methods

Subjects
Adult male Wistar-Imamichi rats (weighing 350–450 g) bred in our laboratory were used as subjects. They were maintained in an air-conditioned room (22 ± 2°C) with the lights on from 0700–2100 h and were given free access to food and water. The subjects were treated with appropriate care and respect according to the Guidelines for Animal Experimentation of Azabu University.

Elicitation and measurement of ultrasonic vocalization
The procedure for the elicitation of USV responses in adult rats by air-puff stimuli was a modified version of those described by Knapp and Pohorecky [7]. In brief, air controlled under 5.2 kg/cm² by a pressure valve of a cylinder was routed via a 1.2 mm ID Tefron tube to a computer-controlled electromagnetic valve (CKD, AB31-02-2-DC12V, Japan). Air-puffs were delivered from a 1.2 mm ID Tefron tube aperture located 3 cm distal from the electromagnetic valve and were aimed at the dorsolateral head and neck region of the rat. The duration of the delivery of the air-puffs was set at 120 ms, and one air-puff was delivered every 15 s until the rat began to vocalize or until 16 air-puffs had been delivered. In order to deliver the air-puff stimuli to these regions of the rat, we used a semi-restraining cage (5 × 18 × 7 cm) in which the rat was not able to turn in a different direction and which did not induce any pain in the rats during handling and restraining. A microphone was installed above the cage. Only those rats which began to vocalize within the delivery of 16 air-puffs were used for assessing the effect of anxiolytic drugs.

The procedure for electric foot-shock stimulation is described elsewhere [12, 13]. In brief, an individual rat was placed in an inescapable cage (17 × 21 × 18 cm) with a grid floor and then was received a series of electric foot-shocks (2 mA, 1 s) at intervals of 1 min (no more than 10 shocks were applied). If a rat squeaked or emitted USV for longer than 1 min during the delivery of foot-shocks, the delivery of stimulation was interrupted in order to improve the acquisition of USV to avoid the stimuli. If a rat did not emit USV, another series of stimuli were delivered the next day. Only those rats that had responded by the time five series of foot-shocks had been delivered and showed an USV response of longer than 3 min after being put in the stimulation cage or after receiving the first foot-shock were used in assessing the effect of anxiolytic drugs.

Ultrasonic waves transduced by a condenser microphone (Kunitachi Acoustic Lab., ACM-20F) were amplified and fed to an electronic device (Diamedical System, DMP-350) measuring the average frequency and average amplitude of sine waves in 5-msec time bins which were successively output to a personal computer in real-time through an A/D converter. Sound production with frequencies higher than 20 kHz was continuously monitored on a computer screen. For the waves with frequencies higher than 20 kHz, the average amplitude in a 5-msec time bin was collected for 3 min after starting the measurement. The data are therefore combined products of the intensity and duration of the emission of USV. The data are expressed in arbitrary units. Moreover, sonagrams of both paradigms were monitored on the computer display for a total duration of 12 s according to the procedures described previously [11, 17].

Assessment of effects of drugs
Both aversive stimuli-elicited USVs in adult rats were measured for 3 min in the morning. Two hours later, diazepam or buspirone was subcutaneously injected. Diazepam (Horizon, Yamanouchi Seiyaku Co., Japan) was used as an anxiolytic benzodiazepine receptor agonist and was injected subcutaneously at a dose of 0.25–1.0 mg/kg body weight (0.5 mg/ml suspension in 0.9% saline with 1% Tween 80). Buspirone (RBI, USA), a 5-HT1A receptor agonist, was dissolved in 0.9% saline and injected at a dose of 0.1–1.0 mg/kg. The volume injected was 2 ml/kg. Measurement of ultrasonic sounds was repeated 30 and 60 min after the injection. Moreover, the effects of diazepam, buspirone, 8-hydroxy-2-(di-n-propyl-amino)-tetralin hydrobromide (8-OH-DPAT, RBI, USA), haloperidol (RBI, USA), and imipramine (RBI, USA) on the USV responses elicited by air-puff stimuli were also examined. Diazepam was orally administered at a dose of 1–10 mg/kg in a volume of 5 ml/kg, 30 min before testing. Buspirone was also orally administered at a dose of 1–100 mg/kg. 8-OH-DPAT is a 5-HT1A receptor agonist and was dissolved in 0.9% saline and injected subcutaneously at a dose of 0.01–0.5 mg/kg. Haloperidol, an antipsychotic, or imipramine, an antidepressant, were injected subcu-
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Simultaneously at a dose of 0.2–1.0 mg/kg, respectively, and their appropriate vehicles were injected subcutaneously in a volume of 2 ml/kg and 30 min before testing.

The effects of the drugs on the USV responses were expressed quantitatively as percentage inhibition of vocalization activity after injection as compared to that before injection in each rat.

Statistics

The effects of drugs in the USV test were analysed using four-way and one-way analysis of variance and Student’s t-test. Significance was defined as the 0.05 level.

Results

In the present study, USV response inducibility (percentage of rats emitting ultrasounds) to air-puff stimuli was 58.2% (64/110) without requiring any pretreatments and painful stimuli, whereas USV response was induced in 53.3% (64/120) after the delivery of five series of foot-shocks. The vocalization activities elicited by air-puff stimuli (2014.2 ± 312.6, n=64) during the 3-min recording period were comparable to those elicited by foot-shocks (2354.6 ± 235.4, n=64). Typical sonagrams of the USV responses elicited by air-puffs and electric foot-shocks are shown in Fig. 1. During vigorous vocalization, the ultrasounds consisted of pulses longer than 1,000 msec with a frequency between 22 and 28 kHz without any frequency shift and intermissions of about 200 msec separated the pulses. Frequency pattern, duration, and interpulse interval of air-puff-elicited USVs are similar to those elicited by foot-shocks.

Figure 2 shows the effects of diazepam on the USV responses elicited either by air-puff stimuli or by foot-shocks. Diazepam dose-dependently attenuated USV,
and statistical differences were observed between dosages (air-puff: F(3,63)=126.48, p<0.01; foot-shock: F(3,63)=87.25, p<0.01). Buspirone also dose-dependently attenuated the USV responses elicited either by air-puff stimuli or by foot-shocks, and statistical differences were observed between dosages (air-puff: F(3,63)=140.57, p<0.01; foot-shock: F(3,63)=126.48, p<0.01), as shown in Fig. 3. However, no statistical differences were observed between recording times following the injection of each drug. The effects of both anxiolytics were comparable in both paradigms and no statistical difference was observed between them.

As shown in Table 1, orally-administered diazepam and buspirone, and subcutaneously injected 8-OH-DPAT attenuated the USVs elicited by air-puff stimuli in a dose-dependent manner, and statistical differences were observed between dosages. Haloperidol weakly attenuated the USV response. Buspirone slightly enhanced the USV response at 1.0 mg/kg p.o., and imipramine did not affect the USV response elicited by air-puff stimuli.

**Discussion**

In the present study, 58.2% of the rats exposed initially to air-puff stimuli emitted USV without any pretreatment procedures. Tonoue et al. [16] and Kaltwasser [5] reported that USV responses were elicited in only 50–70% of all rats by either electric foot-shocks or by acoustic stimuli, though Knapp and Pohorecky [7] reported that nearly all rats tested were able to elicit the USV response by air-puff stimuli. The cause of these differences in USV response inducibility was not clarified, though the high-emotionality of the rat may act as an important factor in the elicitation of the USV response as indicated in our previous findings [13]. The vocalization pattern and the vocalization activity in response to air-puff stimuli were identical to

![Fig. 3. Effects of buspirone on the ultrasonic vocalization responses elicited either by air-puff stimuli or by foot-shocks in adult rats. Ultrasonic vocalization was recorded 30 and 60 min after injection in each rat. Effects of vehicle and drug on the USV responses was expressed quantitatively as percentage inhibition (mean ± S.E.M.) of vocalization activity after injection as compared to that before injection in each rat. Eight animals were tested at each dose. Asterisks indicate significant (p<0.01) effects compared to vehicle injection.](image)

**Table 1. Effects of psychotropic drugs on air-puff-elicited ultrasonic vocalization in adult rats**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Route of administration</th>
<th>Dose (mg/kg b.w.)</th>
<th>% inhibition (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diazepam</td>
<td>p.o.</td>
<td>Vehicle</td>
<td>-0.5 ± 4.5 (7)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>29.6 ± 16.8** (8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10.0</td>
<td>73.8 ± 7.9** (8)</td>
</tr>
<tr>
<td>Buspirone</td>
<td>p.o.</td>
<td>Vehicle</td>
<td>0.3 ± 3.1 (6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>-28.6 ± 38.9** (6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10.0</td>
<td>84.6 ± 11.5** (6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100.0</td>
<td>99.4 ± 0.5** (6)</td>
</tr>
<tr>
<td>8-OH-DPAT</td>
<td>s.c.</td>
<td>Vehicle</td>
<td>-1.5 ± 3.1 (6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.01</td>
<td>0.4 ± 7.1 (6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.05</td>
<td>99.0 ± 0.4** (6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.5</td>
<td>100.0 ± 0.0** (6)</td>
</tr>
<tr>
<td>Haloperidol</td>
<td>s.c.</td>
<td>Vehicle</td>
<td>0.2 ± 5.1 (6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.2</td>
<td>13.6 ± 7.3* (6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>57.3 ± 4.6** (6)</td>
</tr>
<tr>
<td>Imipramine</td>
<td>s.c.</td>
<td>Vehicle</td>
<td>0.5 ± 3.8 (6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.2</td>
<td>2.6 ± 5.4 (6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>0.2 ± 4.3 (6)</td>
</tr>
</tbody>
</table>

Drug or vehicle was given 30 min before test. Data are expressed as percentage inhibition (mean ± S.E.M.) of vocalization activity after administration as compared to that before administration. Asterisks indicate significant drug effects compared to vehicle treatment (**P<0.01, *P<0.05).
those in response to electric foot-shocks. Therefore, the air-puff stimuli may act as an aversive stimuli, though air-puff stimuli contain an acoustic component as Knapp and Pohorecky [7] indicated.

Although Knapp and Pohorecky [7] demonstrated that gepirone, a 5-HT1A agonist, inhibited the USV response elicited by air-puff stimuli, they did not test diazepam in their study. The findings of the present study demonstrate that not only electric foot-shock-elicited USV but also air-puff-elicited USV are equally attenuated by diazepam (Fig. 2) and the 5-HT1A agonist buspirone (Fig. 3). The anxiolytic effects of many benzodiazepines on USVs have been previously shown in adult rats [1, 2, 10, 16] and in rat pups [3, 4, 20], though the active doses were close to those that produce muscle relaxation and sedation. The dosage of diazepam (0.25–1.0 mg/kg, s.c.) used in the present study was supposed to have an anxiolytic effect, with neither muscle relaxation nor sedation occurring. Our results are in agreement with previous findings [1] showing that the attenuation of USV response elicited by unavoidable aversive stimuli are not due to a sedative or muscle-relaxant activity of diazepam (0.5–1.0 mg/kg, i.p.).

The air-puff-elicited USV response may be sensitive to a wide range of anxiolytics, such as benzodiazepines and 5-HT1A agonists. Moreover, several psychotrophic drugs containing anxiolytic properties attenuated the USV response in this paradigm, as shown in Table 1. The present experimental procedure detected the USV response in this paradigm, as shown in Table 1. The present study demonstrated that not only electric foot-shock-elicited USV but also air-puff-elicited USV are equally attenuated by diazepam (Fig. 2) and the 5-HT1A agonist buspirone (Fig. 3). The anxiolytic effects of many benzodiazepines on USVs have been previously shown in adult rats [1, 2, 10, 16] and in rat pups [3, 4, 20], though the active doses were close to those that produce muscle relaxation and sedation. The dosage of diazepam (0.25–1.0 mg/kg, s.c.) used in the present study was supposed to have an anxiolytic effect, with neither muscle relaxation nor sedation occurring. Our results are in agreement with previous findings [1] showing that the attenuation of USV response elicited by unavoidable aversive stimuli are not due to a sedative or muscle-relaxant activity of diazepam (0.5–1.0 mg/kg, i.p.).

The air-puff-elicited USV response may be sensitive to a wide range of anxiolytics, such as benzodiazepines and 5-HT1A agonists. Moreover, several psychotrophic drugs containing anxiolytic properties attenuated the USV response in this paradigm, as shown in Table 1. The present experimental procedure detected the anxiolytic-like profile of haloperidol in a weakly attenuating the USV response, though several findings have demonstrated that haloperidol had no attenuating effect on the USV response [1, 2]. Sánchez recently presented that haloperidol had a weak inhibitory effect on the USV elicited by foot-shocks in adult rats [15].

The USV response elicited by foot-shocks was effectively attenuated by many anxiolytics and has already been validated as an animal model of anxiety [2, 16, 18]. The present findings suggest that the air-puff-elicited USV response specifically detects anxiolytic properties of psychotrophic drugs. Therefore, USV in response to air-puff stimuli may provide a novel animal model of anxiety and may constitute a valid and quantifiable amount of anxiety.

Consequently, air-puff-elicited vocalizations as well as foot-shock-elicited vocalizations may provide simple and reliable tools in the study of anxiety.

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References


