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High-Frequency (35–70 kHz) Ultrasonic Vocalizations in Rats Confronted With Anesthetized Conspecifics: Effects of Gepirone, Ethanol, and Diazepam

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**Bekesy Laboratory of Neurobiology, 1993 East-West Road, Honolulu, HI 96822 and*
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BLANCHARD, R. J., E. B. YUDKO, D. C. BLANCHARD AND H. K. TAUKULIS. *High-frequency (35–70 kHz) ultrasonic vocalizations in rats confronted with anesthetized conspecifics: Effects of gepirone, ethanol, and diazepam.* PHARMACOL BIOCHEM BEHAV **44**(2) 313–319, 1993.—The effects of three anxiolytics—gepirone, diazepam, and ethanol—on high-frequency ultrasonic vocalizations elicited from rats via a new method are described. Subjects confronted with an anesthetized, same-sex conspecific in a neutral test cage emitted ultrasonic vocalizations in the 35- to 70-kHz range. The great majority of these were calls with frequencies higher than 40 kHz; of these, short calls (< 50 ms) occurred significantly more frequently than long calls (> 50 ms). Female subjects emitted far more of these high-short and high-long vocalizations than males did. In females, but not males, these calls were reduced in number by gepirone, 5-hydroxytryptamine$_{1A}$ (5-HT$_{1A}$) agonist, at both 1.0- and 10.0-mg/kg doses and by diazepam, a benzodiazepine, at 3.0 but not 1.0 mg/kg. Ethanol (0.6 and 1.2 g/kg) had no detectable effect. The utility of this method, both for the study of ultrasounds and assessment of serotonergic anxiolytics, is discussed.

Ultrasonic vocalizations Gepirone Diazepam Ethanol Anesthetized conspecific Sex differences

ADULT rats emit ultrasonic vocalizations in a variety of contexts [for reviews, see (21,25,29,40)]. These calls fall into two basic categories based upon wave frequency: those with frequencies falling largely within the 20- to 29-kHz range (sometimes referred to as "22-kHz" vocalizations) and those within the 30- to 70-kHz range (known as "50-kHz" vocalizations). The latter have been recorded during mating encounters (3,20,27,33,42), in situations involving conspecific aggression, defense, and submission (21,28,30,32,34), and in same-sex conspecific encounters while undergoing morphine withdrawal (41). In infant rats, high-range ultrasounds have been detected during periods of acute isolation (8,17,19,23,29).

Recent studies in this laboratory (R. J. Blanchard et al., in preparation) have shown that both male and female adult rats vocalize in the 35- to 70-kHz range when reunited with a same-sex sibling after a period of separation. Calls of this type also occur when a rat encounters a same-sex anesthetized conspecific. This condition is especially interesting for two reasons. First, animals produce *only* high-range calls without the accompanying 20- to 29-kHz calls found to occur in other circumstances such as sexual or aggressive encounters. Second, the presentation of an anesthetized conspecific provides an experimental context in which ultrasounds generated by a single animal can be recorded without interference from those produced by another animal. In the usual experiments involving dyadic encounters, it is impossible to tell which of the two animals is emitting the ultrasounds unless one of them is surgically devocalized.

Because ultrasounds are often recorded in situations presumed to be stressful for the vocalizing animals, investigators have been interested in the effects of anxiolytic drugs on these calls. Most focused primarily on the 22-kHz type, except for studies of the high-range isolation cries emitted by infant rats. The latter demonstrated the attenuating effects of a variety

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of anxiolytics, including benzodiazepines (9,14,15,19,22) and some newer agents known to interact with various serotonin [5-hydroxytryptamine (5-HT)] receptor subtypes (16,22,43). Although few studies of pharmacological effects on 50-kHz vocalizations in adult rats have been conducted, it has been shown that these calls, when emitted by an animal in a situation of social stress, can be altered by diazepam (a benzodiazepine) and clonidine (an α_2-adrenoreceptor agonist) but not by gepirone (classified as a 5-HT_{1A} type of anxiolytic) (21). Miczek et al. (21) and Tornatzky and Miczek (37,38) reported that diazepam (1.0 and 3.0 mg/kg) reduced the number of 50-kHz calls emitted by a defeated intruder rat during a 10-min period before it again encountered (but was protected from) the dominant resident; at the same time, 22-kHz calls remained unaffected. In contrast, low doses of gepirone (0.3 and 0.6 mg/kg), an agonist at 5-HT_{1A} receptors, suppressed the rate and duration of 22-kHz calls by male intruder rats in the same context, while these and even much higher doses of the drug (3.0 and 6.0 mg/kg) did not alter the emission of 50-kHz vocalizations.

In the present experiments, the effects of three anxiolytics—gepirone, ethanol, and diazepam—on the high-range vocalizations selectively elicited in rats by an anesthetized conspecific were explored. Two additional variables examined were: a) the gender of the rat being tested and b) the subtype of high-range call being emitted. White et al. (42) modified a high-range call classification scheme originally suggested by Sales (27) to include four subtypes based upon frequency and duration: a) high-short (>40 kHz, <50 ms); b) low-short (30–40 kHz, <50 ms); c) high-long (>40 kHz, >50 ms); and d) low-long (30–40 kHz, >50 ms). This scheme, with minor variations, was adopted here.

METHOD

Subjects

Thirty-three male and thirty-three female Long-Evans rats, 120 days old, were used as subjects. Animals were bred in colonies maintained by the University of Hawaii Laboratory Animal Services. At the time of testing, they were housed individually in polycarbonate cages in a room maintained on a 12 L : 12 D cycle. Food and water were available ad lib.

Apparatus

All animals were tested individually in a wire-topped polycarbonate cage (45 × 26 × 21 cm) illuminated by overhead red lights. Test sessions were videotaped with a camera positioned to one side. Ultrasounds were recorded with a QMC Model 200 bat detector, or an Ultrasound Advice Model S25 bat detector, and a Racal Store 4DS instrumentation recorder (Racal, Irvine, CA). The detector microphone was suspended 1 in. above the cage top.

Drugs

Gepirone (Bristol-Myers, Syracuse, NY) was administered at doses of 1.0 and 10.0 mg/kg. It was prepared in an isotonic saline vehicle in concentrations that permitted injections at a volume of 2.0 ml/kg body weight. Diazepam (Sigma Chemical Co., St. Louis, MO) was administered at doses of 1.0 and 3.0 mg/kg. It was dissolved in saline to which polyoxyethylene sorbitan monooleate (Tween-80; Sigma) had been added. Concentrations were adjusted so that the injection volume for each dose was 1.0 ml/kg. Ethanol (200 proof, Quantum Chemical Corp.) was administered at doses of 0.6 and 1.2 g/kg. It was diluted with saline to concentrations that permitted an injection volume of 10.0 ml/kg.

Procedure

The study was comprised of three separate experiments, one for each of the drugs tested. In each experiment, equal numbers of male and female subjects (10 of each in the gepirone and ethanol experiments and 13 of each in the diazepam experiment) were injected IP on three occasions, once with the lower dose of the test drug, once with the higher dose, and once with isotonic saline. The three injections were spaced either 4 (diazepam and ethanol) or 5 (gepirone) days apart, and their sequence varied randomly between subjects.

Thirty minutes after injection, a subject was placed into the test cage, which contained wood chips and a same-sex conspecific anesthetized beforehand with sodium pentobarbital. The subject remained in this cage for 8 min while ultrasounds and video images were recorded.

The wood chip litter was changed between test sessions. Different test cages were used for male and female subjects.

Analysis of Ultrasounds

For the studies with gepirone and ethanol, ultrasonic vocalizations were classified into four categories on the basis of frequency and duration: high (>40 kHz) or low (30–40 kHz) and long (>50 ms) or short (<50 ms).

Statistical Methods

For each experiment, a three-way analysis of variance (ANOVA) (drug dose × sex of subject × subtype of ultrasound) was performed. When appropriate, subsequent pairwise comparisons were carried out using the Newman-Keuls method, and the obtained probabilities are given in parentheses.

RESULTS

In each of the three experiments, the distribution of ultrasounds under the control (saline injection) condition was similar. High-frequency (>40 kHz) calls far outnumbered low-frequency (30–40 kHz) subtypes; and, of the latter none fell below 35 kHz. Among the high-frequency calls, more were of short (<50 ms) rather than long (>50 ms) duration.

Gepirone

Figure 1 illustrates the mean number of calls of each type emitted by male and female subjects after an injection of saline or gepirone (1.0 and 10.0 mg/kg). Gepirone significantly reduced vocalizations of all types across all groups, $F(2, 36) = 7.00$, $p < 0.01$; both drug groups differed reliably from the saline control group with $p < 0.01$. Females vocalized more than males, $F(1, 18) = 7.67$, $p < 0.02$, and the sexes differed in terms of the subtypes of ultrasound they produced, $F(3, 54) = 6.23$, $p < 0.01$. Females generated more high-short ($p < 0.001$) and high-long ($p < 0.01$) ultrasounds, while no significant differences were found for the less common low-short and low-long subtypes. An interaction emerged between drug dose and subtype of ultrasound, $F(6, 108) = 6.05$, $p < 0.001$, reflecting the fact that both doses of gepirone significantly ($p < 0.05$ and $p < 0.001$) reduced the number of high-short vocalizations while the other subtypes remained unaffected. A significant sex × dose interaction, $F(2, 36) = 3.79$, $p < 0.05$, indicated that the effect of dose was significant for females but not for males. Finally, a significant

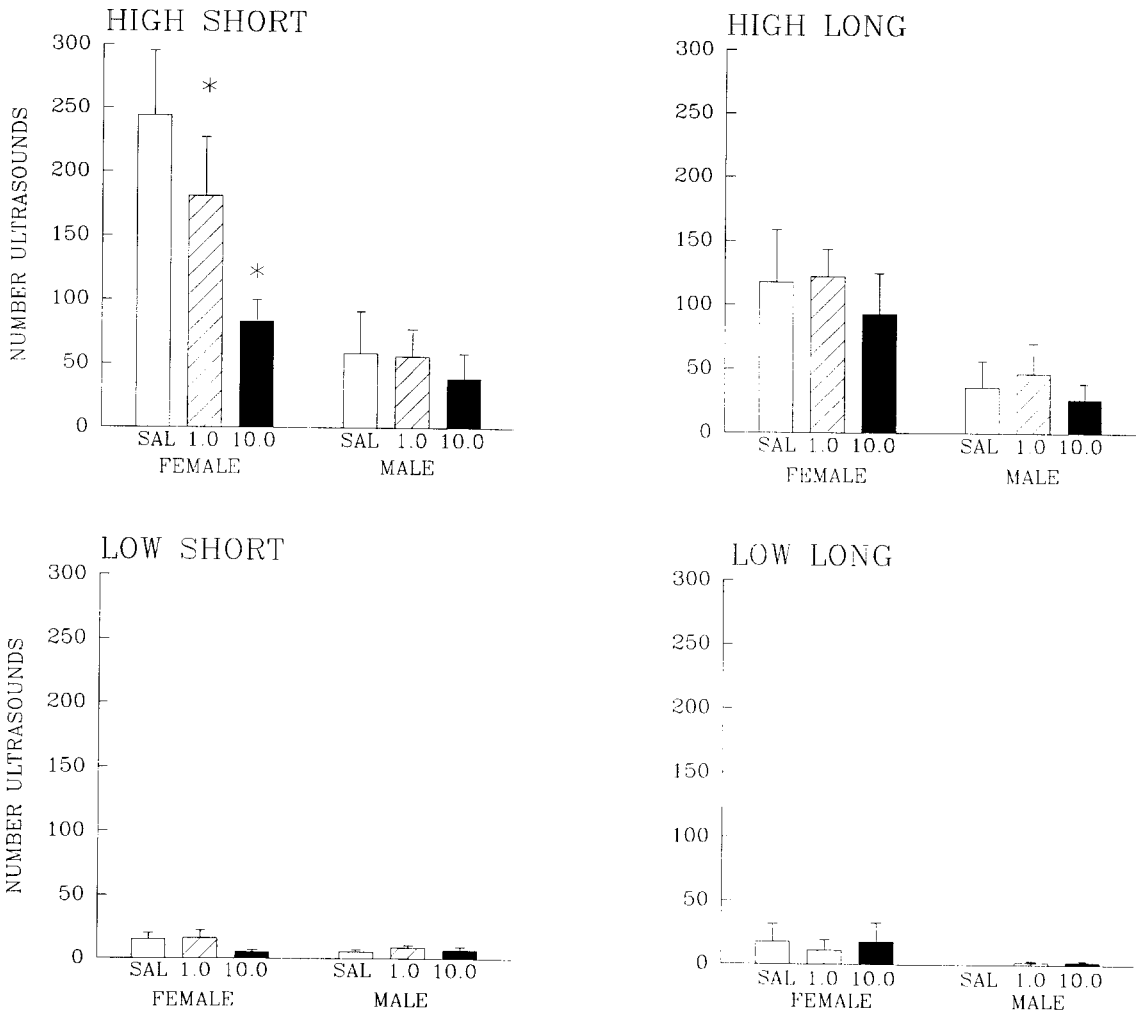

FIG. 1. Effects of gepirone (1.0 and 10.0 mg/kg) on the mean (±SEM) numbers of four subtypes of high-range ultrasounds recorded from male (n = 10) and female (n = 10) subjects. *Within-subject comparisons of drug treatment vs. the saline control condition were significant at $p < 0.05$ or less.

three-way interaction was obtained, $F(6, 108) = 3.55$, $p < 0.01$, because the dose × sex interaction occurred only with the high-short ultrasounds and not with the other subtypes.

Ethanol

Figure 2 shows the effects of ethanol (0.6 and 1.2 g/kg) and sex of subject on ultrasonic vocalizations. As in the previous experiment, a main effect of ultrasound subtype appeared, $F(3, 54) = 12.17$, $p < 0.001$, with both high-frequency subtypes occurring more frequently than the two low-frequency subtypes. Females vocalized more than males, $F(1, 18) = 5.46$, $p < 0.05$; and a sex × subtype interaction, $F(3, 54) = 4.50$, $p < 0.01$, occurred because the difference between sexes was apparent only in high-short calls ($p < 0.001$). Ethanol had no significant effect on these vocalizations, $F(2, 360 < 1$.

Diazepam

Figure 3 illustrates the effects of diazepam (1.0 and 3.0 mg/kg) on four of the six subtypes of high-range ultrasound in male and female subjects. A significant main effect of diazepam was not obtained, $F(2, 46) = 2.31$, $p > 0.10$. However, a significant three-way interaction did emerge, $F(10, 230) = 1.95$, $p < 0.05$. In female subjects only, diazepam at 3.0 mg/kg reduced the number of high-short ultrasounds relative to saline controls ($p < 0.001$). No dose or sex effects were observed in the other subtypes. As in the previous two experiments, females vocalized more than males, $F(1, 23) = 15.87$, $p < 0.001$, depending upon ultrasound subtype [sex × subtype interaction, $F(5, 115) = 19.06$, $p < 0.001$]. Females produced more vocalizations of the high-short ($p < 0.001$) and high-long ($p < 0.01$) varieties relative to males. A main effect of subtype, $F(5, 115) = 40.36$, $p < 0.001$, emerged because the high-short and high-long calls occurred more frequently than any of the other types of calls (for all pairwise comparisons, $p < 0.05$ or less).

DISCUSSION

Presentation of an anesthetized conspecific proved a reliable method of eliciting specific subtypes of high-range ultrasounds from Long-Evans rats, especially females. In all three

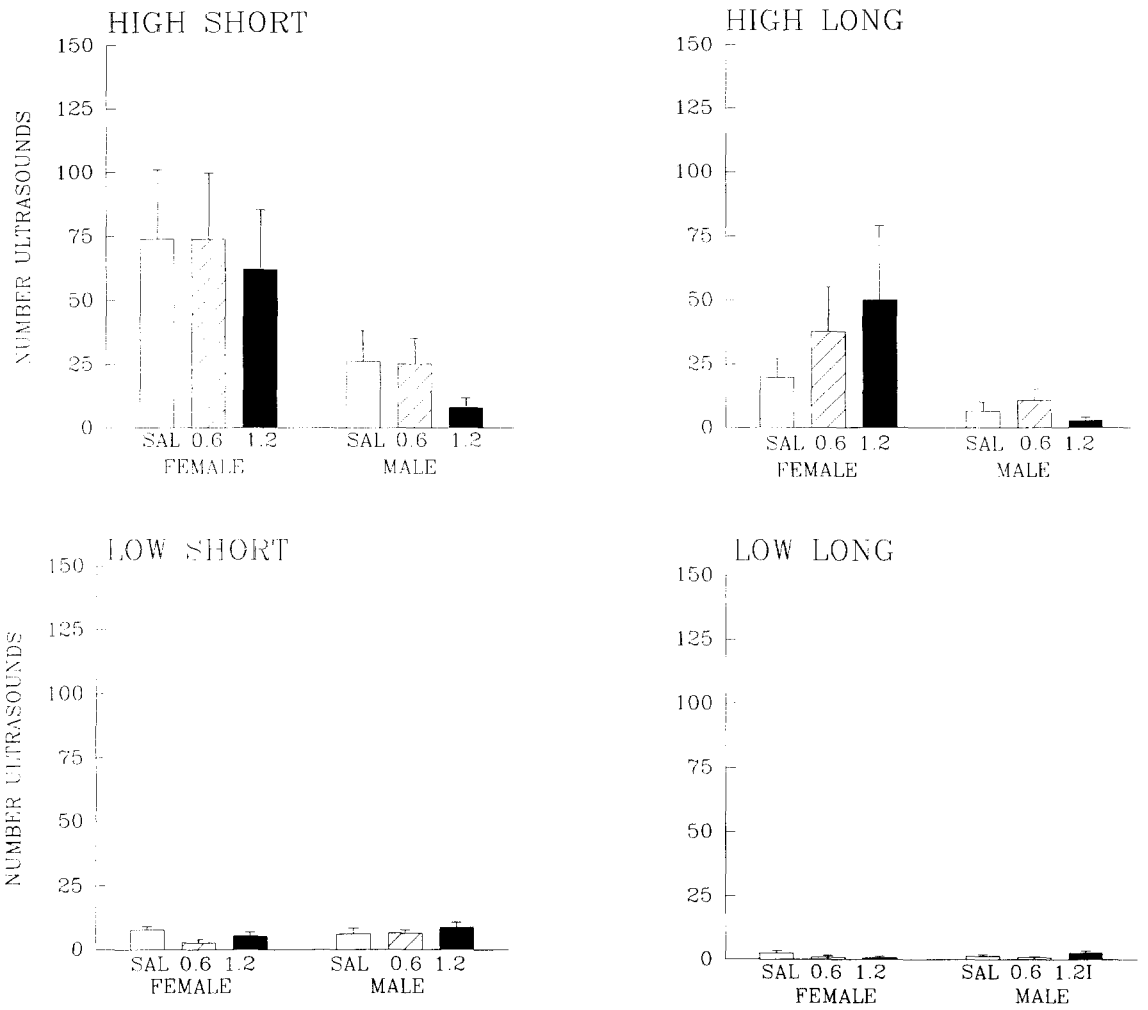

FIG. 2. Effects of ethanol (0.6 and 1.2 g/kg) on the mean (±SEM) numbers of four subtypes of high-range ultrasounds recorded from male (n = 10) and female (n = 10) subjects. No within-subject comparisons were statistically significant.

experiments, females emitted many high-frequency (>40 kHz) calls of both short (<50 ms) and long (>50 ms) duration but few calls of 30–40 kHz. Males produced far fewer calls of all types, but the distribution of these in terms of frequency and duration was similar to that of females. Rats of both sexes have been shown to produce high-range (30–70 kHz) ultrasounds in the context of reproductive encounters (3, 33,35), and male rats produce them during interactions with conspecifics that involve defense and submission (21,28,30). The present context was novel in that it involved neither reproductive activity nor overt agonistic behaviors and the sexual dimorphism it yielded was dramatic.

Females' high-short (>40 kHz, <50 ms) vocalizations were reduced by both doses of gepirone (1.0 and 10.0 mg/kg) and by the higher of the two doses of diazepam (3.0 mg/kg) but remained unaffected by either dose of ethanol. In contrast, Miczek et al. (21) and Tornatzky and Miczek (37,38) reported that the high-range calls they obtained from "protected" intruder rats during a period of threat from a resident conspecific were decreased by diazepam but not by gepirone. It may be that the present model is in particular sensitive to serotonin-targeting drugs like gepirone. Alternatively, the difference between the studies may reflect a gender × procedure interaction. Miczek and colleagues studied male rats, and recent findings suggested that, at least in the context of defensive behavior, males may be relatively more responsive to drugs like diazepam, which affect GABA systems, while females may be more affected by serotonergic agents (6,13). Further, the number of male calls in response to the anesthetized conspecific in the present experiments may have been so low as to preclude the detection of drug effects.

It is known that some anxiolytic drugs reduce muscle control, and the possibility that this effect could have been responsible for the decrease in ultrasonic vocalizations noted in these experiments was considered but was discounted for several reasons. First, evidence indicates that gepirone has little effect on musculature (39). Second, if gepirone were to disrupt ultrasound in this fashion then a similar effect should be seen in other circumstances; however, Miczek et al. (21) reported that gepirone had no effect on high-range ultrasounds in male rats in their experiments. Finally, ethanol impairs muscle control (1) but had no effects on ultrasounds in the present study.

The three drugs tested in the present studies are known for their shared capacity to reduce anxiety in human subjects.

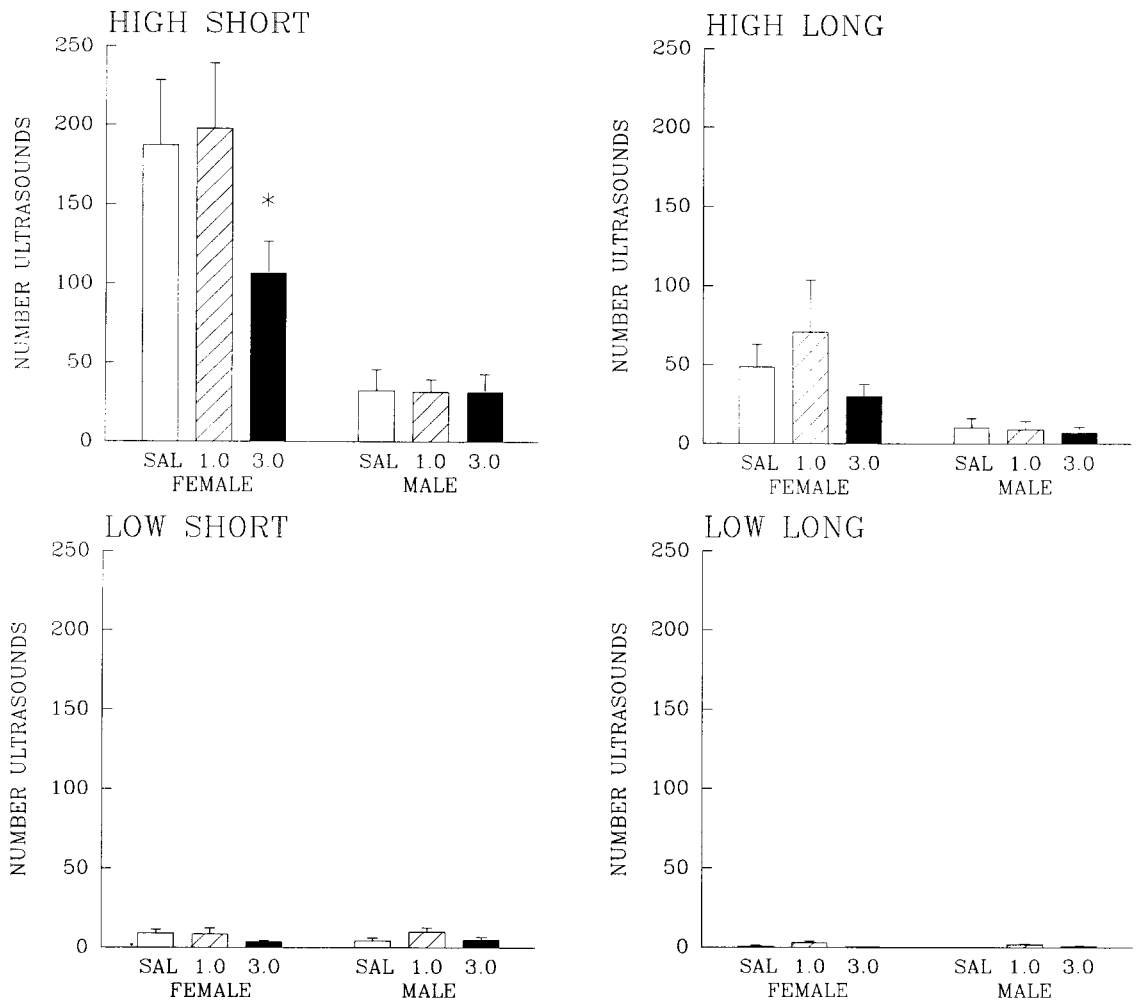

FIG. 3. Effects of diazepam (1.0 and 3.0 mg/kg) on the mean (± SEM) numbers of four of the six subtypes of high-range ultrasounds recorded from male (n = 13) and female (n = 13) subjects. *Within-subject comparison of drug treatment vs. saline control condition was significant at $p < 0.001$.

Yet, in animal models it is becoming increasingly clear that their effects can be strikingly different. For example, Blanchard et al. (4,5) found that in wild rats (*Rattus rattus*) gepirone and buspirone (another pyramidinyl piperazine derivative with a high affinity for 5-HT_{1A} sites) altered various aspects of animals' defensive responses to threatening stimuli in a fear/defense test battery. In particular, these drugs attenuated a variety of defensive threat/attack behaviors in situations in which flight was not possible. In comparison, diazepam and two other benzodiazepines, chlordiazepoxide and midazolam, tended to reduce only defensive threat, leaving defensive attack intact. Ethanol (7) at higher doses (1.8 g/kg) reduced a variety of defensive behaviors, but at lower doses differed from all the other anxiolytics tested in that it potentiated audible vocalizations and jump-attacks.

Given the differences in the behavioral characteristics of these drugs in other contexts, it is not surprising that they differed in terms of their effects on ultrasonic vocalizations, in particular if the anesthetized conspecific was perceived as a threatening stimulus. The studies cited above showed that various behavioral responses to a variety of threatening stimuli can be differentially affected by chemically disparate anxiolytics. Ultrasonic calls, despite superficial similarities, may be controlled by different neurophysiological systems as a function of the characteristics of the eliciting event. These systems may vary considerably in terms of the types of chemical disturbances to which they are sensitive.

The efficacy of gepirone in the attenuation of calls evoked by an anesthetized conspecific suggests that serotonergic systems may play a role in the expression of this behavior, although further confirmational studies with subcategories of 5-HT agonists and antagonists are necessary. Winslow and Insel (43) already explored serotonergic involvement in isolation-induced ultrasonic calls by rat pups, and a similar analysis of high-range vocalizations in the adult is warranted. Because ontogenetic changes in response to serotonin-targeting drugs have been reported in the context of other behaviors (12), age-related differences in their effects on ultrasound may also be anticipated. Diazepam, although its primary site of action is the benzodiazepine-GABA-chloride ionophore complex, is known to modify serotonergic activity as well (11, 24,26,44), and this property may account for its efficacy in

the present context. Ethanol, which had no discernible effect on the number of ultrasonic vocalizations, is also believed to exert many of its behavioral effects through its interaction with the benzodiazepine-GABA-chloride ionophore complex (36). However, while systemic ethanol can stimulate serotonin release from several central sites (18,45) no clear correlates with behaviors associated with the production of ultrasounds have been established.

Low-range (20-29 kHz) and high-range (30-70 kHz) calls can occur separately or in combination, as the situation dictates. For example, White et al. (42) reported that the vocalizations produced by male rats over several ejaculatory series were almost exclusively of the high-range variety. At other times during the pre- and postejaculatory phases of reproductive activity, low-range calls predominated or a mixture of the two were noted (2). Assuming that most ultrasonic vocalizations serve a communicatory function, the "message" a given ultrasound conveys may depend upon the context of other ultrasounds emitted in the same time frame. For instance, a 50-kHz call emitted alone may not convey the same message as the same call when it is temporally associated with calls in the 20- to 29-kHz range. For purposes of functional analysis, it will be useful to discover behavioral contexts that only elicit calls falling within a narrowly defined class. The anesthetized conspecific technique seems to serve this purpose nicely. In the three experiments herein described, not one call in the 20- to 29-kHz range was detected.

The functional role of the high-frequency calls emitted by rats when they encounter the anesthetized animal is open to conjecture, although preliminary hypotheses may be drawn from earlier analyses of other circumstances that elicit ultrasound (21,31). They may be indicative of the subject's affective state. The presence of the conspecific, or perhaps its unusual behavior (or lack thereof), may be mildly aversive; and, aversive events are known to correlate with ultrasonic vocalizations (21). Alternatively, the calls may have the more specific function of inhibiting aggression under normal circumstances (28), although the state of the conspecific in this situation precludes an agonistic encounter. Finally, the calls may be species recognition cries that serve a social or reproductive purpose, a role that vocalizations of various kinds play in many diverse species (10).

Functional questions aside, this technique presents a simple, reliable method for eliciting high-range ultrasounds from rats, especially females. It avoids the problems associated with agonistic or reproductive dyads, in which it is usually difficult to determine which animal is emitting the calls unless one of the two is surgically devocalized. And, finally, if future research proves it to be in particular sensitive to 5-HT_{1A} compounds it may serve as a useful screening method for novel anxiolytics of this class.

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