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Inhibition of Ultrasonic Vocalizations by Beta-Adrenoceptor Agonists

ABSTRACT: *Infant rat ultrasonic vocalizations (USVs) are widely believed to result from the induction of an emotional state of anxiety or distress. This perspective, however, is not easily reconciled with the demonstration by W. J. Farrell and J. R. Alberts (2000) that norepinephrine, a nonselective beta-adrenoceptor agonist with anxiogenic properties, inhibits production of USVs. Here, Farrell and Alberts' finding was replicated and extended with 12-day-old rats using a conventional isolation paradigm. First, treatment with norepinephrine (1 mg/kg) significantly inhibited ultrasound production while also increasing body temperature. Next, treatment with the beta-2 agonist terbutaline (1 mg/kg) and the beta-3 agonist CL-316243 (1 mg/kg), but not the beta-1 agonist dobutamine (1 mg/kg), inhibited ultrasound production; only CL-316243 increased body temperature. The unexpected inhibition of USVs by terbutaline, a potent bronchodilator, was replicated using a slightly modified procedure; again, body temperature was unaffected by terbutaline administration. In no experiment was inhibition of USVs related to changes in motor activity. Altogether, these results suggest either that ultrasound production is not a valid indicator of anxiety or that anxiety in infant rats is produced by neuropharmacological mechanisms that differ fundamentally from those in adults.* © *2005 Wiley Periodical, Inc.* Dev Psychobiol 47: 66–76, 2005.

Keywords: *body temperature; cardiac rate; brown adipose tissue; bronchodilation; asthma; anxiety; infant; rat*

The ultrasonic vocalization (USV) of infant rats has been variously interpreted as a signal of the emotional state of anxiety (Shair, Brunelli, Masmela, Boone, & Hofer, 2003) and as an acoustic by-product of physiological mechanisms that promote cardiovascular and respiratory homeostasis (Blumberg & Alberts, 1990; Blumberg & Sokoloff, 2001). These two perspectives are not necessarily mutually exclusive. Nonetheless, two different paradigms have evolved that reflect the research questions of interest to different investigators. On one hand, the isolation paradigm has been favored by the majority of investigators who believe that pups vocalize as a result of the induction of a causative emotional state, and that this state is most robustly induced when pups are rapidly isolated from the home cage and littermates (Hofer & Shair, 1980; Kehoe & Blass, 1986; Miczek, Weerts, Vivian, & Barros, 1995; Winslow & Insel, 1991b). On the other hand, because the isolation paradigm makes recording of physiological variables more difficult, a second paradigm was developed in which pups can be instrumented, stabilized at thermoneutrality, and then exposed in a controlled fashion to subthermoneutral temperatures that reliably evoke USVs (Blumberg & Alberts, 1990; Blumberg, Sokoloff, & Kent, 1999; Blumberg & Stolba, 1996). Although the latter controlled-cooling paradigm has yielded useful information concerning physiological changes that accompany USVs, the ease and usefulness of the isolation paradigm for exploring how social and related factors modulate USVs contribute to its continued popularity (Shair et al., 2003).

Temperature has long been recognized as a primary factor in the stimulation of USVs (Allin & Banks, 1971;

Received 30 August 2004; Accepted 8 February 2005
Correspondence to: M. S. Blumberg
Contract grant sponsor: NIH
Contract grant numbers: HD38708, MH50701, MH66424
Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/dev.20070

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Okon, 1971), even when the isolation paradigm is used (Blumberg, Efimova, & Alberts, 1992a, 1992b; Kraebel, Brasser, Campbell, Spear, & Spear, 2002; Shair et al., 2003). Though it is often assumed that temperature merely acts as a signal to the pup that it is no longer in the nest (Hofer & Shair, 1993), the effects of cooling on cardiac rate, oxygen consumption, respiratory rate, arterial pressure, and blood viscosity suggest that USVs, produced by the forced expiration of air through a constricted larynx, are produced as part of a coordinated physiological response to homeostatic challenges (Blumberg, 2001). Although critics of this perspective correctly point out that at least some of the social factors that modulate USVs are not mediated through thermal mechanisms (Hofer, Brunelli, & Shair, 1993; Shair et al., 2003), the nature of the isolation paradigm has thus far limited the monitoring of other physiological parameters during these experiments.

Pharmacological manipulations provide a means of investigating the mechanisms underlying ultrasound production as well as afford the opportunity to test the efficacy of anxiolytics in infant rats (Gardner, 1985; Miczek et al., 1995; Winslow & Insel, 1991a, 1991b). With these goals in mind, the contributions of opioids (Carden, Barr, & Hofer, 1991; Moles, Kieffer, & D'Amato, 2004; Winslow & Insel, 1991a), benzodiazepines (Gardner & Budhram, 1987; Insel, Gelhard, & Miller, 1989), and other well-established or presumptive anxiolytic agents to ultrasound production have been examined. But what are we to make of the fact that clonidine, an anxiolytic in adults, dramatically increases USVs in infants (Blumberg et al., 2000b; Hård, Engel, & Lindh, 1988; Kehoe & Harris, 1989)? Similarly, how do we explain the recent finding of Farrell and Alberts (2000) that norepinephrine (NE), an anxiogenic in adults (Brunello et al., 2003; Gorman, Hirschfeld, & Ninan, 2002; Tanaka, Yoshida, Emoto, & Ishii, 2000), inhibits infant USVs? In our view, such counterintuitive findings demand greater attention if we are serious about testing the hypothesis that USVs signal anxiety in infant rats.

Farrell and Alberts (2000) attributed NE's ability to inhibit USVs to its stimulatory action on brown adipose tissue (BAT) thermogenesis and the resulting increase in body temperature; moreover, they interpreted their finding within the context of the by-product perspective. Proponents of the view that USVs are evoked by an underlying emotional state, however, might feel justified in dismissing their finding for one or more of the following reasons. First, it could be argued that enhancement of BAT thermogenesis by NE inhibited ultrasound production by decreasing the saliency of the isolation for the pups; according to this perspective, a warmer pup's isolation would be less salient than a cooler pup's, resulting in diminished anxiety and fewer USVs. Second, Farrell and Alberts used 7-day-old (P7) rats, and it could be argued that pups at P7 are more sensitive to thermal manipulations than older pups whose USVs are more easily modulated by social stimuli; for example, P12 seems to be an age of peak expression of the phenomenon known as maternal potentiation (Hofer, Masmela, Brunelli, & Shair, 1998). Finally, Farrell and Alberts isolated pups by placing them in a plastic container at thermoneutrality and then moving the container to a cool environment, a procedure that blunts the impact of the isolation and leads to a more gradual vocal response. In other words, their procedure resembled more the controlled-cooling procedure than the conventional isolation procedure, and it could be argued that the use of the favored isolation paradigm would yield different results.

Therefore, in the present series of experiments, we examine the effects of nonselective and selective beta-adrenoceptor agonists on ultrasound production and motor behavior in P12 rats. In these experiments, we follow closely the preferred method described by those who favor the isolation paradigm for investigating USVs (Hofer et al., 1998). In Experiment 1, we replicate the finding of Farrell and Alberts (2000) that NE inhibits USVs. In Experiment 2, we find that the beta-2 agonist terbutaline and the beta-3 agonist CL-316243, but not the beta-1 agonist dobutamine, inhibit ultrasound production. Finally, the surprising and novel USV-inhibiting effect of terbutaline, a bronchodilator that does not increase either body temperature or cardiac rate at the dose used here, is replicated in Experiment 3 using a longer delay between injection and isolation testing. Altogether, although these findings do not directly address the notion that USVs are acoustic by-products, they do raise further doubts regarding the conventional notion that these vocalizations are caused by a central state of anxiety.

METHODS

Experiment 1: Effect of a Nonselective Beta-Adrenoceptor Agonist on Ultrasound Production

Subjects. Twelve P12 Harlan Sprague-Dawley male ($n = 6$) and female ($n = 6$) rat pups from six litters were used. Body weights ranged from 24.0 to 36.5 g. All pups were born to females in the animal colony at the University of Iowa. The pups were raised in litters culled to 8 pups within 3 days after birth (day of birth = Day 0). Mothers and their litters were housed in opaque polypropylene cages ($48 \times 20 \times 26$ cm); food and water were available ad libitum. All animals were maintained on a 12:12 hr light:dark schedule, with lights on at 7:00 a.m.

Test Environment. All pups were tested inside a glass chamber (height = 17 cm; inner diameter = 12.5 cm). Air temperature within the chamber was unregulated; room temperature was

approximately 22°C. Pups were allowed to move freely inside the chamber on a platform constructed of polyethylene mesh. A microcamera was placed above the chamber for recording of behavior to videotape.

USVs. USVs were detected using a bat detector (Model SM100, QMC, Ltd., London, UK) tuned to 41 ± 5 kHz. The microphone was placed approximately 12 cm above the surface of the testing chamber. The output of the bat detector was connected to the audio input of a videotape recorder.

Physiological Temperatures. Immediately after each test, interscapular temperature (T_{is}) was measured using a digital thermometer (Omega, Stamford, CT). A chromel-constantan thermocouple (Omega Engineering, Stamford, CT) was held in place against the skin until a stable reading was obtained. Thermocouples were calibrated before the experiment using a mercury thermometer with an accuracy of 0.1°C.

Drugs. The nonselective beta-adrenoceptor agonist NE (arterenol hydrochloride; Sigma, St. Louis, MO) was dissolved in isotonic saline to a concentration of 1 mg/ml.

Procedure. On the day of testing, a dam with a litter of at least 7 pups was removed from the colony and placed in the testing room. The dam was removed from the nest, and the home cage containing the litter was placed on a heating pad maintained at 30°C. Two same-sex littermates were weighed and randomly assigned to an experimental group (In this and all subsequent experiments, latex gloves were always worn when handling or transferring pups.) Pups were then placed back into the nest. Ten min later, the pups were injected sc with saline or NE at a dose of 1 mg/kg in a volume of 1 µl/g body weight. All pups were then placed back into the nest with littermates during a 30-min postinjection period. (The drug dosage and delay between injection and testing are similar to those used by Farrell & Alberts, 2000.) Then, 1 pup was removed from the litter and placed in the testing chamber. Behavior and USVs were recorded for 6 min, after which the second pup was tested; order of testing followed the order of the injections and was counterbalanced across litters. After the test, T_{is} was measured and the pup was returned to its home cage. After the chamber was cleaned with alcohol, the next pup was tested.

Data Analysis. USV data were scored offline from videotape by an experienced observer. The observer pressed the key of an event recorder each time a USV was detected. Rearing behavior was scored by the same observer during a separate pass through the videotape. A single rearing event comprised the pup standing on its two hindlegs with its forepaws touching the side of the glass chamber; this event was terminated only when the pup had regained a four-legged stance on the floor of the chamber.

Differences in ultrasound production between the two groups were tested using a Wilcoxon matched-pairs signed ranks test. Rearing and temperature data were analyzed using paired *t* tests. For all tests, $\alpha = .05$. Unless otherwise indicated, all means are presented with their *SE*s.

Experiment 2: Effects of Selective Beta-Adrenoceptor Agonists on Ultrasound Production

The methods used in Experiment 2 were identical to those in Experiment 1 except for the changes indicated below.

Subjects. Forty-eight P12 Harlan Sprague-Dawley male ($n = 24$) and female ($n = 24$) rats from 12 litters were used. Body weights ranged from 24.4 to 35.1 g.

Drugs. The beta-1 adrenoceptor agonist dobutamine (hydrochloride; Sigma, St. Louis, MO), the beta-2 adrenoceptor agonist terbutaline (hemisulfate salt; Sigma, St. Louis, MO), and the beta-3 adrenoceptor agonist CL-316243 (disodium salt; Wyeth-Ayerst Research, Pearl River, NY) were dissolved in isotonic saline to a concentration of 1 mg/ml.

Procedure. After separation from the dam, 4 same-sex littermates were weighed and returned to the nest. Ten min later, pups were injected sc with saline or with one of the three beta-adrenoceptor agonists at a dose of 1 mg/kg in a volume of 1 µl/g body weight. Thirty min after the first injection, each pup was isolated in the test chamber for 6 min. Order of testing followed the order of the injections and was balanced across litters. At the end of each test, T_{is} was measured and the pup was returned to its home cage. After the chamber was cleaned with alcohol, the next pup was tested.

Data Analysis. The Wilcoxon matched-pairs signed ranks test was used to assess differences in ultrasound production between the saline group and each of the other three; to correct alpha for the use of multiple tests, the Bonferroni procedure was used. Analysis of variance (ANOVA) was used to test for group differences in rearing and T_{is}; the post hoc test was Fisher's protected least significant difference.

Experiment 3: Effect of the Selective Beta-2-Adrenoceptor Agonist Terbutaline on Ultrasound Production after a 60-Min Delay between Injection and Isolation Testing

The methods used in Experiment 3 were identical to those in Experiment 1 except for the changes indicated below.

Subjects. Twenty-four P12 Harlan Sprague-Dawley male ($n = 12$) and female ($n = 12$) rats from 12 litters were used. Body weights ranged from 25.3 to 35.4 g.

Test Environment. All pups were tested in an open-air, standard mouse cage ($25 \times 20 \times 14$ cm).

Drugs. The beta-2 adrenoceptor agonist terbutaline (hemisulfate salt; Sigma, St. Louis, MO) was dissolved in isotonic saline to a concentration of 1 mg/ml.

USVs. A different bat detector was used in this experiment (Anabat, Titley Electronics, Ballina, Australia). As in Experiment 1, the output of the bat detector was connected to the audio input of a videotape recorder.

Procedure. After separation from the dam, 2 same-sex littermates were weighed and returned to the nest. Ten min later, the pups were injected sc with saline or terbutaline at a dose of 1 mg/kg in a volume of 1 µl/g body weight. Both pups were again placed back into the nest with littermates for a 60-min postinjection period. Then, 1 pup was isolated at room temperature for at least 6 min (and no more than 10 min). At the end of each test, T_{is} was measured and the pup was returned to its home cage. After the cage was cleaned with alcohol, the second pup was tested. Order of testing was counterbalanced across litters.

Data Analysis. USV data were analyzed as in Experiment 1. In addition, because of the larger isolation cage used in this experiment, the pups' motor behavior consisted of shuttles from one end of the cage to the other. Therefore, as a measure of activity over the 6-min test, the number of times that a pup's entire body crossed over the center line of the cage was counted. A paired *t* test was used to test for differences in motor activity.

RESULTS

Experiment 1: Effect of a Nonselective Beta-Adrenoceptor Agonist on Ultrasound Production

The nonselective beta-adrenoceptor agonist NE inhibited isolation-induced ultrasound production throughout the 6-min test (Figure 1A). The box plots in Figure 1C indicate that the total number of USVs was significantly lower in the NE-treated group in relation to the control group, $z = 2.2$, $p < .05$. In contrast, as shown in Figure 1D, rearing behavior was not significantly different between the two groups, $t(5) = 1.2$, n.s.

At the end of the test, mean T_{is} was 37.9 ± 0.2°C and 36.0 ± 0.1°C in the NE- and saline-treated pups, respectively, $t(5) = 8.2$, $p < .0005$ (Figure 1B). Such an increase in temperature was expected because norepinephrine activates BAT thermogenesis in infant rats (Blumberg, 2001; Cannon, Jacobsson, Rehnmark, & Nedergaard, 1996; Nedergaard, Connolly, & Cannon, 1986).

Experiment 2: Effects of Selective Beta-Adrenoceptor Agonists on Ultrasound Production

The effects of three selective beta agonists on isolation-induced ultrasound production are shown in Figure 2A. Dobutamine, a beta-1 agonist, did not significantly inhibit isolation-induced ultrasound production in relation to controls, $z = 0.2$, n.s., despite the fact that 10 of the 12 dobutamine-treated pups vocalized less than their saline-

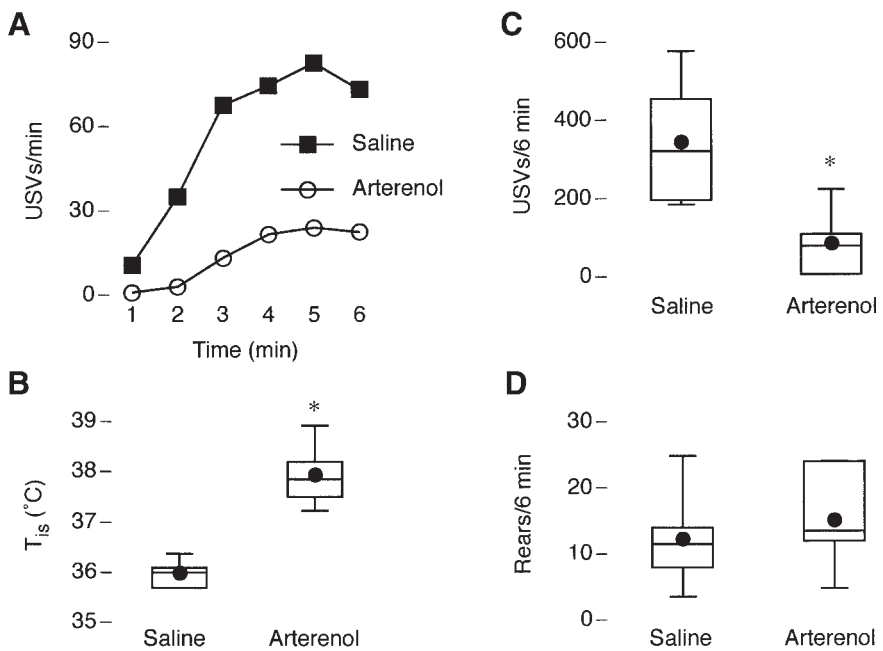

FIGURE 1 (A) Number of USVs emitted each min during the 6-min isolation test for the P12 rats in Experiment 1. Pups were injected 30 to 40 min before the test either with saline (filled squares) or the nonselective beta-adrenoceptor agonist NE (open circles; 1 mg/kg). For clarity, error bars are not shown. Box plots depict interscapular temperature (T_{is}) at the end of the test (B), as well as the total number of USVs emitted (C) and the number of rears exhibited (D) during the 6-min isolation tests. The top, middle, and bottom horizontal lines of the box represent the 75th, 50th (median), and 25th percentiles, respectively. The thin vertical lines above and below the box represent the 90th and 10th percentiles, respectively. Filled circles are means. *Significant difference from saline. $n = 6$ pups per group.

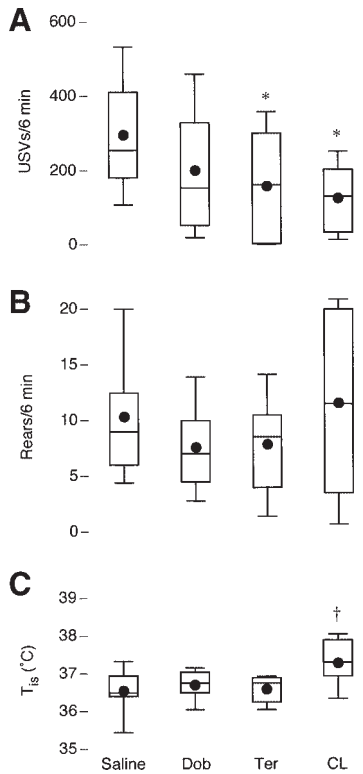

FIGURE 2 Box plots depicting the total number of USVs emitted (A) and the rears exhibited (B) during the 6-min isolation test for the P12 rats in Experiment 2. Interscapular temperature (T_{is}) at the end of the test also is depicted (C). Pups were injected 30 to 50 min before the test with saline or with one of three beta-adrenoceptor agonists: the beta-1 agonist dobutamine (Dob), the beta-2 agonist terbutaline (Ter), or the beta-3 agonist CL-316243 (CL). All agonists were injected at a dose of 1 mg/kg. *Significant difference from saline. †Significant difference from all other groups. $n = 12$ pups per group.

treated littermates. In contrast, the beta-2 and beta-3 agonists terbutaline and CL-316243, respectively, did significantly inhibit USVs, $z > 2.7$, $p < .008$. Finally, as in Experiment 1, none of the agonists significantly affected rearing behavior in relation to controls, $F(3, 44) = 1.2$, n.s.

Mean T_{is} was 36.6 ± 0.2°C in the saline-treated group, 36.7 ± 0.1°C in the dobutamine-treated group, 36.6 ± 0.1°C in the terbutaline-treated group, and 37.3 ± 0.2°C in the CL-316243-treated group (Figure 2C). ANOVA revealed a significant main effect of experimental group on T_{is}, $F(3, 44) = 4.3$, $p < .01$, and post hoc tests indicated that the pups treated with CL-316243 differed from each of the other three groups. Nonetheless, the effect of CL-316243 on T_{is} was only one third as large as that caused by NE in Experiment 1.

Experiment 3: Effect of the Selective Beta-2-Adrenoceptor Agonist Terbutaline on Ultrasound Production after a 60-Min Delay between Injection and Isolation Testing

In a pilot study using urethanized P12 rats, terbutaline administration at a dose of 1 mg/kg did not increase body temperature; however, it did produce a small and brief (i.e., <30 min) reflex tachycardia that appeared to result from transient vasodilation (Blumberg, Johnson, & Middlemis-Brown, 2004, unpublished data). Therefore, the effect of terbutaline on USVs was reexamined using a 60-min delay between injection and isolation. Replicating terbutaline's inhibitory effects on ultrasound production after this longer delay would strengthen the conclusion that terbutaline's actions are unrelated to acute changes in cardiovascular function.

The box plots in Figure 3A indicate that the total number of USVs was significantly lower in the terbutaline-treated group in relation to the control group, $z = 2.6$, $p = .01$. Ten of the 12 terbutaline-treated pups vocalized less over the 6-min test than their saline-treated littermates. The box plots in Figure 3B indicate that

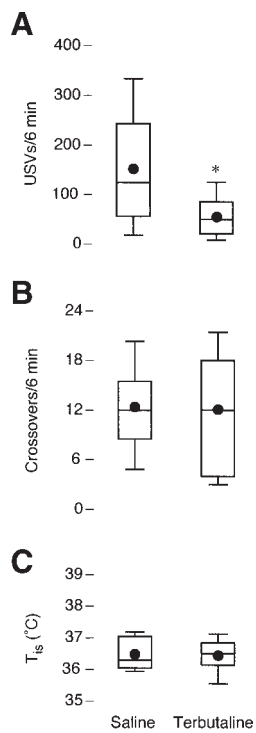

FIGURE 3 Box plots depicting the total number of USVs emitted (A) and crossovers performed (B) during the 6-min isolation test for the P12 rats in Experiment 3. Interscapular temperature (T_{is}) at the end of the test also is depicted (C). Pups were injected 60 to 70 min before the test either with saline or the beta-2 agonist terbutaline (1 mg/kg). *Significant difference from saline. $n = 12$ pups per group.

locomotor behavior, as measured by the number of crossovers from one side of the cage to the other, did not differ between the two groups, $t(11) = 0.1$, n.s.

At the end of the test, mean T_{is} was $36.4 \pm 0.2°C$ and $36.5 \pm 0.2°C$ in the terbutaline- and saline-treated pups, respectively, $t(11) = 0.2$, n.s. (Figure 3C).

DISCUSSION

The present series of experiments replicates and extends the seemingly paradoxical finding of Farrell and Alberts (2000) that NE, a nonselective beta-adrenoceptor agonist with anxiogenic properties, decreases ultrasound production. Specifically, using P12 rats and a standard isolation paradigm, Experiment 1 demonstrated again the USV-inhibiting effects of NE. Experiment 2 demonstrated that at the dose used here, selective beta-2 and beta-3 agonists (i.e., terbutaline and CL-316243, respectively) also inhibit ultrasound production whereas a beta-1 agonist (i.e., dobutamine) does not. Experiment 3 replicated the USV-inhibiting effects of terbutaline using a longer delay between injection and isolation testing.

None of the drugs used in these experiments affected motor activity in relation to control subjects (as measured by rearing in Experiments 1 and 2 and crossovers in Experiment 3), thus indicating that these drugs do not inhibit ultrasound production by producing general debilitation. Moreover, the fact that pups treated with beta-agonists were as motorically active as controls, even as they vocalized less, does not support the notion that these pups vocalized less because they somehow failed to perceive that they had been isolated from their littermates.

Thermal and Cardiovascular Considerations

The finding that both NE and CL-316243 inhibit ultrasound production and increase body temperature is consistent with the well-established finding that temperature is a critical variable in the evocation and modulation of the vocal response of isolated infant rats (Allin & Banks, 1971; Blumberg et al., 1992a, 1992b; Blumberg et al., 1999; Farrell & Alberts, 2000; Okon, 1971). The prevalence of beta-3 receptors in BAT also is well established, as is the primary role of this receptor in the thermogenic response of BAT (Bronnikov et al., 1999; Cannon et al., 1996). Thus, selective beta-3 agonists such as CL-316243 (Bloom et al., 1992) stimulate BAT thermogenesis, thereby increasing interscapular temperature.

Interactions between thermal and cardiovascular factors also appear to play a role in USV modulation (Blumberg, Kreber, Sokoloff, & Kent, 2000a; Blumberg et al., 1999). Beta-1 receptors are prominent in cardiac smooth muscle, and beta-1 agonists such as dobutamine increase cardiac rate (Hoffman & Lefkowitz, 1996). From this perspective, it may seem surprising that dobutamine did not inhibit USVs in Experiment 2. Of course, it is possible that testing only a single dose of dobutamine was insufficient to reveal a modulatory effect on USVs. It also is possible that the enhanced suppression of USVs by NE is attributable in part to its combined stimulation of thermogenesis and cardiac rate via its effects on beta-1 and beta-3 receptors; moreover, any drug that increases an infant's body temperature, such as NE and CL-316243, also will increase cardiac rate due to the direct metabolic effects of temperature on heart muscle (Sokoloff, Kirby, & Blumberg, 1998). In contrast, although dobutamine—once considered a selective beta-1 agonist—has been shown to activate beta-3 receptors in BAT (Bronnikov et al., 1999; Hoffman & Lefkowitz, 1996), it did not increase body temperature in Experiment 2; thus, its effect was likely restricted to increasing cardiac rate. Determining the relative importance of these factors for the modulation of USVs may be achieved through simultaneous evaluation of these drugs' effects on body temperature, cardiac rate, and isolation-induced USVs at different doses.

Through What Mechanism Does Terbutaline Inhibit Ultrasound Production?

The suppression of USVs by terbutaline was surprising because beta-2 adrenoceptors have never been implicated in the modulation of USVs. Moreover, unlike NE and CL-316243, terbutaline did not stimulate BAT thermogenesis, consistent with the lack of beta-2 receptors in BAT (Cannon et al., 1996). Instead, beta-2 receptors are associated most prominently with bronchial, vascular, gastrointestinal, and genitourinary smooth muscle as well as skeletal muscle (Hoffman & Lefkowitz, 1996). The predominant action of beta-2 stimulation on smooth muscle is dilatation, thus accounting for the widespread clinical use of agonists such as terbutaline as a bronchodilator in the treatment of chronic obstructive lung disease and asthma (Hoffman & Lefkowitz, 1996; Nelson, 1995; Papiris, Kotanidou, Malagari, & Roussos, 2002; Serafin, 1996).

In human adults, the side effects of terbutaline are numerous and include muscle tremor (due to stimulation of beta-2 receptors in skeletal muscle), tachycardia, vasodilation, and anxiety (Nelson, 1995). Tachycardia can result from direct stimulation of the relatively small population of beta-2 receptors on heart muscle or from reflexive tachycardia in response to vasodilation. In addition, on the venous side of the circulation, beta-2 stimulation can cause either dilation or constriction depending on the particular vascular bed being stimulated (Cyong, Tanaka, & Horiguchi, 1982; Lee, Raya, Gay,

Olajos, & Goldman, 1987; Leenen & Reeves, 1987; Wang & Lung, 2003). Beta-2 stimulation with terbutaline also has been associated with increased venous return (Cyong et al., 1982; Lee et al., 1987; Leenen & Reeves, 1987). As mentioned earlier, although injection of urethanized P12 rats with terbutaline does appear to produce vasodilation and reflex tachycardia, those effects were relatively brief and likely did not influence the USV-inhibiting effect of terbutaline observed in Experiment 2. Such an influence seems even less likely in light of the findings of Experiment 3, in which terbutaline was injected at least 60 min before isolation testing.

It is possible that terbutaline's suppressive effects on USVs resulted from its actions as a bronchodilator, which would imply that isolation and cold exposure trigger bronchoconstriction and thereby evoke USVs. Several pieces of evidence make this implication plausible. First, cooling is one of the primary triggers of bronchoconstriction in animals as well as in asthmatic and nonasthmatic humans (Jammes, Barthelemy, & Delpierre, 1983; Koskela & Tukiainen, 1995; Yuan & Nail, 1995; Zeitoun et al., 2004). Cool, dry air triggers bronchoconstriction by stimulating receptors in the upper airway, including the larynx (Jammes et al., 1983; Jammes, Barthelemy, Fornaris, & Grimaud, 1986; Sant'Ambrogio, Matthew, Sant'Ambrogio, & Fisher, 1985); facial cooling also contributes to bronchoconstriction (Koskela & Tukiainen, 1995; Zeitoun et al., 2004).

Second, a prominent response in humans with asthma and chronic obstructive lung disease is pursed-lip breathing, in which air is forcibly expelled against the resistance produced by the pursed lips (Barach, 1973). This maneuver resembles laryngeal braking, a respiratory maneuver that has been hypothesized to underlie the production of USVs (Blumberg & Alberts, 1990). In laryngeal braking, the resistance to expiration is provided by adducted laryngeal folds rather than pursed lips and has the same effect of producing back pressure within the lungs, thereby preventing alveolar collapse and maintaining functional residual capacity (FRC; i.e., the volume of air remaining in the lungs at the end of expiration) (Higenbottam, 1980; Higenbottam & Payne, 1982).

The possibility that terbulatine acted centrally to inhibit USVs cannot be excluded at this time. Interestingly, one study showed that terbutaline inhibits the laryngeal chemoreflex and the trigeminal diving reflex in newborn lambs (Grogaard & Sundell, 1983). Moreover, inhibition of these reflexes—which entail apnea and bradycardia—occurred more rapidly after intrathecal injection than after intravenous infusion. Thus, terbutaline has known central and peripheral effects on respiratory function, either of which might be related to its ability to inhibit USVs in infant rats.

Are USVs Acoustic By-Products of a Cardiopulmonary Maneuver?

Infant USVs are produced by the forced expulsion of air through a constricted larynx, not the vibration of the vocal cords as is typical of most mammalian vocalizations (Roberts, 1975). The functional role of the larynx, however, goes far beyond the production of sound (Negus, 1929). In infants, adduction of the laryngeal folds is used often to completely (i.e., apnea) or partially (i.e., laryngeal braking) impede expiratory flow (Dorion & Praud, 2003; Mortola, 1987). Because air flow does not cease during laryngeal braking, it is often associated with an audible grunt, especially in infants (Barach, 1973; Davis & Bureau, 1987; Diaz, Kianicka, Letourneau, & Praud, 1996; Harrison, de V. Hesse, & Klein, 1968; Johnson, Harding, McClelland, & Whyte, 1977; Mortola, 1985, 1987).

Several functions have been attributed to laryngeal braking in infants, including lung expansion and clearing of lung fluid after birth, maintenance of FRC, and preventing alveolar collapse in infants deficient in lung surfactant (as in Respiratory Distress Syndrome) (Harrison et al., 1968; Mortola, 1985; Mortola, Magnante, & Saetta, 1985). In lambs, laryngeal braking is closely associated with temperature-induced changes in respiratory drive and is thought to play a key role in the regulation of respiratory rhythmogenesis (Andrews, Symonds, & Johnson, 1991a, 1991b). Thus, if USVs are analogous to the grunts produced during laryngeal braking, then it is likely that many manipulations that compromise respiratory function trigger USVs, including those that result in the modulation of respiratory drive or that challenge maintenance of FRC. Consistent with this notion is the finding that pups recovering from deep hypothermia emit USVs even while ''unconscious,'' a phenomenon that was attributed to pulmonary edema (Hofer & Shair, 1992). Interestingly, in lambs, pulmonary edema triggers laryngeal braking, most likely to help maintain FRC (Diaz et al., 1996).

It also has been hypothesized that USVs are acoustic by-products of the abdominal compression reaction (ACR) (Kirby & Blumberg, 1998). The ACR entails abdominal contractions during or at the end of expiration to propel blood back to the heart when venous return has been compromised (Gilfoil, Youmans, & Turner, 1959; Youmans et al., 1963). The likelihood that venous return is compromised during extreme cooling in infant rats served as a basis for the hypothesis that the ACR is activated to improve venous return, producing USVs as a by-product (Blumberg & Sokoloff, 2001; Kirby & Blumberg, 1998).

The two maneuvers—laryngeal braking and the ACR—would be incompatible if the increased intrathoracic pressure produced during laryngeal braking retards venous return, such as occurs during a Valsalva maneuver

(Porth, Bamrah, Tristani, & Smith, 1984); however, note that intraabdominal pressure also is a key determinant of venous return (Kitano et al., 1999; Takata, Wise, & Robotham, 1990). Thus, it is possible that the increase in intraabdominal pressure that accompanies USVs produces a sufficient pressure gradient to increase venous return, as was recently found during USVs in adult rats (Blumberg & Bjelica, 2002). If, in infant rats, venous return is increased during laryngeal braking (or, alternatively, if pulmonary function is enhanced during the abdominal compression reaction), then it may be that the maneuver that underlies USVs is a cardiopulmonary maneuver that contributes to the maintenance of FRC and airway patency while also helping to match ventilation and perfusion of the lungs. The physiological event that initiates USVs, however, remains unclear: One recent study indicated that decreased venous return is not sufficient to evoke USVs (Shair & Jasper, 2003), although this issue is not yet fully resolved.

CONCLUSIONS

Regardless of the viability of any by-product interpretation of USVs, the present results and those of Farrell and Alberts (2000) are not easily explained within the context of the widely preferred anxiety hypothesis. The fact that beta-adrenoceptor agonists suppress USVs suggests that the vocalizing rat pup is not a valid model of anxiety as conventionally understood in adults. Alternatively, if it could be shown using independent measures that beta agonists do indeed have anxiolytic properties in infants, then anxiety in pups must be governed by distinct neuropharmacological mechanisms as compared with adults. In other words, the seeming paradox of beta agonists exhibiting anxiolytic properties can be reconciled either by seeking alternative explanations regarding the underlying causes of USVs or by positing anxiolytic properties for NE in infants. [As suggested earlier, an analogous argument pertains to the ability of clonidine, an alpha-2-agonist with anxiolytic properties in adults, to evoke USV responses in infants (Blumberg et al., 2000b; Hård et al., 1988; Kehoe & Harris, 1989).] If the latter option has any merit, then the hope of using the isolated infant rat as a model for testing the efficacy of novel anxiolytics would be significantly diminished (Winslow & Insel, 1991b).

Note that the previous arguments do not exhaust the basis for doubt regarding the anxiety hypothesis. Indeed, when the standards of necessity and sufficiency are applied to the anxiety hypothesis, as they have been applied to by-product hypotheses (Hofer & Shair, 1991a; Shair & Jasper, 2003), it appears that anxiety is neither necessary nor sufficient for ultrasound production. Specifically, to the extent that anxiety is a state expressed only in awake, conscious animals, the finding that pups emit USVs while in a state of deep hypothermia indicates that anxiety is not necessary for emission of the vocalization (Hofer & Shair, 1992). Several lines of evidence also indicate that anxiety is not sufficient for emission of the vocalization: (a) Pinching the tail of a pup maintained at thermoneutrality evokes a powerful motor response, including the emission of audible voiced vocalizations, but does not evoke USVs; and (b) neither prolonged starvation nor hypoxia evokes USVs (Blumberg & Alberts, 1991; Hofer & Shair, 1991a).

It must be emphasized that the mechanisms that evoke USVs need not be identical to the mechanisms by which nest odors and related stimuli suppress USVs. In support of this notion, combined olfactory and trigeminal nerve cuts do not prevent normal rates of emission of USVs during isolation, but do prevent their suppression by nest odors (Hofer & Shair, 1991b). Therefore, to fully understand the relationship between these two aspects of the vocal response of infant rats, we must learn more about the physiological changes that occur during isolation and in response to ''social'' stimuli. It may be, as discussed by Farrell and Alberts (2000), that ''integrated responses of the autonomic nervous system may become associated with olfactory perception in ways that might modulate the emission of ultrasonic vocalization by pups'' (p. 812). Important information for addressing this possibility includes changes in respiratory drive and airway patency during isolation and the possible conditioned modulation of these variables by olfactory and other nest-related stimuli.

At present, it seems reasonable to conclude that USVs cannot yet—and may never—be fully explained by any single mechanism or captured by any single theoretical construct. Moreover, it is important to remember that communicatory signals, emotional states, and acoustic by-products are not mutually exclusive categories. Thus, at present, we agree with the statement of Kraebel et al. (2002) that ''the acceptance of the 'emotional state' viewpoint to the complete exclusion of the equally viable 'acoustic by-product' interpretation seems premature and risky'' (p. 158).

NOTES

This research was supported by National Institutes of Health Grants HD38708, MH50701, and MH66424 to M.S.B. We thank Ralph Fregosi and Jean-Paul Praud for many helpful suggestions.

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