

# Ultrasonic Vocalizations by Rat Pups After Adrenergic Manipulations of Brown Fat Metabolism

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Rat pups emit ultrasonic vocalizations (USVs) during cold challenge. R. F. Kirby and M. S. Blumberg (1998) suggested that when brown adipose tissue (BAT) thermogenesis fails to compensate for body heat loss and heart rate declines, infant pups maintain venous return to the heart with a mechanical maneuver that is accompanied by ultrasonic emissions. Thus, manipulations that attenuate or enhance BAT thermogenesis should have inverse effects on cold-induced USVs. The authors found that hexamethonium (10 mg/kg) and propranolol (1 and 20 mg/kg) attenuated BAT metabolism while enhancing USV production, and norepinephrine (NE, 800  $\mu$ g/kg) enhanced BAT metabolism while ultrasonic emissions decreased. The findings are consistent with the hypothesis that BAT metabolism influences USVs during cold challenge by affecting cardiac rate and inducing compensatory, homeostatic responses.

Mother–pup interactions in the Norway rat are composed of a set of stereotyped, species-typical activities, one form of which is the mother’s retrieval of pups that have strayed from the nest. The mother rat’s retrieval of a lost pup appears to be facilitated, at least in part, by high-frequency (40–50 kHz) ultrasonic vocalizations (USVs) emitted by the infant. Whereas a pup’s USVs are inaudible to humans, these sounds can be detected by adult rats, which have peak cochlear and inferior collicular sensitivities corresponding to the frequency range of these vocalizations (Brown, 1973; Crowley, Hepp-Reymond, Tabowitz, & Palin, 1965). Furthermore, it has been demonstrated that USVs can elicit and direct maternal search behavior (Allin & Banks, 1972), particularly when combined with pup-appropriate olfactory stimuli (Smotherman, Bell, Starzec, Elias, & Zachman, 1974).

Over the last three decades, USVs have been analyzed and used in diverse research, including behavioral, pharmacological, and physiological studies. Across much of this research is reflected an attitude that USVs are “distress calls” signaling a state, or even levels, of isolation anxiety. It has also long been recognized that cold is a reliable stimulus for USVs (Allin & Banks, 1971). Because an isolated infant pup is almost invariably cold or cooling, the pup’s thermal condition is embedded in the general state of isolation distress. Emphasis has remained on the presumed state of anxiety or distress, however, and not on thermal variables. Indeed, USVs have been incorporated into pharmacological screens for anxiolytic compounds (e.g., Insel & Winslow, 1991) and as a behavioral marker in studies examining how maternal and home

nest cues regulate different kinds of arousal and quiescence in the young, developing infant (e.g., Hofer & Shair, 1987).

More recently, however, there have been efforts to reframe the analysis of USV production. The common theme of these alternative hypotheses is that USVs are an aspect, specifically a *coincidental* aspect or *by-product*, of other primary responses to cold or cooling. These are views in which USVs are seen as noises made when an infant rodent recruits physiological mechanisms to defend homeostasis in the face of thermal challenge. The putative “noisy” mechanisms include those for maintaining arterial oxygenation during nonshivering thermogenesis (Blumberg & Alberts, 1990), as well as strategic maneuvers that protect blood pressure and venous return to the heart by varying blood flow in the face of cold-induced decreases in heart rate (Kirby & Blumberg, 1998).

Often in the studies that invoke a regulatory framework, there are hypothesized links between cold-induced changes in infant physiology and the emission of USVs (see Blumberg & Alberts, 1990; Blumberg, Efimova, & Alberts, 1992; Blumberg & Sokoloff, 1998). Cold-induced activation of brown adipose tissue (BAT) metabolism is a salient theme. Nonshivering thermogenesis by way of BAT metabolism is the rat pups’ sole source of heat production (Hull, 1973). BAT metabolism is activated by the sympathetic nervous system and mediated by  $\beta$ -adrenergic receptors (see Girardier & Seydoux, 1986). BAT is strategically located so that its thermogenesis can warm both the cervical spinal cord and organs within the thoracic cavity, including the heart.

Observations such as pups remaining silent when isolated in a thermoneutral environment ( $\approx 35^\circ\text{C}$ ) and then showing concurrent increases in USV production and BAT metabolism when ambient temperature is reduced (to  $20\text{--}23^\circ\text{C}$ ) led Blumberg and Alberts (1990) to propose that USVs are the by-product of respiratory adjustments that provide the oxygen required to support BAT thermogenesis. Recent research retains the thermoregulatory aspect of this hypothesis but incorporates cardiovascular homeostasis into a new, refined view of USV production.

In a series of studies, Blumberg and associates examined thermogenesis, heart rate (Blumberg, Sokoloff, & Kirby, 1997; Sokoloff, Kirby, & Blumberg, 1998), blood pressure (Kirby & Blumberg, 1998), and USV production (Blumberg & Stolba, 1996;

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Sokoloff & Blumberg, 1997) during exposure to a variety of different subthermoneutral temperatures. They found that a pup's response to cold is highly dependent on the severity of the thermoregulatory challenge. When a week-old rat pup is exposed to a moderate cold challenge ( $\approx 34-25$  °C), which initiates a submaximal BAT response, the pup remains silent and its heart rate remains constant. If, however, a pup is exposed to an "extreme" cold challenge ( $< 25$  °C), exceeding that required to elicit maximal BAT thermogenesis, heart rate falls and USV production is initiated. Surprisingly, blood pressure remains stable during exposure to extreme cold despite the decline in heart rate (Kirby & Blumberg, 1998).

It now appears clear that USVs are not directly and inextricably linked to BAT metabolism. The observation that USVs are produced only during cold challenges sufficient to cause decreased heart rate, combined with the finding that pups maintain stable blood pressure when faced with extreme cold, led to the proposition that USVs are the by-product of a mechanical maneuver, the abdominal compression response (ACR), which helps maintain arterial pressure and venous return to the heart during cold-induced reductions in cardiac rate (Kirby & Blumberg, 1998). The ACR involves the rapid contraction of abdominal muscles accompanied by an increase in intra-abdominal pressure. The hypothesized link between the ACR and USVs is supported by the finding that USVs are accompanied by transient increases in intra-abdominal pressure and elevations in mean arterial pressure (Kirby & Blumberg, 1998).

BAT metabolism may be capable of modulating USV production through its thermal effects on heart rate. When the ganglionic blocker chlorisondamine is used to eliminate both BAT metabolism and neural input to the heart, and ambient temperature is progressively reduced, pups exhibit simultaneous declines in both interscapular temperature ( $T_{is}$ ) and heart rate, even at moderate temperatures that normally fail to elicit bradycardia (Blumberg et al., 1997), thus suggesting that heart rate is normally protected by the heat derived from BAT thermogenesis. Furthermore, if BAT metabolism is pharmacologically enhanced at thermoneutral temperatures, pups exhibit increases in both heart rate and  $T_{is}$ , an effect that can be reversed by cooling the interscapular region with a cutaneous thermode (Sokoloff et al., 1998).

If USVs are a by-product of physiological adjustments that help maintain cardiovascular homeostasis during periods of cold-induced bradycardia, and  $T_{is}$  is directly related to heart rate, then BAT metabolism (and consequently  $T_{is}$ ) should be inversely related to USV production during cold exposure. To investigate this possibility, we examined the emission of cold-induced USVs following pharmacological manipulations designed to uncouple BAT metabolism from ambient temperature. We hypothesized that pharmacological manipulations that decrease BAT metabolism (hexamethonium and propranolol administration) would enhance the production of USVs, whereas manipulations that increase BAT metabolism (norepinephrine administration) would attenuate USV production during cold exposure.

#### Experiment 1: Ultrasound Emission by Hexamethonium-Treated 7-Day-Old Rat Pups

An initial test of the hypothesis that  $T_{is}$  is inversely related to USV production sought to determine whether blocking the initiation of BAT metabolism during cold exposure would increase

USV production. In Experiment 1, pups received either systemic injections of the ganglionic blocker hexamethonium, a compound understood to inhibit BAT metabolism (Hofer & Shair, 1991; Hsieh, Carlson, & Gray, 1957), or saline vehicle. Hofer and Shair (1991) previously examined USV production following the administration of hexamethonium (20 mg/kg) and found a trend toward increased vocalizing ( $p < .07$ ). They used only a single dose, however, so in Experiment 1 we expand on their work by examining the effect of both lower and higher doses. We hypothesized that reductions in BAT metabolism caused by hexamethonium would be accompanied by increased vocalizing.

#### Method

**Subjects.** The subjects in Experiment 1 were 22 male and 22 female 7-day-old Sprague-Dawley rat pups. Pups were bred in the rat colony at the Indiana University Animal Behavior Laboratory, from stock originally purchased from Taconic Laboratories (Germantown, NY). Litters were born and reared in standard, plastic maternity cages (45 × 25 × 20 cm) on hardwood chip bedding. Litters were culled to a total of 8 pups (4 male, 4 female) at 3 days of age (day of birth = Day 0). Animals were maintained on a 12-hr light-dark cycle, with lights on at 0800 hr; the temperature of the animal colony was maintained at  $22$  °C  $\pm$  2 °C. Food and water were available ad libitum. All pups used in this experiment had milk bands at the beginning of the experimental session, signifying recent ingestion of milk.

**Apparatus.** Testing was conducted in a  $35$  °C  $\pm$  1 °C environment, maintained in a custom-built incubator (52 × 45 × 34 cm interior dimensions). The walls and ceiling of the incubator were constructed of Plexiglas (0.64 cm thick), and the floor was aluminum. The air inside the incubator was heated by resistive heat coils and circulated by a small fan, both of which were located beneath a vent in the aluminum floor. There were two holes (12.8 cm diameter) cut in the walls of the incubator to facilitate placement of animals and movement of equipment. These holes were covered with flexible plastic sheeting when not in use, so that a stable ambient temperature could be maintained. A third hole (10.2 cm diameter) was located in the roof of the incubator to allow placement of an ultrasound microphone and a thermal scanner. All of the experimental equipment inside of the incubator was placed on a ventilated Plexiglas floor (44.5 × 38 cm), which was elevated 4.5 cm above the aluminum floor of the incubator.

A second, cool (25 °C) environmental chamber was located within the confines of the larger, warm (35 °C) incubator. This second chamber was a double-walled glass cylinder (8.5 cm i.d., 10.5 cm o.d., 18 cm high). The ambient temperature inside the cylinder could be precisely controlled by altering the temperature of the water circulating between the inner and outer walls.

Air temperature inside the incubator was measured and controlled using a thermistor (Model 405, Yellow Springs Instruments, Yellow Springs, OH) connected to a temperature controller (YSI Model 73A). Air temperature inside the cool environmental chamber was monitored using a Type K thermocouple and thermocouple reader (Omega Engineering, Model HH23, Stamford, CT).

Skin temperature was measured ( $\pm 0.1$  °C resolution) with an infrared thermography system (Thermovision 870, Agema Infrared Systems, Danderyd, Sweden). This system consisted of an infrared scanner, an IBM-compatible 386 computer (Compaq), and accompanying computer interfaces and software. In brief, this system digitizes infrared radiation emitted from the surface of the unrestrained pup and presents this information on the computer monitor as a pseudocolor image. Different colors represent different skin temperature ranges. The lens of the thermal scanner was positioned 38 cm above the subject, using the hole in the roof of the incubator.

USVs were detected using a microphone with a Mylar diaphragm positioned 26 cm above the dorsal surface of the subject. The microphone's

output passed through an ultrasound detector (Ultrasound Advice, U.K., Model S-25) that converted the signal to audible sounds.

**Procedure.** On the day of testing, a home cage containing a mother rat and her litter was removed from the colony and placed in the testing room. Just prior to the beginning of a session, a pup was removed from the litter, weighed, and voided of urine. Subjects were injected with hexamethonium HCl (3, 10, 30 mg/kg sc; Sigma Chemical Co., St. Louis, MO) or saline vehicle. All injections were administered subcutaneously at a volume of 4 ml/kg under the loose skin around the thigh, to avoid direct injection into the interscapular BAT pad. Each treatment group contained 12 pups from different litters except for the group receiving 30 mg/kg of hexamethonium, which contained 8 pups. Each group was composed of an equal number of male and female pups.

Immediately following injection, rat pups were transferred to a small plastic mesh cup (7 cm diameter) and placed in the warm (35 °C) incubator where they remained for the next 30 min. The 35 °C ambient temperature and the 30-min time period were chosen because they were sufficient to cause a cessation of BAT metabolism and USVs. In addition, the 30-min period in the warm environment allowed the hexamethonium to take effect prior to cold exposure.

Following 30 min in the warm (35 °C) incubator, the rat pups were transferred to the cool (25 °C) environmental chamber. Heat loss was minimized during the transfer procedure by moving the pup in the same plastic cup used in the warm environment, thereby avoiding contact between the experimenter and pup that might cause conductive heat loss. The pup remained in the cool environment for 45 min, during which time BAT metabolism and USVs were monitored.

**Quantification of BAT metabolism.** To estimate BAT metabolism, we programmed the thermal scanner to capture a "snapshot" of the pup at the beginning of each minute of the test. BAT metabolism was estimated by measuring the average skin temperature in a 0.25 cm<sup>2</sup> area over the pup's interscapular region ( $T_{is}$ ), an area which contains BAT, and an equivalent area over the sacral region of the pup's back ( $T_b$ ), an area which lacks this tissue. The difference between these two measures ( $T_{is} - T_b$ ) was used as an estimate of BAT thermogenesis. We collected and report absolute  $T_{is}$  values.  $T_{is} - T_b$  was chosen as an estimator of BAT metabolism, however, because this measure provides a solution to the problem of overall body cooling caused by exposure to cool ambient temperatures and allows us to evaluate relative changes in local temperatures. Numerous studies examining BAT thermogenesis in infant rats have demonstrated that  $T_{is} - T_b$  values correlate well with increased oxygen consumption during BAT thermogenesis (Blumberg & Alberts, 1990; Blumberg & Stolba, 1996; Kirby & Blumberg, 1998).

**Quantification of USV production.** Percentage of time spent vocalizing was estimated by recording the presence or absence of USVs during 2-s bins on a check sheet. Vocalizations were monitored for the first 6 min of cold exposure and for the first 30 s of each minute thereafter.

**Statistical analysis.** BAT metabolism and USV data are presented as mean ( $\pm$  SEM) values for 9-min bins. Missing values in the raw BAT metabolism data were interpolated by calculating the mean value of the two adjacent data points. If this was impossible, such as when the first or last data point in a session was missing, the group mean for that time point was used. Missing values were infrequent and were typically the result of pup movement or the dorsal surface of the pup being obscured from view when the thermal "snapshot" was taken. Planned comparisons between mean values obtained for each drug dose and saline were conducted using two-factor (Treatment  $\times$  Time) analyses of variance (ANOVAs). Treatment was designated as a between-subjects variable, and time was analyzed as a within-subjects variable. Probability values obtained from ANOVAs were multiplied by 3, yielding a probability value that was corrected for the three sets of planned comparisons. Select post hoc comparisons between means were conducted using unpaired *t* tests, and the obtained probability values were multiplied by 2 to correct for two sets of comparisons. Differences were considered significant if the corrected *p* was less than .05.

### Results and Discussion

Hexamethonium dose dependently attenuated BAT metabolism as estimated by the difference between  $T_{is}$  and  $T_b$ . ANOVAs revealed significant main effects of treatment and time for all doses of hexamethonium, as well as significant Treatment  $\times$  Time interactions following the administration of 10 and 30 mg/kg of hexamethonium. See Table 1 for specific ANOVA results.

Differences between treatment groups emerged over time (see Figure 1A). During the first 9 min of testing, saline-treated subjects had mean  $T_{is} - T_b$  values of  $0.36 \text{ }^\circ\text{C} \pm 0.05 \text{ }^\circ\text{C}$ , whereas subjects receiving the highest dose of hexamethonium (30 mg/kg) had  $T_{is} - T_b$  values of  $0.19 \text{ }^\circ\text{C} \pm 0.04 \text{ }^\circ\text{C}$ . By the final 9 min of the test session, saline-treated subjects had a  $1.46 \text{ }^\circ\text{C} \pm 0.04 \text{ }^\circ\text{C}$  difference between  $T_{is}$  and  $T_b$ , whereas subjects receiving the highest dose of hexamethonium (30 mg/kg) had a  $T_{is} - T_b$  value of  $0.35 \text{ }^\circ\text{C} \pm 0.13 \text{ }^\circ\text{C}$ . Also consistent with a hexamethonium-induced reduction in BAT metabolism were our observations that treated pups' interscapular regions cooled more rapidly than did the controls'. At the beginning of the test session, mean  $T_{is}$  values for all groups were between  $35.5 \text{ }^\circ\text{C}$  and  $34.7 \text{ }^\circ\text{C}$ . After 18 min of cold exposure,  $T_{is}$  values leveled off at  $\approx 33.4 \text{ }^\circ\text{C}$  for the saline-treated group. In contrast,  $T_{is}$  values continued to fall following treatment with 30 mg/kg of hexamethonium until they reached a low of  $29.13 \text{ }^\circ\text{C} \pm 0.29 \text{ }^\circ\text{C}$  during the final 9 min of testing.

In addition to reducing BAT metabolism, hexamethonium administration led to increased USV production (see Figure 1B). The

Table 1

Results of Two-Way Analyses of Variance (Treatment  $\times$  Time) Conducted on BAT Metabolism and USV Data After Administration of Saline (sal) or Hexamethonium (hex; 3, 10, or 30 mg/kg)

Comparison	Treatment		Time		Treatment $\times$ Time	
	<i>F</i>	<i>df</i>	<i>F</i>	<i>df</i>	<i>F</i>	<i>df</i>
BAT metabolism						
3 mg/kg hex vs. sal	8.89*	1, 22	252.24**	4, 88	0.99	4, 88
10 mg/kg hex vs. sal	39.60**	1, 22	127.59**	4, 88	7.89**	4, 88
30 mg/kg hex vs. sal	238.70**	1, 18	82.94**	4, 72	34.67**	4, 72
Percentage of time vocalizing						
3 mg/kg hex vs. sal	5.29	1, 22	20.36**	4, 88	2.81	4, 88
10 mg/kg hex vs. sal	7.48*	1, 22	4.45**	4, 88	8.44**	4, 88
30 mg/kg hex vs. sal	2.56	1, 18	0.81	4, 72	17.18**	4, 72

Note. BAT = brown adipose tissue; USV = ultrasonic vocalization.

\* *p* < .05. \*\* *p* < .01.

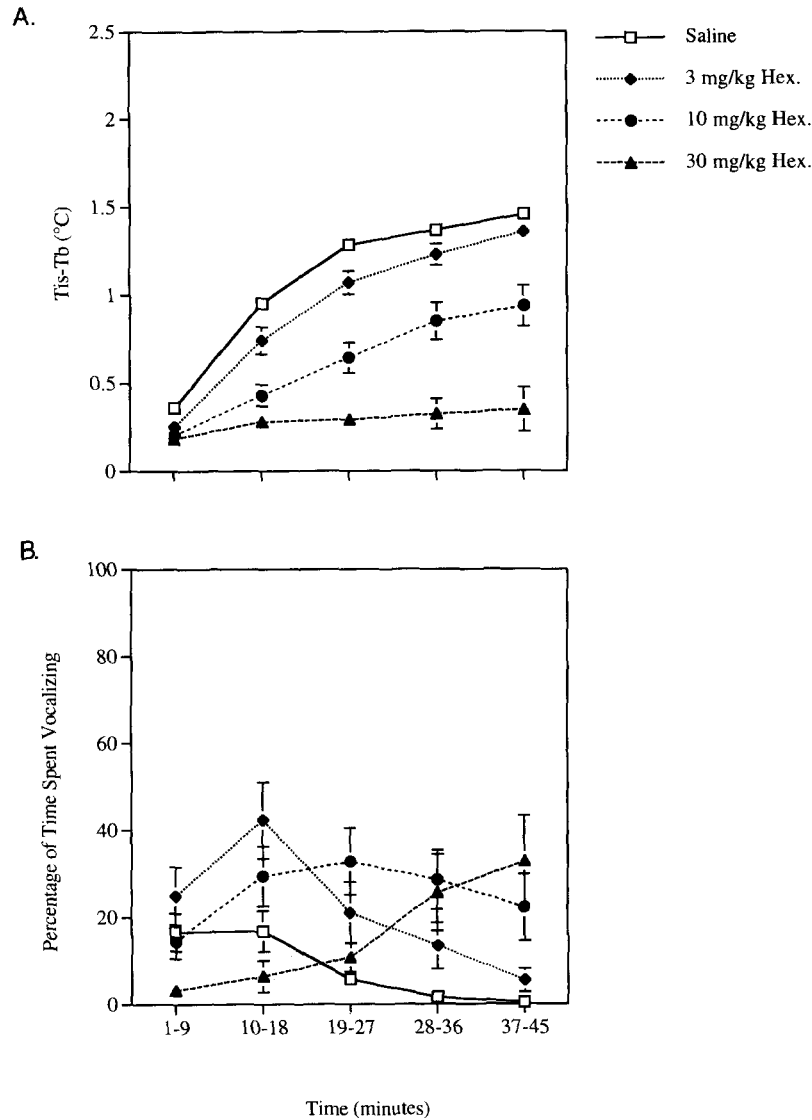


Figure 1. Brown adipose tissue metabolism (A) and ultrasonic vocalization production (B) during 45 min in a cool (25 °C) environment following hexamethonium (Hex.) administration (3, 10, or 30 mg/kg). Data points represent mean values for five consecutive 9-min time bins. Error bars denote the standard error of the mean. T<sub>is</sub> = interscapular temperature; T<sub>b</sub> = temperature of the pup's back.

ANOVA comparing the mean percentage of time spent vocalizing following treatment with 10 mg/kg of hexamethonium with that obtained following saline administration yielded a significant main effect of treatment, indicating enhanced vocalizing. The USV-enhancing effect of hexamethonium was not strictly dose dependent; there were no main effects of treatment or time for the 30 mg/kg dose. There was, however, a significant Treatment  $\times$  Time interaction, and post hoc *t* tests revealed enhanced vocalizing during the final 18 min of testing,  $t(18) = 3.35$  and  $3.85$  for Minutes 28–36 and Minutes 37–45, respectively,  $p < .01$ . Although we did not systematically measure overall activity following drug administration, we observed that the highest dose of hexamethonium (30 mg/kg) had an overt sedative effect at the beginning of testing, possibly precluding increased USV production. It is possible that the enhanced USV production at the end of

testing corresponds to increases in activity over time, presumably due to reduced sedation. This observation may explain why Hofer and Shair (1991) found only a trend for increased USV production following the administration of 20 mg/kg of hexamethonium.

#### Experiment 2: Ultrasound Emission by Propranolol-Treated 7-Day-Old Rat Pups

Hexamethonium's dichotomous effects on BAT metabolism and USV production are consistent with the hypothesis that the heat produced by BAT attenuates USV production during cold exposure. Nevertheless, the observation that the 30 mg/kg dose of hexamethonium appeared to have a sedative effect raises the possibility that hexamethonium acted centrally to increase USV

production rather than altering USV production through its peripheral thermal effects as we had proposed.

In Experiment 2, we examined USV production following the administration of a second compound, propranolol, which inhibits BAT metabolism (Heim & Hull, 1966) by blocking  $\beta$ -adrenergic receptors. To assure that propranolol's effects on USVs would be thermally mediated and not the result of central effects of the compound, we added an additional control group in which we assessed USV production following propranolol administration under thermoneutral conditions (35 °C). We hypothesized that reductions in BAT metabolism caused by propranolol would be accompanied by increased USV production during exposure to a cool (25 °C) environment. Because BAT metabolism is minimal at thermoneutrality, propranolol should have negligible thermal effects under these conditions and increased vocalizing would be the result of nonthermal effects of the compound. Therefore, we expected that propranolol would fail to enhance USV production under thermoneutral conditions.

**Method**

The subjects were 21 male and 19 female 7-day-old rat pups. The methods used in Experiment 2 were similar to those used in the first experiment except that pups received subcutaneous injections of either d,l-propranolol (0.3, 1, or 20 mg/kg; Sigma Chemical, St. Louis, MO) or saline ( $n = 8$  per group). In addition, one control group was added ( $n = 8$ ) in which pups received 20 mg/kg propranolol and were tested at a thermoneutral (35 °C) temperature as opposed to the cooler 25 °C temperature. The handling procedures and apparatus used to test this 35 °C control group were the same as those used during the testing of all other experimental subjects. Experimental groups were gender balanced, except for the 35 °C control group, which was composed of 5 male and 3 female pups. Litters were represented only once per treatment group.

**Results and Discussion**

Propranolol dose-dependently attenuated BAT metabolism during cold exposure. ANOVAs comparing BAT metabolism following propranolol administration with values obtained from the saline control group revealed a significant main effect of treatment following the administration of both 1 and 20 mg/kg propranolol and a significant main effect of time at all doses. The Treatment  $\times$

Time interaction was also significant at the 0.3 and 20 mg/kg doses. The specific *F* values and associated probabilities are enumerated in Table 2.

Differences in BAT metabolism following propranolol administration emerged over time (see Figure 2A). On average, during the first 9 min of the test period,  $T_{is} - T_b$  values were minimal for all groups, ranging from a high of  $0.42 \text{ }^\circ\text{C} \pm 0.05 \text{ }^\circ\text{C}$  for the saline-treated subjects to a low of  $0.22 \text{ }^\circ\text{C} \pm 0.05 \text{ }^\circ\text{C}$  for the group that received 1 mg/kg propranolol. By the end of the test session, differences between the propranolol-treated subjects and their saline-treated counterparts had become more apparent. Saline-treated subjects had a mean  $T_{is} - T_b$  value of  $1.40 \text{ }^\circ\text{C} \pm 0.08 \text{ }^\circ\text{C}$ . In contrast, subjects receiving the highest dose of propranolol (20 mg/kg) had only a  $0.55 \text{ }^\circ\text{C} \pm 0.11 \text{ }^\circ\text{C}$  difference between  $T_{is}$  and  $T_b$ . As was the case for pups treated with hexamethonium in Experiment 1,  $T_{is}$  values fell more rapidly in propranolol-treated rat pups than in control subjects. By the final 9 min of testing, saline-treated pups had mean  $T_{is}$  values of  $33.16 \text{ }^\circ\text{C} \pm 0.3 \text{ }^\circ\text{C}$ , whereas pups receiving 20 mg/kg of propranolol had a mean  $T_{is}$  value of  $29.65 \text{ }^\circ\text{C} \pm 0.16 \text{ }^\circ\text{C}$ .

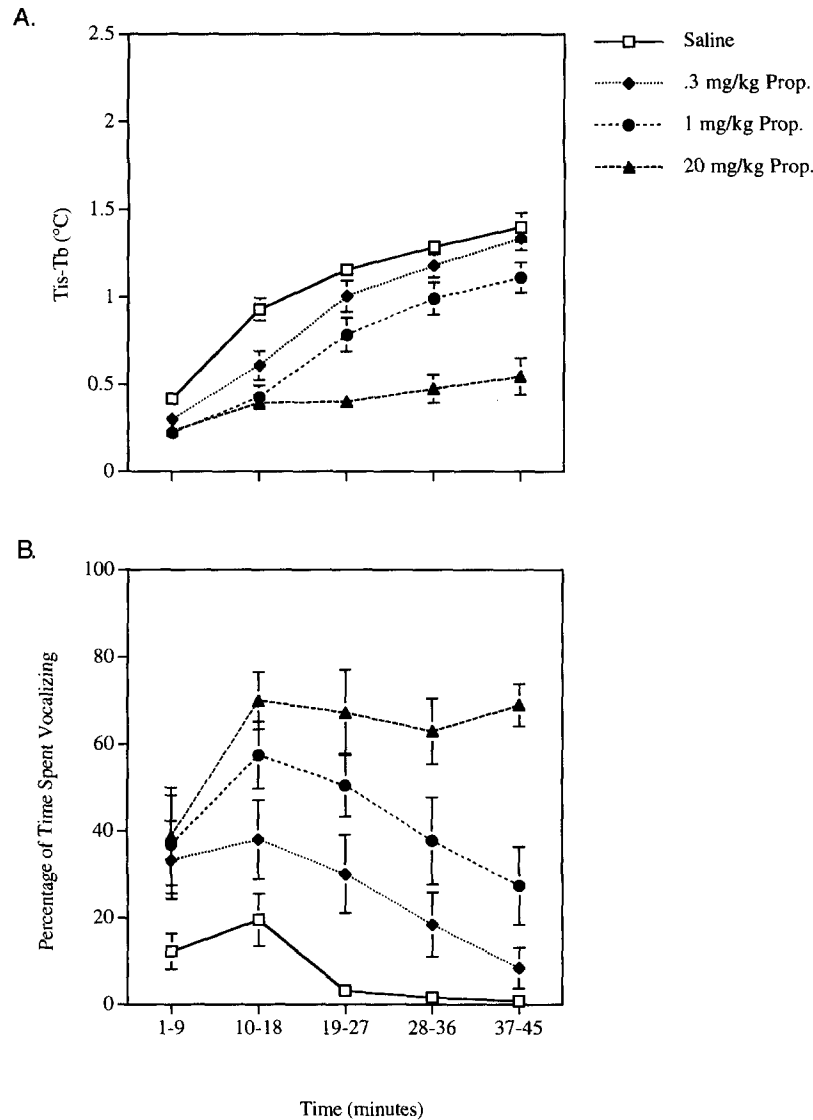
The doses of propranolol that significantly decreased BAT metabolism during cold exposure caused concurrent increases in vocalizing, as confirmed by significant main effects of treatment at dosages of both 1 and 20 mg/kg. ANOVAs also revealed significant main effects of time for all doses and a significant Treatment  $\times$  Time interaction at the two highest doses (1 and 20 mg/kg).

Regardless of experimental treatment, peak rates of USVs were obtained during the second 9 min of the 45-min test session (see Figure 2B). Saline-treated subjects vocalized for  $19.54\% \pm 6\%$  of the time during the second 9 min of the test session, whereas subjects receiving 20 mg/kg of propranolol vocalized  $70.00\% \pm 6.56\%$  of the time during the same time period. Similar to BAT metabolism, differences in USV production increased as the test session progressed. During the final 9 min of the experimental session, saline-treated subjects were virtually silent, vocalizing only  $0.83\% \pm 0.33\%$  of the time. In contrast, subjects receiving 20 mg/kg of propranolol continued to vocalize at a rate that was almost identical to that achieved during the 10th–18th minutes of the test ( $68.98\% \pm 4.84\%$ ). Importantly, propranolol did not elicit

Table 2  
Results of Two-Way Analyses of Variance (Treatment  $\times$  Time) Conducted on BAT Metabolism and USV Data After Administration of Saline (sal) or Propranolol (prop.; 0.3, 1.0, or 20.0 mg/kg)

Comparison	Treatment	Time	Treatment $\times$ Time
	<i>F</i> (1, 14)	<i>F</i> (4, 56)	<i>F</i> (4, 56)
BAT metabolism			
0.3 mg/kg prop. vs. sal	3.67	225.08**	3.38*
1.0 mg/kg prop. vs. sal	16.11**	139.49**	3.17
20.0 mg/kg prop. vs. sal	71.75**	75.20**	22.70**
Percentage of time vocalizing			
0.3 mg/kg prop. vs. sal	7.23	9.89**	1.32
1.0 mg/kg prop. vs. sal	15.70**	13.65**	3.51*
20.0 mg/kg prop. vs. sal	56.34**	5.33**	7.86**

Note. BAT = brown adipose tissue; USV = ultrasonic vocalization.  
\*  $p < .05$ . \*\*  $p < .01$ .



**Figure 2.** Brown adipose tissue metabolism (A) and ultrasonic vocalization production (B) during 45 min in a cool (25 °C) environment following propranolol (Prop.) administration (0.3, 1, or 20 mg/kg). Data points represent mean values for five consecutive 9-min time bins. Error bars denote the standard error of the mean.  $T_{is}$  = interscapular temperature;  $T_b$  = temperature of the pup's back.

USVs independent of exposure to cool ambient temperatures. Rats observed at 35 °C following the administration of 20 mg/kg propranolol remained virtually silent. The peak vocalization rate for these rats was obtained during the first 9 minutes of the test session, when on average they vocalized only  $0.37\% \pm 0.19\%$  of the time (data not shown).

### Experiment 3: Ultrasound Emission by Norepinephrine-Treated 7-Day-Old Rat Pups

The results of Experiments 1 and 2 support the idea that USV production is sensitive to  $T_{is}$  or a direct correlate of  $T_{is}$ , as both propranolol and hexamethonium administration caused a reduction in BAT metabolism that was accompanied by an increase in USVs. To complement the manipulations that blocked BAT thermogen-

esis, Experiment 3 was designed to determine whether a pharmacological manipulation that increases BAT metabolism would cause a concurrent reduction in USVs. Infant rats were treated with the adrenergic agonist norepinephrine (NE) or saline prior to testing. NE dramatically increases BAT metabolism (Hofer & Shair, 1991; Hsieh, Emery, & Carlson, 1971). If the heat produced by BAT metabolism is capable of attenuating cold-induced USVs, then the enhanced BAT metabolism caused by NE administration should be accompanied by decreased vocalizing.

### Method

The subjects were 16 male and 16 female 7-day-old rat pups. The methods and procedures were similar to those used in the previous two experiments except pups received subcutaneous injections of either 800

$\mu\text{g/kg}$  NE (arterenol; Sigma Chemical, St. Louis, MO) or saline vehicle. To minimize irritation, we injected NE at a concentration of 0.1 mg/ml. Saline was administered in a similar volume. To accommodate the larger injection volume (8 ml/kg), we administered NE and saline by way of two subcutaneous injections under the loose skin on both flanks. In addition, in an attempt to increase vocalization rates, we decided to examine BAT metabolism and USV production at both 22 °C and 15 °C ( $n = 8$  saline-treated rats and 8 NE-treated rats at each temperature) as opposed to the warmer 25 °C environmental temperature used in the two previous experiments.

### Results and Discussion

Results showed that 800  $\mu\text{g/kg}$  NE enhanced BAT metabolism at both 22 °C and 15 °C (see Figure 3A); NE-treated subjects maintained mean  $T_{\text{is}} - T_{\text{b}}$  values between 1.71 °C and 2.02 °C throughout the duration of the test. The ANOVA conducted on the data obtained at 22 °C revealed significant main effects of treat-

ment,  $F(1, 14) = 32.43, p < .01$ , and time,  $F(4, 56) = 33.43, p < .01$ , as well as a significant interaction,  $F(4, 56) = 17.05, p < .01$ . The ANOVA conducted on data obtained at 15 °C also revealed significant main effects of treatment,  $F(1, 14) = 11.88, p < .01$ , and time,  $F(4, 56) = 48.47, p < .01$ , and a significant interaction,  $F(4, 56) = 17.67, p < .01$ .

$T_{\text{is}}$  values declined more rapidly in the NE-treated subjects tested at 15 °C compared with those tested at 22 °C. At the beginning of testing, both NE-treated groups had  $T_{\text{is}}$  values  $\approx 38.5$  °C. During the final 9 min of testing, NE-treated subjects tested at 22 °C had a mean  $T_{\text{is}}$  value of  $32.57 \text{ °C} \pm 0.32 \text{ °C}$ , whereas subjects tested at 15 °C had a mean  $T_{\text{is}}$  value of  $26.86 \text{ °C} \pm 0.66 \text{ °C}$ .

Consistent with our hypothesis, NE dramatically attenuated the production of USVs at both 22 °C and 15 °C (see Figure 3B). The

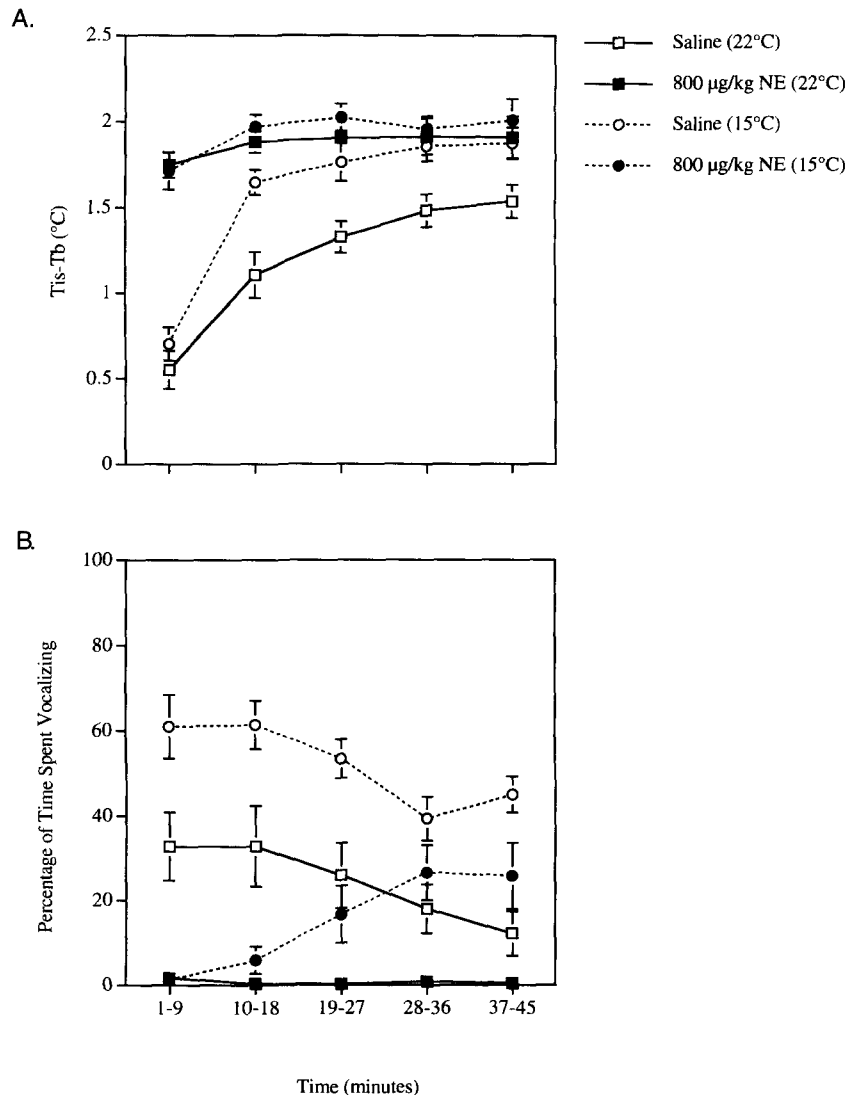


Figure 3. Brown adipose tissue metabolism (A) and ultrasonic vocalization production (B) during 45 min in a cool (22 °C or 15 °C) environment following norepinephrine (NE) administration (800  $\mu\text{g/kg}$ ). Data points represent mean values for five consecutive 9-min time bins. Error bars denote the standard error of the mean.  $T_{\text{is}}$  = interscapular temperature;  $T_{\text{b}}$  = temperature of the pup's back.

ANOVA conducted on the USV data obtained at 22 °C revealed significant main effects of treatment,  $F(1, 14) = 13.08, p < .01$ , and time,  $F(4, 56) = 5.21, p < .01$ , as well as a significant interaction effect,  $F(4, 56) = 4.79, p < .01$ . An ANOVA conducted on data collected at 15 °C also revealed a main effect of treatment,  $F(1, 14) = 37.28, p < .01$ , and a significant interaction effect,  $F(4, 56) = 13.19, p < .01$ . The main effect of time was not statistically significant,  $F(4, 56) = 0.35, p < .84$ .

Pups in both saline groups vocalized vigorously throughout the test session, with maximal vocalization rates being obtained during the first 18 min. NE-treated pups observed at both 22 °C and 15 °C were nearly silent during the first 9 min of the test session, vocalizing less than 2% of the time. However, as the test session progressed, the vocalization rates of the pups observed at 15 °C increased, peaking between 28 and 36 minutes ( $26.57\% \pm 6.58\%$ ). NE-treated pups observed at 22 °C remained virtually silent (vocalized less than 2% of the time) for the duration of the test session. An ANOVA comparing the vocalization rates of NE-treated pups tested at 22 °C and 15 °C revealed significant main effects of temperature,  $F(1, 14) = 15.17, p < .01$ , and time,  $F(4, 56) = 5.34, p < .01$ , as well as a significant interaction,  $F(4, 56) = 5.87, p < .01$ .

### General Discussion

The results of the present series of experiments support our hypothesis that BAT thermogenesis influences USV production. Excluding the results obtained following the administration of the highest dose of hexamethonium (30 mg/kg), all three of the pharmacological manipulations used to uncouple the excitation of BAT from ambient temperature had inverse effects on BAT metabolism and USV production. Both hexamethonium and propranolol significantly reduced BAT metabolism and caused a concurrent increase in USV production. NE, on the other hand, increased BAT metabolism while dramatically attenuating USVs. Furthermore, it appears that USV production was influenced primarily by the thermal effects of our pharmacological manipulations, because propranolol administration (20 mg/kg) failed to elicit USV production in a thermoneutral environment, and NE's ability to attenuate USVs could be lessened by exposure to a cooler (15 °C) ambient temperature.

The finding of an inverse relationship between BAT metabolism and USVs is consistent with the general hypotheses that USVs are the by-product of regulatory processes involved in maintaining physiological homeostasis. Moreover, the present results are compatible with the specific hypothesis that USVs are emitted during efforts to maintain blood pressure and venous return when cooling decreases heart rate. As was outlined in the introduction, the initiation of USV production typically accompanies a reduction in heart rate (Kirby & Blumberg, 1998), and there appears to be a direct positive relationship between the maintenance of  $T_{is}$  and heart rate during cold exposure (Blumberg et al., 1997; Sokoloff et al., 1998), both in unmanipulated pups and following ganglionic blockade. On the basis of these findings, it appears likely that the pharmacological manipulations used here to uncouple BAT metabolism from ambient temperature influenced not only BAT metabolism and consequently  $T_{is}$  but also had thermal effects on heart rate that influenced USV production. It is important to note, however, that we did not monitor heart rate. Because all three of the compounds we used are likely to have not only thermal effects

on heart rate but also nonthermal effects due to their impingement on sympathetic cardiac control, further research simultaneously examining USVs, heart rate, blood pressure, and  $T_{is}$  will be required to fully assess the validity of the hypothesis relating cardiovascular homeostasis to USVs.

Although it has long been established that cooling is a potent stimulus for the initiation and maintenance of USV production, there have been numerous reports that stimuli associated with the nest environment, such as exposure to an anesthetized dam, are capable of either attenuating or potentiating USV production without exerting thermal effects. Studies examining the effect of nest stimuli on USV production typically involve observing pups in a novel, often cold, test environment, both alone and in the presence of a dam or littermates. Pups vigorously emit USVs when alone, and these vocalizations are dramatically reduced in the presence of the dam or sibling. Although many of the early studies examining "contact comfort" were thermally confounded because the pup could huddle with the target stimulus, thereby gaining heat through conduction while retarding convective heat loss, there are also some indications that nest stimuli can attenuate USVs through nonthermal mechanisms. Hofer, Brunelli, and Shair (1993) reported that a dead dam cooled to 10 °C below room temperature quieted pups, and they interpret this observation as evidence of nonthermal regulation of USVs. We have found that the odors of soiled home cage bedding attenuate cold-induced USVs without altering cutaneous temperature (Farrell & Alberts, 1996). In addition, whereas exposure to an anesthetized dam quiets pups, removal of the dam potentiates isolation-induced USV production, provided that exposure to the dam is brief and occurs in the context of the novel test arena (Hofer, Brunelli, & Shair, 1994; Shair, Masmela, Brunelli, & Hofer, 1997).

Hypotheses relating USVs to cold-induced changes in infant physiology cannot directly explain the quieting effect of nest stimuli or the potentiation seen following brief maternal exposure. Nevertheless, the possibility of a link between the maintenance of cardiovascular homeostasis and USV production may provide interesting avenues for exploration. For instance, it would be instructive to examine  $T_{is}$ , heart rate, blood pressure, and USVs simultaneously during cold challenge, both in isolation from and during exposure to nonthermal stimuli that either attenuate or potentiate USV production. There is a report that both USV and heart rate are elevated during isolation in a novel test arena compared with the home cage (Hofer & Shair, 1987). There is also evidence, however, that stimuli associated with the nest environment can elicit increases in heart rate, and isolation can cause cardiac deceleration in young pups. Hofer and Grabie (1971) found that 12- to 19-day-old rat pups exhibit an increase in heart rate while nursing that is independent of both motor activity and temperature. In addition, Hofer and Reiser (1969) reported that during the 2nd postnatal week pups exhibit cardiac deceleration following removal from the home cage and placement in a novel test box, a response that changes to cardiac acceleration late in the 3rd week of life. These observations suggest that integrated responses of the autonomic nervous system may become associated with olfactory perception in ways that might modulate the emission of ultrasonic vocalizations by pups. Indeed, learned associations between odors and physiological responses can be readily formed (Graham & Desjardins, 1980; Martin & Alberts, 1982).

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