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Research report

Effects of antihypertensive drugs on ultrasound production and cardiovascular responses in 15-day-old rats

Mark S. Blumberg *, Greta Sokoloff, Robert F. Kirby, Tricia G. Knoot, Sean J. Lewis

Program in Behavioral and Cognitive Neuroscience, Department of Psychology, The University of Iowa, E11 Seashore Hall, Iowa City, IA 52242, USA

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Abstract

When exposed to extreme cold or injected with the α_2-adrenoceptor agonist, clonidine, infant rats emit ultrasonic vocalizations (USVs). Based upon the cardiovascular changes that accompany these two manipulations, especially decreased venous return, it was hypothesized that USVs are the acoustic by-product of the abdominal compression reaction (ACR), a maneuver that increases venous return. If this hypothesis is correct, then other anithypertensive drugs that decrease venous return should evoke USVs. In Experiment 1, sodium nitroprusside (SNP, 400 μg/kg), a direct-acting dilator of arteries and veins, was administered to 15-day-old rats under thermoneutral conditions while cardiac rate and ultrasound production were monitored. In Experiment 2, femoral artery pressure was monitored after SNP administration. Infants responded to SNP administration with decreased arterial pressure and tachycardia and, in addition, significantly increased ultrasound production. In Experiment 3, chlorisondamine (5 mg/kg), a ganglionic blocker that causes vasodilation and bradycardia, and hydralazine (20 mg/kg), a selective dilator of arteries, was administered to 15-day-olds. As predicted, chlorisondamine evoked ultrasound production and hydralazine did not. These results introduce SNP and chlorisondamine as only the second and third known agents capable of independently evoking USVs in thermoneutral conditions, and provide further support for the notion that ultrasound production is triggered by decreased venous return.

Keywords: Sodium nitroprusside; Hydralazine; Chlorisondamine; Clonidine; Ganglionic blocker; Baroreceptor; Venous return; Vasodilation; Bradycardia

1. Introduction

For many years the ultrasonic vocalization of infant rats has been interpreted as a behavioral marker of psychological distress [14,30]. To utilize this model of distress, investigators typically stimulate ultrasound production by removing a pup from its home cage and isolating it in a novel environment for a brief period of time. The investigator then assesses the ability of pharmacological agents (usually administered before a pup is isolated) or contextual cues to modulate emission of the vocalization during the isolation period. The popularity of this methodological approach, and of the isolation procedure in particular, can be attributed to at least two factors: (a) confidence that the procedure reflects the circumstances that evoke the vocalization under natural conditions; and (b) the assumption that the vocalization reflects a unitary psychological process (i.e. distress) and that the isolation procedure is sufficient for examining those factors—sensory, perceptual, social, pharmacological—that modulate emission of the vocalization [19–22,35].

Although isolation from the nest is a multisensory stimulus that involves olfactory, vestibular, tactile, and

* Corresponding author. Tel.: +1-319-335-2424; fax: +1-319-335-0191.

E-mail address: mark-blumberg@uiowa.edu (M.S. Blumberg).

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thermal cues, it is the last factor that is acknowledged to be of primary importance for the stimulation of ultrasound production in infant rats [2,4,5,29]. For example, if the isolation procedure is not accompanied by significant cold exposure, the vocal responses of infants are significantly blunt [4]. In recent years, our understanding of this process has been refined by employing a controlled cooling procedure in which pups are removed from the nest, acclimated to a warm environment, and then exposed to cold [13]. This procedure has revealed that ultrasound production increases dramatically across the transition from moderate to extreme cold exposure when heat production can no longer compensate for heat loss, resulting in hypothermia. This transition is also marked by increased blood viscosity and dramatic decreases in cardiac rate [7]. These physiological consequences of extreme cooling suggest an animal whose ability to maintain adequate venous return is severely compromised [9].

The insights gained from these controlled cooling experiments inspired the hypothesis that the ultrasonic vocalization is the acoustic by-product of a physiological maneuver, called the abdominal compression reaction (ACR, [38]) that functions to enhance venous return during extreme thermal challenges [25]. Accordingly, it has been proposed that the vocalization is, like a sneeze or a cough, the acoustic by-product of a physiological process—in this case, the coupling of forceful abdominal contractions with laryngeal constriction as a means of propelling venous blood out of the abdomen and back to the heart. In support of this hypothesis, ultrasonic vocalizations are accompanied by pulsatile increases in intra-abdominal pressure and central venous pressure [12,25].

Although extreme cooling is a highly reliable method for stimulating ultrasound production in a controlled fashion, it is not the only such method. Administration of the α_2-adrenoceptor agonist, clonidine, provokes intense and prolonged ultrasound production in infant rats, even in thermoneutral conditions [17,23]. Clonidine's ability to evoke ultrasound increases with age until it peaks at 15 days postpartum, after which it declines rapidly [10]. Moreover, recent experiments have provided strong evidence that clonidine-induced ultrasound production results from its effects on cardiovascular function, beginning with a centrally-mediated withdrawal of sympathetic outflow that leads to decreases in cardiac rate and stroke volume as well as relaxation of venous and arterial vessels [6,10]. Therefore, clonidine shares with extreme cold exposure the ability to promote pooling of venous blood and inhibit venous return.

Our working hypothesis, implied by the above discussion, is that decreased venous return underlies emission of the vocalization regardless of the experimental context [8]. If correct, this hypothesis demands that any pharmacological agent that, like clonidine, significantly decreases venous return should stimulate ultrasound production. Since clonidine is classified as an antihypertensive drug, it is useful to consider other agents within that class and how they might be expected to influence the expression of the ACR and ultrasound production. Antihypertensive drugs are classified as sympatholytics, vasodilators, and diuretics [28], and each of these three classes is divided into sub-categories. For example, clonidine is classified as a centrally acting sympatholytic.

In the present study, we examined the ability of three other anti-hypertensive drugs to evoke ultrasound production in rats at 15 days of age, when clonidine has its maximal effects. First, in Experiment 1, sodium nitroprusside (SNP) was administered while cardiac rate and ultrasound production was monitored. SNP, classified as an arterial and venous vasodilator, is a nitric oxide donor that directly relaxes the smooth muscles in arteries and veins, resulting in venous pooling and decreased venous return. Since Youmans and his colleagues [38], in their seminal investigations of the ACR in anesthetized adult dogs, were able to stimulate the ACR using nitroglycerine (a compound that shares a number of functional properties with SNP), we predicted that SNP administration would evoke ultrasound production in 15-day-old rats. In Experiment 2, the effects of SNP on blood pressure were measured to ensure that its cardiovascular effects are similar in infants and adults.

In Experiment 3, we examined the effects of two other antihypertensive drugs. First, chlorisondamine hydrochloride, a ganglionic blocker that, like clonidine, is classified as a sympatholytic, was tested to determine its ability to evoke ultrasound production. Second, hydralazine, a selective arterial vasodilator [1,28], was also tested. Since hydralazine's effects are restricted to dilation of arterial vessels, it has a negligible effect on venous return and therefore provides a useful contrast to the effects of SNP. Thus, on the basis of the ACR hypothesis, we predicted that chlorisondamine would evoke ultrasound production while hydralazine would not.

2. Materials and methods

2.1. Subjects

In total, 48 male and female rat pups from 29 litters were used. Pups were tested at 15 days of age (hereafter designated PD15). Pups were born to Harlan Sprague–Dawley females maintained in the animal colony at the University of Iowa. On the day of testing, PD15 rats weighed 28.3–42.1 g. Mothers and their litters were housed in standard laboratory cages ($48 \times 20 \times 26$ cm)

in which food and water were available ad libitum. Litters were culled to eight pups within 3 days after birth (day of birth = Day 0). All animals were maintained on a 12-h light:12-h dark schedule with lights on at 06:00 h.

2.2. Apparatus and physiological measures

The methods used here for the measurement of cardiac rate, femoral artery pressure, and ultrasound production have been described elsewhere [11,25].

All pups were tested inside a double-walled glass chamber. Air temperature (T_a) within the chamber was controlled by pumping temperature-controlled water through the chamber walls using a water circulator (Neslab, Portsmouth, NH). The air temperature in the chamber was regulated at 32 °C, which is within the thermoneutral range of pups at 15 days of age [33]. Pups were allowed to move freely inside the chamber on a platform constructed of polyethylene mesh. Compressed air flowed through the chamber at a rate of 300 ml/min.

For the measurement of cardiac rate in Experiments 1 and 3, pups were lightly anesthetized with ether and three electrodes were attached, two on either side of the thoracic cavity and a ground electrode attached near the base of the tail (this procedure was typically completed in less than 10 min). Collodion was used to secure the electrodes and to improve the electrical connection. The ECG leads from the pup were connected to an impedance pneumograph (UFI, Morro Bay, CA). The ECG analog signal was digitized at a rate of 1000 samples per s and interbeat intervals (IBIs) were determined using a customized software program employing a peak threshold detector [32]. IBI data were acquired at a rate of 30 samples per min.

For recording of arterial pressure in Experiment 2, the femoral artery was catheterized under isoflurane anesthesia. The catheter was constructed from Micro-Renathane tubing (MRE-040; Braintree Scientific, Inc, Braintree, MA) and filled with heparinized (50 IU/ml) isotonic saline. For catheter implantation, the left femoral artery was exposed and the catheter was introduced into the artery and advanced approximately 0.9–1.5 cm into the artery. This distance placed the tip at the branching of the femoral artery from the abdominal aorta. After checking for adequate blood flow, the catheter was sutured in place and stabilized with a drop of cyanoacrylate at the juncture of the catheter and femoral artery. The incision was then sutured closed and the pup was transferred to the chamber to recover.

After recovery from surgery, polyethylene tubing was used to connect the arterial catheter to a pressure transducer (Argon, Athens, TX). The entire length of tubing from pup to transducer was filled with heparinized saline. The output of the transducer passed through an analog-to-digital converter, whose signal was then fed into a computerized data acquisition system. Before each pup was tested, the system was calibrated using a sphygmomanometer with a resolution of 1 mmHg.

Ultrasonic vocalizations (USVs) were made audible using a microphone sealed inside the lid of the metabolic chamber and connected to a detector (Model SM100, UltraSound Advice, London, UK) tuned to a ± 5 kHz range centered on 35 kHz. Ultrasonic vocalization data were scored by an experienced observer. To do this, the observer used an event recorder written in HyperCard for the Macintosh and pressed a computer key each time an ultrasonic vocalization was detected. Each key press recorded the time at which the ultrasonic pulse occurred.

2.3. Drugs

In Experiments 1 and 2, sodium nitroprusside (Roche, Milan, Italy) was dissolved in isotonic saline and administered at a dose of 400 μg/kg. In Experiment 3, chlorisondamine hydrochloride (Ciba-Geigy, Summit, NJ) was dissolved in saline and administered at a dose of 5 mg/kg, and hydralazine hydrochloride (Sigma, St. Louis, MO) was dissolved in isotonic saline and administered at a dose of 20 mg/kg. The dosages for SNP [26], chlorisondamine [11], and hydralazine [3] were identical or within the range of dosages used by others in previous reports.

2.3.1. Procedure and data acquisition

For Experiment 1, 20 PD15 rats were used. On the day of testing a pup was removed from its cage, lightly anesthetized with ether, and placed on a heating pad. After ECG leads were attached the pup was transferred to the metabolic chamber. The pup recovered in the chamber for at least 45 min, after which baseline recording of cardiac data and monitoring of ultrasound production began for a period of 1 min. Cardiac data were recorded by computer on-line and ultrasound production was scored in real-time. After the 1-min baseline period, the experimenter, wearing latex gloves to prevent conductive cooling of the pup, injected it subcutaneously with SNP or saline in a volume of 1 μl/g. After the injection, recording of cardiac rate data and ultrasound production continued for 15 min, after which the test ended and the pup was returned to its home cage. For the majority of subjects, same-sex littermates were assigned to one of the two conditions at each age.

For Experiment 2, four PD15 rats were used. As noted above, the pup recovered from surgery (which lasted 45–70 min) in the chamber maintained at thermoneutrality. This period of recovery varied between 90 and 150 min, depending on the status of the animal.

Acquisition of blood pressure data was initiated for a 1-min baseline period, after which the pup received its first subcutaneous injection of either SNP or saline in a volume of 1 μl/g. Data were acquired at the rate of 200 samples per s for 15 min, after which the pup remained undisturbed for another 15 min so that blood pressure could return to baseline levels. At that time, the data acquisition protocol was repeated except a pup initially injected with SNP was now injected with saline, or vice versa. Order of injections was counterbalanced.

For Experiment 3, 24 PD15 rats were used. Pups were prepared for measurement of cardiac rate as described above for Experiment 1. All other procedures were identical to those in Experiment 1 except pups were injected with chlorisondamine, hydralazine, or vehicle control.

2.4. Data analysis

All statistical analyses were performed using StatView 4.5 for the Macintosh. For all three experiments, IBIs were converted to cardiac rate (CR) in beats per min (bpm) before analysis. For visualization of cardiac rate responses across ages, the percent change in CR from baseline values (ΔCR) was determined at each of the post-injection time points. For statistical analysis, the maximum percent ΔCR was determined for each of the 15-min tests and an analysis of variance (ANOVA) or an unpaired t-test was performed when appropriate.

The number of USVs was calculated for every 30 s post-injection period. A pup was excluded from analysis when data for that pup exceeded the group mean $\pm$ 2 standard deviations (S.D.). Such exclusions occurred for one pup in Experiment 1 and for two pups in Experiment 3. In Experiment 1, differences in cumulative ultrasound production between conditions across the 15-min tests were tested using the Mann–Whitney U-test. In Experiment 3, the Kruskal–Wallis one-way analysis of variance by ranks was used and the Mann–Whitney U-test was used for post hoc comparisons.

Blood pressure data were imported into DataDesk 6.0 for the Macintosh. A scatterplot of the data was produced for each session, and each point in the scatterplot was linked to a row number that represented the passage of 1/200 s. The time at which maximum systolic and minimum diastolic pressures occurred were determined and, from these data, interbeat interval (IBI) was determined to a resolution of $\pm$2.5 ms. Mean arterial pressure (MAP) was calculated as $P_d + 1/3(P_s - P_d)$, where P_d and P_s are diastolic and systolic pressure, respectively. At each of eight periods of the test (i.e. baseline, Min 1–5, Min 10, Min 15), 11 pressure waves were identified to determine mean MAP and IBI. In addition, the percent change in MAP from baseline values (ΔMAP) was determined at each of the post-injection time points.

For all tests α was set at 0.05. All means are presented with their standard errors.

3. Results

3.1. Experiment 1: sodium nitroprusside and ultrasound production

Mean baseline cardiac rates for the PD15 rats were 422.2 ± 6.9 bpm, within the range of values reported previously for rats at this age at thermoneutrality [10,18]. There was no significant difference in baseline cardiac rate between the SNP and vehicle conditions ($t_{17} = 0.5$).

As shown in Fig. 1A, administration of SNP evoked pronounced tachycardias that, as is typical of SNP's rapid initiation and termination [28], were immediate and brief. For maximum ΔCR, the drug and vehicle conditions differed significantly from each other ($t_{17} = 5.3$, $P < 0.0001$).

As shown in Fig. 1B, SNP administration also evoked the rapid onset of ultrasound production. Ultrasound production peaked within 30 s of SNP administration and returned to control levels by 7 min post-injection. Over the 15-min test, cumulative ultrasound production was significantly greater in the SNP group ($z = 2.4$, $P < 0.05$, see Fig. 3). Specifically, of the 11 pups in the SNP group, four emitted more than 100 vocalizations and only two emitted fewer than 20. In contrast, of the eight pups in the Vehicle group, only one emitted more than 100 vocalizations and the remaining seven emitted fewer than 20.

3.2. Experiment 2: sodium nitroprusside and arterial pressure

Since the cardiovascular effects of SNP have not previously been assessed in infant rats, it was important to verify that the SNP-induced tachycardia observed in Experiment 1 was accompanied by decreased blood pressure. Therefore, four PD15 rats were instrumented with a femoral artery catheter and blood pressure was monitored after administration of SNP (400 μg/kg) or vehicle. As shown in Fig. 1D, SNP evoked a rapid decrease in MAP that triggered a baroreceptor-mediated increase in cardiac rate (Fig. 1C) that was similar to that found in Experiment 1 (Fig. 1A). The change in MAP peaked within 30 s post-injection and returned to baseline values within approximately 5 min. Interestingly, the patterns of ultrasound production (Fig. 1B) and changes in arterial pressure (Fig. 1D) mirror each other, both in terms of the rapidity of the peak response and recovery time.

3.3. Experiment 3: chlorisondamine, hydralazine and ultrasound production

Having demonstrated in Experiment 1 that SNP can, like clonidine, evoke ultrasound production in infants at thermoneutrality, we next investigated the ability of two other antihypertensive drugs to evoke ultrasound production. First, we used chlorisondamine, a non-selective ganglionic blocker whose ability to dilate blood vessels and slow cardiac rate bears some resemblance to clonidine. Based on the ACR hypothesis, it was expected that chlorisondamine, like clonidine, would evoke ultrasound production. Second, we used hydralazine, a drug that is similar to SNP in that it is a vasodilator. Hydralazine differs in its action from SNP, however, in an important way: hydralazine is a specific dilator of arterial smooth muscle while SNP is a non-specific dilator of both arterial and venous smooth muscle [24,28]. Therefore, if the ultrasonic vocalization is a by-product of the ACR, and if the ACR is primarily a response to venous pooling and decreased venous return [38,39], then hydralazine should evoke less ultrasound production than SNP even while triggering a baroreceptor-mediated tachycardia.

Fig. 2 presents ΔCR and ultrasound production across the 15-min test for the three conditions. First, subjects injected with vehicle did not exhibit consistent changes in cardiac rate and did not vocalize. Second, subjects injected with chlorisondamine exhibited an initial increase in cardiac rate, followed rapidly by a large and sustained decrease in cardiac rate; these subjects also exhibited high rates of ultrasound production immediately after injection that decreased over the course of the 15-min test. Finally, hydralazine evoked a rapid and sustained tachycardia, most likely the result of arterial dilation and decreased blood pressure [3]; these subjects, however, did not vocalize.

Fig. 3 provides a direct comparison of the effects of SNP, chlorisondamine, and hydralazine on cardiac rate and ultrasound production in PD15 rats from Experiments 1 and 3 (the statistical analyses for the SNP data are reported in Section 3.1). The top panel of the figure shows that maximum ΔCR differed significantly between the three conditions in Experiment 3 ($F_{2,19}$ =

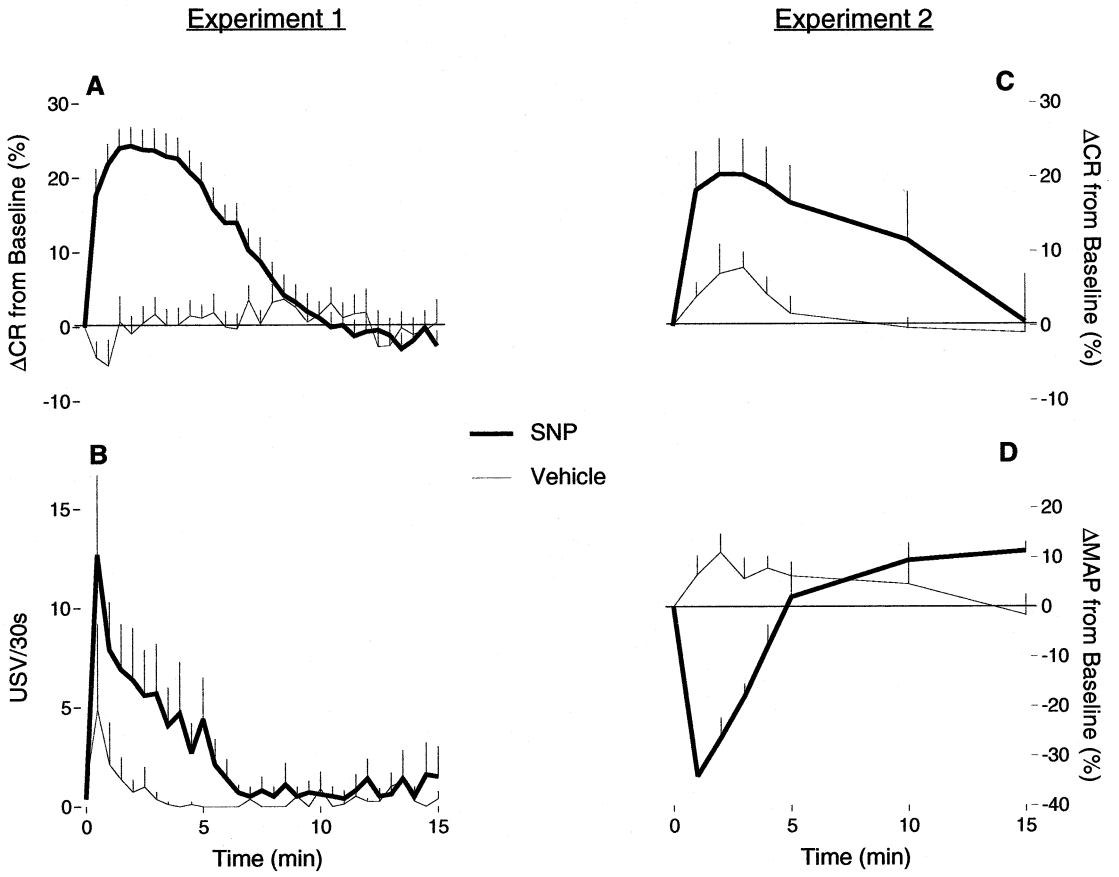

Fig. 1. (A) Percent change in cardiac rate (ΔCR) from baseline and (B) number of ultrasonic vocalizations (USVs) per 30 s interval (bottom row) for the 15-day-old rats in Experiment 1 after administration of sodium nitroprusside (400 μg/kg, $N = 11$)) or vehicle ($N = 8$). (C) Percent change in cardiac rate (ΔCR) from baseline and (D) change in mean arterial pressure (MAP) from baseline for the 15-day-old rats in Experiment 2 after administration of sodium nitroprusside (400 μg/kg) or vehicle. Each subject in Experiment 2 was injected with both drugs in counterbalanced order. $N = 4$. Mean + S.E.

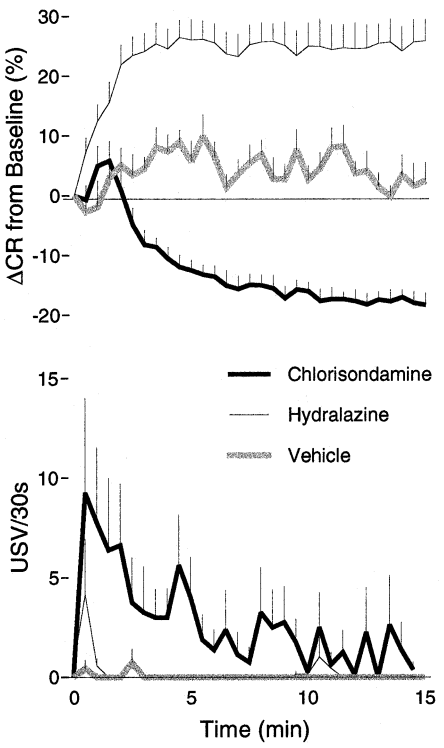

Fig. 2. Percent change in cardiac rate (ΔCR) from baseline (top) and number of ultrasonic vocalizations (USVs) per 30 s interval (bottom) for the 15-day-old rats in Experiment 3 after administration of chlorisondamine (5 mg/kg, $N = 8$), hydralazine (20 mg/kg, $N = 7$) or vehicle ($N = 7$). Mean + S.E.

39.0, $P < 0.0001$) and that all three groups differed significantly from each other. The bottom panel of the figure shows that these three groups also differed from each with respect to cumulative ultrasound production over the 15-min test ($H = 7.4$, $P < 0.05$), with the chlorisondamine subjects vocalizing at higher rates than the hydralazine and vehicle subjects.

4. Discussion

The experiments reported here differ importantly from the majority of pharmacological investigations of ultrasound production in infant rats. Specifically, whereas most investigations have focused on the effect of pharmacological manipulation on ultrasound production after transfer of a pup from the nest to a novel environment [15,35,36], the approach adopted here was to examine those agents that are able to evoke ultrasound production independently of the multisensory stimulation inherent in the transfer protocol.

The present experiments are also unique in that the drugs tested were selected for their known cardiovascular effects rather than for their presumed anxiolytic or analgesic properties. Thus, most studies of ultrasound production in infant rats have focussed on the modulatory role of benzodiazepine and opioid agonists and antagonists upon separation of a pup from the nest [22,27,37]. It must be stressed, however, that not only have some of these studies produced mixed results [36], but also no one has reported the ability of any agent from these drug classes to evoke, rather than modulate, ultrasound production. The sole exception, prior to the present report, was clonidine [10,17]; in fact, clonidine's effects are so profound that pups continue to vocalize even when returned to the nest [16,23]. It may prove interesting to examine the cardiovascular response to pharmacological agents, as well as social contexts [31], that have been implicated in the modulation of ultrasound production.

The hypothesis that ultrasound production is the acoustic by-product of the ACR makes specific predictions regarding the kinds of factors that should stimulate and inhibit ultrasound production. In the present study we tested some of these predictions by focussing on two classes of antihypertensive drugs: the vasodilators and sympatholytics. The vasodilators are divided into two sub-groups: one sub-group includes agents such as SNP that are equally effective at relaxing smooth muscle in arteries and veins; the second subgroup includes agents such as hydralazine that specifically relax arterial smooth muscle [24,28]. Since the ACR is thought to be primarily a response to decreased venous return [38,39], it was predicted that SNP, but not hydralazine, would evoke ultrasound production in infant rats. As Experiments 1 and 3 indicate, both of these predictions were borne out. It should be stressed, however, that while cardiac rate can be used as a bioassay of these drugs' effects on baroreceptor-mediated responses, it is not sufficient to distinguish between these drugs' effects on venous return. Such effects can only be determined through direct measurement of venous blood flow, a very difficult procedure in infant rats.

The class of antihypertensive drugs known as sympatholytics also includes a number of sub-groups, including centrally acting agents (e.g. clonidine and ganglionic blockers such as chlorisondamine). As shown in Fig. 2, ultrasound production increased immediately after chlorisondamine administration and declined gradually over the 15-min test. The cardiac rate response to chlorisondamine was biphasic, with a brief tachycardia, perhaps evoked by a baroreceptor-mediated response to rapid vasodilation, followed by a pronounced and sustained bradycardia. Thus, both clonidine and chlorisondamine evoke ultrasound production, although clonidine's effects are much more profound and prolonged than chlorisondamine's. For example, in a previous study it was found that at 15 min post-injection, pups injected with clonidine emitted a mean of 624 USVs [10] as compared with a mean of 84 vocalizations for pups injected with chlorisondamine

in this study. In addition, clonidine elicited a 50% greater decrease in cardiac rate than chlorisondamine. Thus, it appears that centrally mediated withdrawal of sympathetic outflow, as with clonidine, has a more profound impact on ultrasound production and cardiovascular function than peripheral blockade of both sympathetic and parasympathetic function, as with chlorisondamine.

Clonidine's effects on ultrasound production are also more prolonged and profound than SNP's, although the differences between these two agents are not difficult to explain. First, while SNP is a short-acting compound whose direct physiological effects disappear within minutes, clonidine's effects last for hours. Second, while SNP does not inhibit compensatory reactions (such as the baroreflex-mediated tachycardia seen in Fig. 1), clonidine prevents all compensatory reactions that depend upon sympathetic neural outflow. Third, while SNP is a direct-acting vasodilator, clonidine acts centrally to inhibit all sympathetic outflow, resulting in vasodilation as well as decreased cardiac rate and stroke volume [28,34].

Since cyanide ions are produced as a by-product of SNP metabolism, one might suggest that cyanide toxicity contributed to SNP's ability to evoke a non-specific distress response, including ultrasound production. This seems unlikely for the following reasons: first, the patterns of ultrasound production and blood pressure changes after SNP administration are very similar (see Fig. 1), whereas cyanide build-up and clearance likely exhibit a different temporal pattern. Second, because administration of SNP at the same dose used here is ineffective at evoking ultrasound production in 8- and 20-day-old rats (Blumberg, Sokoloff, Kirby, and Lewis,

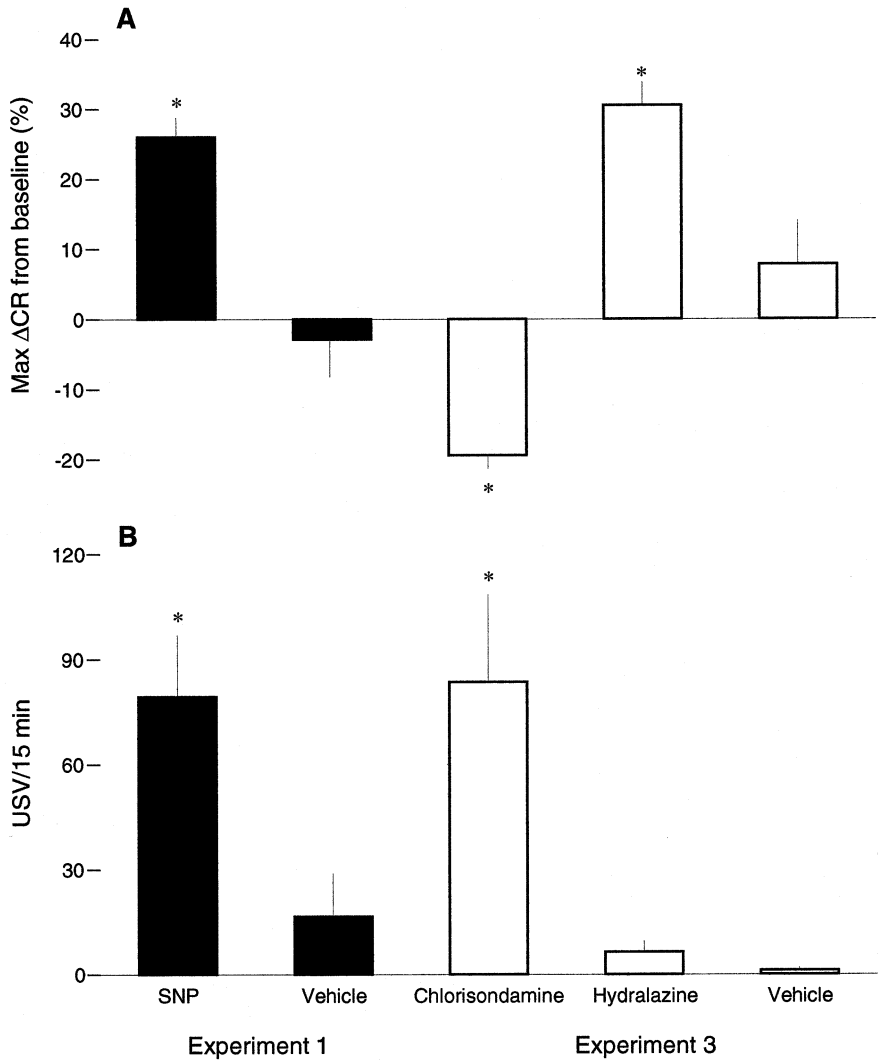

Fig. 3. (A) Maximum percent change in cardiac rate (Max ΔCR) from baseline for the 15-day-old rats tested for 15 min after administration of sodium nitroprusside (400 μg/kg) or vehicle in Experiment 1, or chlorisondamine (5 mg/kg), hydralazine (20 mg/kg), or vehicle in Experiment 3. * $P < 0.01$ in relation to vehicle. (B) Total number of ultrasonic vocalizations (USVs) for the same pups as in the top plot. * $P < 0.05$ in relation to vehicle. Mean ± S.E.

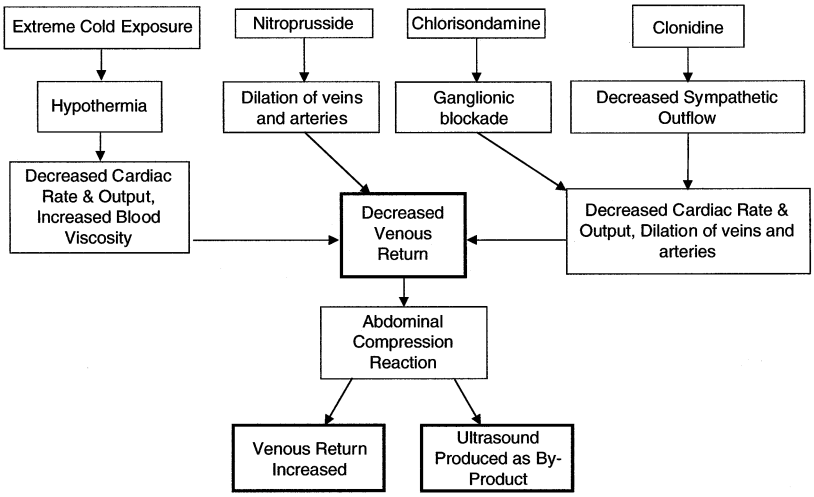

Fig. 4. Working model of the physiological consequences of extreme cold exposure and administration of sodium nitroprusside, chlorisondamine, and clonidine and how they may act through a common pathway to evoke ultrasound production.

unpublished data), it is difficult to explain why cycanide toxicity would stimulate non-specific distress only in 15-day-olds. And third, in pilot studies, we have infused potassium cyanide (KCN) into the arterial and venous circulations of 15-day-old rats in high doses and have failed to stimulate ultrasound production despite the clear behavioral agitation produced by the infusion. This last finding suggests that ultrasound production is insensitive to chemoreceptor stimulation, although more thorough studies are needed before a final conclusion can be drawn.

Other investigators have examined whether modulation of the nitric oxide system influences ultrasound production in infant rats [14,30], although their stated aim was to explore the role of the nitric oxide system in the expression of anxiety. In both of these studies, nitric oxide synthase (NOS) inhibitors were administered subcutaneously or intraperitoneally to infant rats (7–11 days of age) before transfer to a novel environment for 3–5 min. In both studies, ultrasound production was reduced dose-dependently by NOS inhibition, results that are broadly consistent with those reported here in which ultrasound production was increased by a nitric oxide donor.

Does the ACR hypothesis provide insight into the brief burst of ultrasound production that occurs in response to the transfer of a pup from the nest to a novel environment? To answer this question, one must look closely at the methodological details of the isolation procedure as practiced by various laboratories because even seemingly trivial factors may have a significant impact on ultrasound production. For example, given the present and previous findings suggesting that pressor responses and venous return influence ultrasound production, such factors as whether or not the pup is grasped and oriented in a head-up or head-down position during a transfer procedure could significantly affect rates of ultrasound production [8]; indeed, Youmans and colleagues used body tilt as one means of manipulating venous return and thereby modulating expression of the ACR in dogs [38]. Therefore, the brief emission of ultrasound production that follows SNP administration may provide a model of the physiological consequences of the transfer procedure.

Before now, clonidine was the only agent known to be able to evoke ultrasound production under thermoneutral conditions and outside the context of the transfer procedure. The present study adds two new agents to this list, chosen specifically for their cardiovascular effects. That antihypertensives such as clonidine, SNP, and chlorisondamine evoke ultrasound production is difficult to incorporate into the dominant view that these vocalizations are expressions of emotional distress or anxiety. Moreover, one cannot reasonably argue that any drastic change in cardiovascular function triggers a state of emotional distress—and therefore ultrasound production—because infants did not vocalize in response to hydralazine administration and the consequent cardiovascular changes.

Fig. 4 provides an updated version of our working model of ultrasound production in infant rats. On the far left of the figure, the pathway from extreme cold exposure to ultrasound production is summarized; the experiments supporting this pathway were performed primarily in 8-day-old rats. On the far right of the figure, the pathway from clonidine administration to ultrasound production is summarized; the experiments supporting this pathway were performed in 8- and 15-day-old rats. Finally, the figure presents the hypothesized pathways by which SNP and chlorisondamine resulted in ultrasound production in 15-day-old rats in the present study. This figure illustrates how these four

stimuli and pharmacological agents can have a variety of physiological consequences—some unique and some shared—but nonetheless act similarly to impede venous return and thereby trigger ultrasound production. Therefore, although ultrasound production in infant rats has been viewed primarily as a tool for investigations of distress and anxiety, it may prove more useful for basic investigations of the maintenance and development of cardiovascular and cardiorespiratory function.

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