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BRIEF COMMUNICATIONS

Separable Brainstem and Forebrain Contributions to Ultrasonic Vocalizations in Infant Rats

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Competing views persist concerning the functional significance of ultrasonic vocalizations (USVs) emitted by infant rats. One perspective holds that USVs result from an emotional state of fear and anxiety, the adult expression of which depends in part on forebrain mechanisms. Here the authors examine whether pups lacking forebrain input are capable of emitting USVs. Aspirations of neocortex and hippocampus or precollicular decerebrations were performed on 8-day-old rats. After the rats recovered, USV responses were recorded for 10 min at room temperature (Phase 1) followed by enhanced cooling for 20 min (Phase 2). Experimental pups emitted significantly fewer USVs than shams during Phase 1 but vocalized at similar rates during Phase 2. Thus, in infants, brainstem neural circuitry is sufficient to support emission of USVs.

Keywords: Anxiety, fear, periaqueductal gray, amygdala, temperature

The ultrasonic vocalizations (USVs) of infant rats in response to isolation are often interpreted as acoustic signals of fear and anxiety (Shair, Brunelli, Masmela, Boone, & Hofer, 2003). Our current understanding of fear and anxiety, however, derives largely from work in adults. For example, adult rats exhibit unconditioned and conditioned fear responses that include behavioral inhibition (i.e., freezing), increased body temperature, defecation, and emission of USVs at a frequency of 22 kHz (Choi & Brown, 2003; Godsil, Quinn, & Fanselow, 2000; Lee & Kim, 2004). Given that the adult's 22 kHz vocalization shares several features with the infant's 40 kHz "isolation call" (Blumberg & Alberts, 1991), it may be that the infant vocalization is homologous with the adult's.

Investigations of the development of conditioned fear highlight the need for rigorous, systematic analyses that consider the differential ontogenies of sensory and response systems as well as the intrinsic neural circuitry that mediates fear responding (Hunt & Campbell, 1997). For example, in rats, conditioned freezing, heart rate, and potentiated startle emerge sequentially beginning around Postnatal Day 16 (P16), with tests that rely on auditory cues producing conditioned responses several days earlier than tests that rely on visual cues. Freezing in response to unconditioned stimuli (e.g., an unfamiliar adult male) occurs as early as P12; moreover, isolation-induced USVs appear to be suppressed by the presence of an unfamiliar male at this age but not at P9 or younger (Takahashi, 1992). Suppression of a vocal response, however, does not necessarily reveal the motivation for the initiation of the vocalization; for example, if isolation evokes a state of fear in infants that triggers emission of USVs, then does the suppression of the vocalization in the presence of an unfamiliar male—a supposed threatening stimulus—indicate the opposite of fear? Such paradoxes lingering in the literature should spur us to more critically evaluate our guiding assumptions.

There are other reasons to question the widespread assumption that infant USVs signal a state of fear and anxiety akin to that in adults. For example, norepinephrine, a beta-adrenoceptor agonist that is anxiogenic in adults, inhibits USVs in infants (Blumberg, Johnson, & Middlemis-Brown, 2005; Farrell & Alberts, 2000); conversely, the alpha-2 agonist clonidine is anxiolytic in adults, and yet it evokes USVs in infants (Blumberg, Sokoloff, & Kent, 2000; Kehoe & Harris, 1989). Such observations could indicate that infant fear responses are differently organized—pharmacologically and anatomically—than those in adults. Alternatively, and in contrast with the conventional interpretation of infant USVs, the possibility remains that these vocalizations are acoustic by-products of a physiological maneuver that maintains cardiopulmonary homeostasis (Blumberg & Sokoloff, 2001).

Ultimately, resolving questions concerning the functional significance of infant USVs may arise from investigations of their neural substrates, about which little is known. As one step toward this goal, this study addresses the question of whether the forebrain is necessary for the production of infant USVs. Forebrain input to the brainstem was removed in P8 rats either by aspiration of cortical tissue or by precollicular decerebration. After recovery from surgery, USVs were measured as pups were moved from an incubator to a test chamber for 10 min at room temperature and were then exposed to enhanced cooling for an additional 20 min. The results demonstrate that brainstem neural circuits are sufficient for the production of USVs but, in addition, suggest that forebrain mechanisms modulate the vocal response at this age.

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Method

All experiments were performed in accordance with National Institutes of Health guidelines for the care of animals in research and were approved by the Institutional Animal Care and Use Committee of the University of Iowa.

Subjects

Thirty-five P8 Sprague-Dawley male ($n = 19$) and female ($n = 16$) rat pups from 25 litters were used. Body weights ranged from 16.4 g to 23.4 g ($M = 19.6$ g; 1 decerebrated pup was undersized for its age, weighing only 13.7 g, but nonetheless behaved similarly to other pups in its group). All pups were born to females in the animal colony at The University of Iowa. 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 standard laboratory cages (48 cm × 20 cm × 26 cm) where food and water were available ad libitum. All animals were maintained on a 12-hr light–dark schedule with lights on at 7 a.m.

Surgery

On the day of testing, a pup was removed from the litter, weighed, and randomly assigned to one of three groups: aspiration, decerebration, or sham. Under isoflurane anesthesia, aspirations ($n = 15$ from 10 litters) were performed by first drilling two 2-mm trephine holes bilaterally in the skull midway between bregma and lambda and 1–2 mm from midline. A blunted 25-g needle was then inserted into each hole and swept using a caudal-to-rostral motion so as to transect the lateral segments of neocortex. Next, a third hole was drilled approximately 1 mm rostral to bregma, and a blunted 25-g needle was inserted and swept from side to side so as to transect the brain just rostral to the basal forebrain. Finally, neocortex and hippocampus were aspirated by suction through a 16-g needle. In those cases in which heavy bleeding ensued, gel foam soaked in saline or thrombin was inserted into the vacant space within the skull.

Precollicular decerebrations ($n = 11$ from 8 litters) were performed by drilling a single hole just caudal to lambda with an 18-g needle. Then, a blunted 25-g needle was inserted and swept from side to side (Kreider & Blumberg, 2000). Finally, in sham animals ($n = 9$ from 9 litters), holes were drilled in the skull as in either the aspiration or decerebration procedure, but the brain was not manipulated. In all three groups, the skin was then closed using cyanoacrylate adhesive. All surgical procedures were completed in less than 15 min.

In addition to the pups that contributed data to this experiment, 22 pups could not be tested after surgery. Of these, 13 died after the aspiration surgery, and 9 died after the decerebration surgery.

Test Environment

All pups were tested unrestrained inside an empty glass container (22 cm × 12 cm × 7 cm). Air temperature inside this open-air container was the same as room temperature, which was approximately 23.5 °C. A microcamera above the container was used to record behavior to videotape.

USVs

USVs were detected with a bat detector (Anabat, Titley Electronics, Ballina, Australia), the output of which was connected to the audio input of a videotape recorder. The microphone was placed just above the testing chamber.

Physiological Temperatures

Before and immediately after each test, interscapular temperature (T_{is}) was measured with a digital thermometer (Omega Engineering, Stamford, CT). Temperatures were measured by holding a chromel-constantan thermocouple (Omega Engineering, Stamford, CT) against the interscapular skin until a stable reading was obtained. Thermocouples were calibrated before the experiment with a mercury thermometer with an accuracy of 0.1 °C.

Procedure

Pups recovered individually for at least 3 hr in a humidified incubator maintained at thermoneutrality (i.e., 35 °C). Several pups overheated significantly after decerebration (i.e., above 40 °C), most likely due to disinhibition of brown adipose tissue thermogenesis (Blumberg, Schalk, & Sokoloff, 1995). These pups were placed in an incubator at 33 °C, but only 1 pup returned to a normal interscapular temperature as a result; this pup was transferred back into the 35 °C incubator 1 hour prior to testing (the other pups were not tested further). Finally, all pups were then fed intragastrically with 0.2 mL of warm half-and-half at least 30 min before testing.

Before and after each test, the container was cleaned with alcohol. The test began by transferring the pup 1–2 ft (.305–.610 m) from the incubator to the glass container; we always wore latex gloves while handling pups. During the test, which lasted 30 min, behavior and vocalizations were recorded to videotape. For the first 10 min (Phase 1), the pup was tested at room temperature. Then, for the remaining 20 min (Phase 2), an ice pack was carefully placed in direct contact with the underside of the glass container (the floor of the glass container typically cooled to a minimum of 6–8 °C after 10–15 min of contact with the ice pack). After testing, aspirated and decerebrated pups were overdosed with sodium pentobarbital and perfused through the heart with formalin and saline.

Histology

After perfusion, brains were removed from the skull and suspended in sugared formalin. The brains of aspirated pups were examined and photographed for later reference. The brains of decerebrated pups were also examined to assess the location and completeness of the transections.

Data Analysis

Ultrasonic vocalization data were scored from videotape by an experienced observer. The observer, blind to the experimental condition, used an event recorder written in HyperCard for the Macintosh and pressed a computer key each time an ultrasonic vocalization was detected. Group differences in ultrasound production were tested with the Kruskal-Wallis test; post hoc pairwise comparisons were performed with the Mann–Whitney U test. Group differences in T_{is} were tested with a single-factor analysis of variance; the post hoc test was Fisher's protected least significant difference. To examine the relationship between the level of the decerebration and the number of USVs emitted, we used a Spearman rank correlation. For all tests, alpha was set at .05.

There were several occasions when 2–3 littermates were assigned to the same experimental group. Although littermate data are presented individually below, they were averaged before statistical analysis to avoid biases arising from within-litter effects (Abbey & Howard, 1973).

Results

Examination of the aspirated brains revealed that in all cases, the majority of neocortex and all of hippocampus was removed (Figure 1A). In several cases, the ventral portions of the temporal lobe, including the most ventral portions of the amygdala, remained intact. The decerebrations were all precollicular and ranged along the ventral surface from caudal to midhypothalamus (Figure 1B).

Analysis of variance revealed significant group differences in T_{is} both before, $F(2, 18) = 4.0$, $p < .05$, and after, $F(2, 18) = 16.6$, $p < .0001$, the test. As shown in Table 1, decerebrated pups were

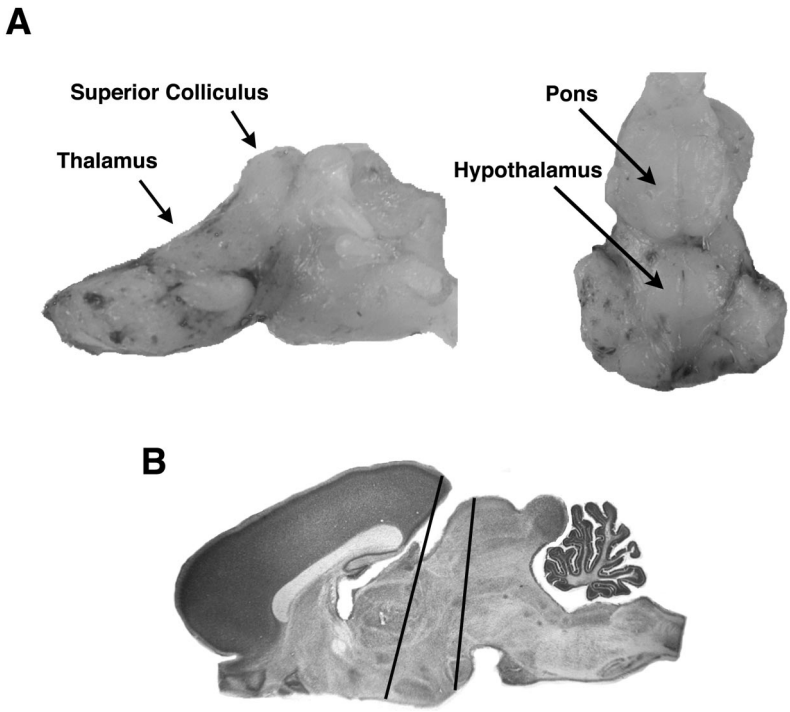

Figure 1. A: Sagittal (left) and ventral (right) views of a representative Postnatal Day 8 (P8) rat brain in which neocortex and hippocampus were aspirated. It can be seen that the most ventrolateral sections of neocortex remain attached. This pup emitted 1 ultrasonic vocalization during Phase 1 and 312 USVs during Phase 2. B: Midsagittal section of a P8 rat brain indicating the rostral-to-caudal range of precollicular decerebrations in this experiment.

significantly warmer than sham controls before the test, which was expected given earlier findings that precollicular decerebrations disinhibit brown fat thermogenesis at this age (Blumberg et al., 1995). In contrast, after the test, pups in both experimental conditions were significantly cooler than the sham controls and were not significantly different from each other. Thus, even though decerebrates began the test with higher T_{is} values, both decerebrated and aspirated pups cooled more rapidly than shams during the test. This likely resulted from a combination of a diminished capacity to regulate heat production physiologically and behaviorally. With regard to behavioral thermoregulation, it was noted that sham pups typically propped themselves up against the wall of the test container (reducing contact with the coldest part of the container surface and thereby diminishing heat loss) whereas the decerebrated and aspirated pups typically remained still on the surface of the container.

Table 1
Mean Interscapular Temperature (T_{is}) Before and After the Test in Aspirated, Decerebrated, and Sham Control Postnatal Day 8 Rats

	T_{is} before test (°C)		T_{is} after test (°C)	
Group	*M*	*SE*	*M*	*SE*
Aspiration	37.7	0.3	22.0	1.2
Decerebration	38.0[a]	0.2	22.2	1.0
Sham	37.0	0.2	29.6[b]	0.7

[a] Significant difference from sham. [b] Significant difference from other two groups.

Mean rates of ultrasound production for each group over the entire 30-min test are shown in Figure 2A. It can be seen that sham controls vocalized at high rates throughout the test. In contrast, both the aspirated and decerebrated pups vocalized at low rates during Phase 1 before increasing their vocalization rates during Phase 2 to the same level as the shams.

These observations are supported by statistical analysis of USV rates during the test. Specifically, there was a significant effect of group on rates of USV during Phase 1, Hypothesis$_2$ (H_2) = 6.8, $p < .05$ (see Figure 2B). Post hoc tests indicated that aspirated pups vocalized significantly less than did shams ($z = 2.5$, $p < .05$), but the difference between decerebrated pups and shams did not reach statistical significance ($z = 1.8$, $p = .07$). Vocalization rates also did not differ between the aspirated and decerebrated pups ($z = 0.3$, *ns*).

In contrast to Phase 1, there were no significant group differences in rates of ultrasound production during the first 10-min period of Phase 2 ($H_2 = 2.8$, *ns*), even though the aspirated and decerebrated pups continued to vocalize at relatively low rates (see Figure 2A). A significant group difference was also not found during the final 10-min period of Phase 2, $H_2 = 1.2$, *ns*, by which time rates of vocalizing by aspirated and decerebrated pups had increased substantially (see Figure 2C).

To examine the relationship between the placement of the decerebration and the total number of USVs emitted, we rank-

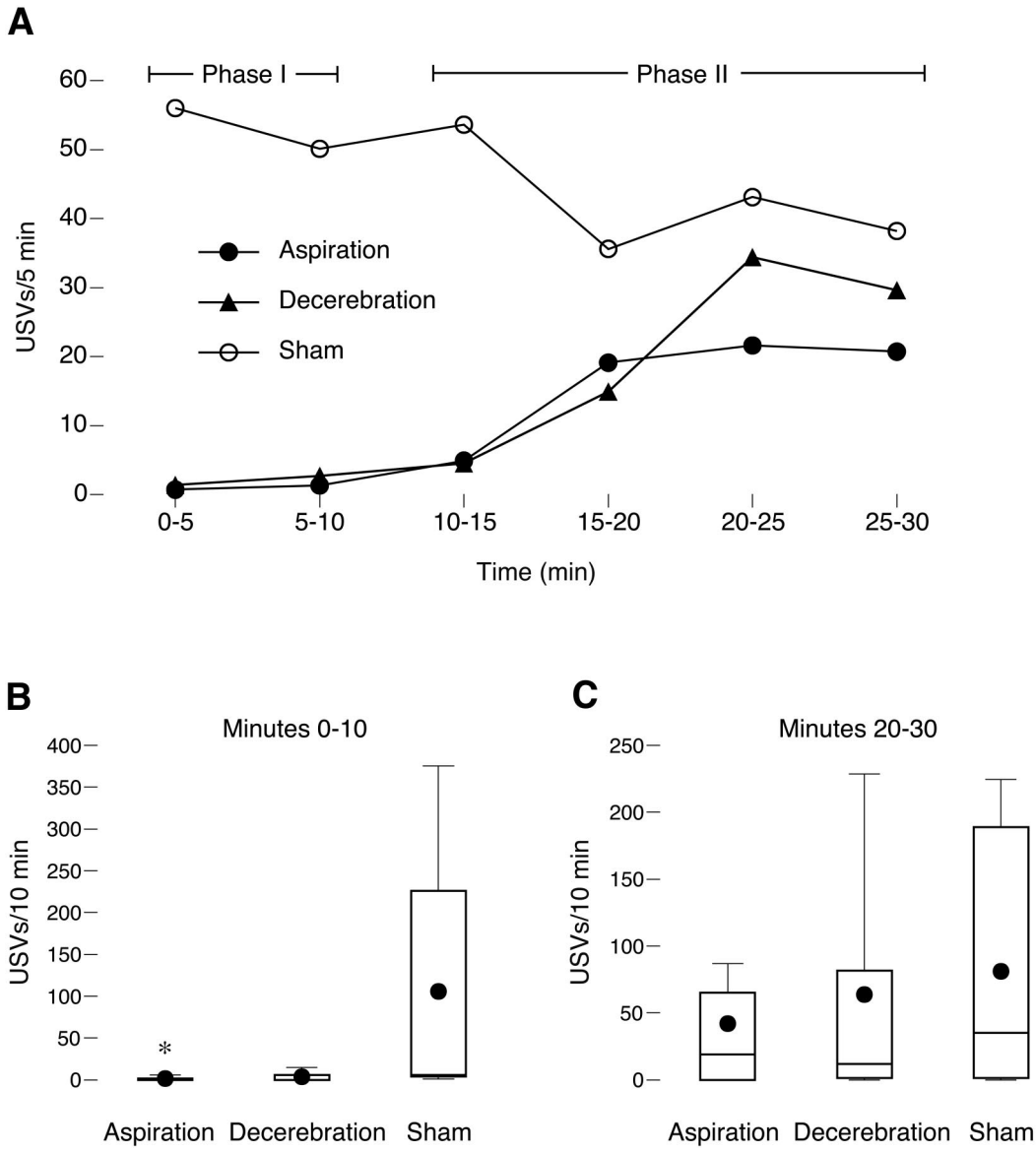

Figure 2. A: Mean number of ultrasonic vocalizations (USVs) emitted by Postnatal Day 8 rats during each 5-min period of the 30-min test. Pups experienced either aspiration of neocortex and hippocampus ($n = 15$), precollicular decerebration ($n = 11$), or sham surgery ($n = 9$). The test consisted of moving the pup from an incubator to a bare glass container for 10 min (Phase 1), after which an ice pack was placed under the container for an additional 20 min (Phase 2). For clarity, error bars are not shown. B: Box plots depicting the number of USVs emitted during Phase 1 (i.e., the first 10 min of isolation). C: The number of USVs emitted during the last 10 min of Phase 2. The top, middle, and bottom horizontal lines of the box represent the 75th, 50th (median), and 25th percentiles, respectively. The vertical lines above and below the box represent the 90th and 10th percentiles, respectively. Filled circles are means. The asterisk indicates significant difference from shams.

ordered the decerebrations from caudal to rostral along the ventral surface of the brain. A Spearman rank correlation revealed no relationship between decerebration placement and USVs emitted ($z = 0.5$, *ns*). Indeed, 4 of the pups with the most caudal decerebrations emitted the most vocalizations; three of these decerebrations began dorsally in the superior colliculus and passed through the caudal hypothalamus. Moreover, the pups with the most caudal and rostral decerebrations, depicted in Figure 1B, emitted 28 and 2 USVs, respectively. When it is considered that these decerebrations result in damage to nearby tissue, it seems unlikely that the caudal hypothalamus played a significant role in the USV responses of these pups.

Discussion

The present results constitute proof that neural circuits at or caudal to the level of the midbrain are sufficient for organizing the week-old rat's vocal response to isolation and cooling. Moreover,

the similar rates of vocalization between the aspirated and decerebrated pups suggests that the diencephalon—or, more precisely, the tissue cut off by the decerebrations but spared by the aspirations—does not contribute on its own to the emission of USVs. In intact pups, however, the diencephalon could contribute via ascending projections to more rostral forebrain structures.

During the initial 10-min test period at room temperature (i.e., Phase 1), both decerebrated and aspirated pups emitted significantly fewer USVs than did shams. Because it has been shown in P12–P13 rats that even combined lesions to the olfactory and trigeminal systems do not disrupt the vocal response to isolation (Hofer & Shair, 1991), it is unlikely that the surgical prevention of olfactory input in the present experiment was responsible for the observed decreases in the emission of USVs. Thus, the present results suggest that the aspirated and decerebrated pups exhibited fewer USVs during Phase 1 because the rostral flow of information from brainstem to forebrain was prevented.

Because combined lesions of the olfactory and trigeminal systems do not suppress USVs in response to isolation, Hofer and Shair (1991) suggested that thermal cues may be sufficient to stimulate USVs. Indeed, cooling has long been known to be a potent stimulus for the elicitation of USVs in infants (Allin & Banks, 1971; Blumberg, Efimova, & Alberts, 1992a, 1992b; Okon, 1971). Thus, it is possible that the aspirations and decerebrations in the present experiment raised the USV activation threshold to thermal stimulation, thereby suppressing USVs during Phase 1; accordingly, the enhanced thermal stimulation during Phase 2 may have evoked USVs by overcoming this raised threshold. This scenario is consistent with the notion that even in adult rats, the brainstem alone contains sufficient circuitry to detect a thermal stimulus and activate appropriate thermoregulatory responses (Rathner, Owens, & McAllen, 2001).

There are, however, other possible explanations for the present results. For example, consider that two response phases can be identified in the vocal behavior of pups isolated at room temperature: First, a rapid USV response occurs before substantial decreases in body temperature are detected, followed by a progressive and prolonged USV response that mirrors the physiological consequences of extreme cooling. Accordingly, perhaps the blunted USV responses of the experimental pups during Phase 1 followed by the increased USV responding during Phase 2 reflect differential interference with these two phases of vocal responding. (In considering this possibility, we should caution that by enhancing cooling during Phase 2, our procedure confounded the effects of time and temperature on emission of USVs; thus, it is possible that the experimental pups would have vocalized sooner had they been cooled more quickly immediately after transfer from the incubator. On the other hand, this confound does not discredit the finding that sham pups vocalized significantly more than experimental pups during Phase 1.)

Thus, it is possible that the USV response to isolation entails an initial conditioned component (in which temperature and other stimuli are conditioned stimuli) and a subsequent unconditioned component (in which cooling is the primary unconditioned stimulus). Indeed, soon after birth, even modest cooling—perhaps occurring during periods when the mother leaves the nest—could act primarily as an unconditioned stimulus, triggering physiological changes in the pup that, in turn, evoke USVs, perhaps as an acoustic by-product of a physiological process (Blumberg & Sokoloff, 2001; Blumberg, Sokoloff, & Kent, 1999). Then, over time, as a pup experiences repeated cooling episodes and as its thermal inertia increases, the salience of temperature as a predictor of the physiological consequences of the cold will also increase; accordingly, the two aspects of the USV response will become increasingly distinguishable. In this way, the role of temperature as conditioned stimulus will differentiate from its role as unconditioned stimulus. Finally, with early experience, odors and other nonthermal stimuli might become associated with thermal stimuli, resulting in the conditioned modulation of USVs and related physiological responses (see Farrell & Alberts, 2000, for discussion). Clearly, for such a learning scenario to be plausible, more information is needed concerning changes in nest and pup temperatures when mothers terminate their nesting bouts (Leon, Croskerry, & Smith, 1978).

The perspective just outlined mirrors the two primary methods currently used to examine USV responses in infants: the *isolation paradigm*, in which pups are isolated from the nest and littermates for several minutes, and the *controlled cooling paradigm*, in which pups are acclimated to a thermoneutral environment and then cooled in a controlled fashion (for review, see Blumberg & Sokoloff, 2001). Not surprisingly, these two methods mirror different perspectives concerning the functional significance of USVs. Perhaps, however, a two-process model like that outlined above can unify these two perspectives under a single conceptual framework.

What form should this framework take? One possibility is to consider cooling—especially extreme cooling that overwhelms homeostatic regulation (Blumberg, 2001)—as a threatening situation for the infant. Accordingly, any reliable predictor of this threat could be viewed within the context of conditioned fear. Given the prominent role of the amygdala in fear conditioning (Davis, 1992), and in light of similarities between infant and adult ultrasonic vocalizations (Blumberg & Alberts, 1991), it is noteworthy that amygdala lesions in adult rats inhibit conditioned fear responses, including USVs and freezing (Koo, Han, & Kim, 2004). Changes in amygdalar function may also underlie developmental changes in conditioned fear, but at this time there is little empirical support for this notion (Hunt & Campbell, 1997), although there is evidence that the amygdala can modulate associative learning during the first postnatal week (Sullivan & Wilson, 1993). Thus, it is an open question as to whether infant USVs are modulated by the amygdala or any other forebrain structure implicated in unconditioned or conditioned fear. Indeed, it remains possible that infant and adult fear responses are mediated by different neural systems.

The neural substrates of adult fear are the subject of intense investigation (Davis, 1992; Graeff, 1990). Central to this system is the periaqueductal gray (PAG), a large midbrain structure capable of organizing complex responses—including freezing and vocalization—to fear-producing stimuli (Behbehani, 1995; Fanselow, 1991). Electrical stimulation of the PAG evokes USVs in adult rats, even after midcollicular decerebration (Yajima, Hayashi, & Yoshii, 1980); infusion with kainic acid also evokes USVs in adults (Depaulis, Keay, & Bandler, 1992). Nonetheless, it is thought that this structure's contributions to the production of fear and anxiety arise from its numerous, dense connections with a variety of forebrain structures, including the amygdala, hippocampus, and neocortex (Behbehani, 1995; Davis, 1992; Graeff, 1990; McNaughton & Corr, 2004). Thus, although conditioned and unconditioned responses to threat are organized within the forebrain,

the PAG serves primarily as the final common path for the expression of organized fear responses.

The PAG was not damaged in pups receiving either the aspirations or decerebrations. Although much less is known about the role of the PAG in infant rats, lesion and stimulation studies indicate a role for this structure in the emission of USVs. Specifically, PAG lesions at P7 resulted in reduced isolation-induced USVs at P10 (Wiedenmayer, Goodwin, & Barr, 2000). The pharmacological mechanism underlying PAG function, however, appears to change with age. For example, in contrast with adults (Depaulis et al., 1992), stimulation of the PAG with kainic acid did not evoke USVs in pups at P7, P14, or P21 (Goodwin & Barr, 1998). In addition, infusion of the kappa opioid agonist U50,488 into the PAG significantly increased USV emissions at P7 but not at P14 or P21 (Goodwin & Barr, 2005). Whether the infant PAG plays a role in the production of USVs during prolonged cooling is not known at this time.

Finally, it is possible that USVs reflect a conditioned response to cooling but that fear and anxiety are not critical factors in this response. To appreciate this possibility, consider that infant rats learn to associate dehydration with the prior consumption of dry food and, in time, begin drinking in close association with eating (Hall, Arnold, & Myers, 2000). However, the fact that drinking can occur as a conditioned response to eating dry food does not, of course, imply that drinking does not serve a physiological function. Similarly, if USVs are, indeed, acoustic by-products of a physiological maneuver, then the notion that the vocalizations may occur as a conditioned response to isolation does not necessarily imply that they do not serve a physiological function.

In pursuing future work, it is important that investigators distinguish between the conditions that evoke USVs and those that modulate them—one is not simply the reversal of the other (Blumberg & Sokoloff, 2001; Hofer & Shair, 1991). In addition, the possibility that the cold can act as both a conditioned and an unconditioned stimulus suggests that body temperature alone cannot be used to rule out thermal influences on emission of USVs; thus, the perception of cold may be as important as the physiological consequences of cooling, especially during the first few minutes of isolation.

The present results do not resolve uncertainties concerning the causes and functions of infant USVs (Blumberg & Sokoloff, 2001). They do indicate, however, that brainstem mechanisms alone are sufficient for producing these vocalizations early in development. Moreover, the present results highlight the need for more detailed developmental investigations of the neural substrates of USVs within the different contexts in which these vocalizations are evoked. Such investigations may help elucidate the degree to which infant USVs should be considered the outward manifestation of fear or anxiety, as well as the degree of homology between infant and adult USVs.

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