

Ultrasonic vocalizations elicit orienting and associative reactions in preweanling mice

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Abstract. On postnatal days (PND) 12 and 13, 90 male Swiss CD-1 mice were tested for orientation to 3 intensities of recorded ultrasounds while climbing an inclined wire grid surface. Motor responses and vocalization to replayed ultrasounds (55-75 kHz) of 20-, 40-, and 60-dB SPL indicated an intensity dependence. In Experiment 2, 138 pups were exposed to either contingent or noncontingent pairings of recorded ultrasounds of 55-75 kHz, averaging 40 dB, and mild inescapable footshocks, or taped vocalizations or footshocks only on PND 12, 14, or 16. At PND 18, subjects were tested for passive avoidance following exposure to the taped ultrasounds only upon entry into the dark side of a black-white compartment. Results suggested only overall, nonspecific effects of pretreatment to elicit responses antagonistic to motor activity. In Experiment 3, 36 pups at PND 15 were tested for passive avoidance with the ultrasound recordings of 40- or 80-dB onset upon entry to the dark compartment; a third group had no ultrasound exposure. A significant intensity effect confirmed that the ultrasounds had prepotent properties.

Key words: auditory development, passive avoidance, motor development, freezing, ontogeny, taxis

INTRODUCTION

Studies of hearing in young animals have produced behavioral and physiological evidence of profound changes in motor and sensory capacity correlated with anatomical development (Rudy and Hyson 1982, Sanes and Rubel 1988, Donaldson and Rubel 1990, Uylings et al. 1990). On the basis of such studies, we know of rapid changes in the relative dominance of various sensory modalities on which the growing rat pup depends (Westerga and Gramsbergen 1990). In the relationship of modalities, prenatal olfactory information can predict postnatal suckling behavior (Pedersen et al. 1983), while postnatal olfactory dependence interacts with tactile stimulation (Pedersen and Blass 1982, Pedersen et al. 1982).

The major auditory event of the early preweanling period centers on ultrasounds emitted by the pup and recognized by the dam as a signal for pup location (Allin and Banks 1972, Noirot 1972, Santucci et al. 1994), although ultrasounds appear to be involved with other functions, including temperature regulation (cf., Blumberg 1992, Hofer et al. 1993, Goodwin et al. 1994). Depending on species and strains, ultrasonic vocalization in rodents have a typical sequence of characteristic ontogenetic development (e.g., Sales and Smith 1978, Elwood and Keeling 1982), increasing in the first postnatal week, reaching peaks of both rate and intensity around PND 7 in mice and PND 10 in rats, followed by a decrease as other sensory systems become functional until nearly total disappearance around PND 14 in mice (Elwood and Keeling 1982) and PND 18 in rats (Fu and Brudzynski 1994).

The study of neural development underlying auditory functions in rodents suggests that, like other sensory systems, the auditory system undergoes dramatic change during ontogeny. In the rat, the auditory meatus, closed at 10 days of age, begins to open by Day 12 and is fully opened at around Day 14 (Rubel 1984, Kelly 1990). In CD-1 mice, meatus opening occurs between PND 12 and 14 (Alleva et al. 1985) and appears to be under the control of polipeptidic circulatory factors (Calamandrei and Alleva 1993). The developing auditory nerve produces changes in threshold levels and tuning curves of auditory nerve fibers (Brugge 1986). Behavioral evidence of proactive reflexive and associative influences from auditory stimuli exposed to rat pups as young as 14 days of age and tested at Day 18 (Rudy and Hyson 1982, 1984, Hyson and Rudy 1984) supports Rubel's (1984) notion

that cochlear sensitivity may shift during early cochlear functioning up to 18-20 days of age. Friauf (1992) used the expression of *c-fos* protooncogene to mark the frequency-place map in developing and adult rats. From PND 14 onward, both low and high auditory frequencies induced strong Fos immunoreactivity in seven brainstem nuclei, while the tonotopic organization assumed an adult-like configuration.

One initial occasion to investigate detection of sound is that period when the physiological capacity for hearing becomes functional - i.e., at the time of opening of the meatus at PND 12 and just after. This developmental period also corresponds to high frequencies of ultrasound emission in altricial rodents. We pursued this relationship through a study of the detection of recorded ultrasounds (a narrow band with medial frequency of 64 kHz) by Swiss albino mice as young as PND 12 and 13. This range of ultrasounds was selected on the basis of studies of the perception of pup ultrasounds by adult mice (Ehret and Haack 1982, Ehret 1992) and by measurements of mouse ultrasounds in comparison to other species (Nyby and Whitney 1978, Sales and Smith 1978, Blake 1992). We hypothesized that the ultrasounds of litter mates are present in the pups' auditory environment at the time of auditory meatus opening when they may serve some still unknown behavioral regulatory role among litter mates.

EXPERIMENT 1. ORIENTATION TO ULTRASOUNDS

An experiment first measured orientation responses to recorded ultrasound stimuli of the range 55- to 75-kHz that differed in intensity (calibrated at 20-, 40-, and 60-dB SPL, when played at a slow speed). The measurement of orientation to ultrasound vocalization rested on the interruption of movement behavior. To elicit movement in young mice of PNDs 12 and 13, which corresponds to the time of initial eye opening, a taxis reflex (Mazur 1994) was used, which involved the geotropism of the pup's orientation with respect to gravity on an inclined surface.

Methods

Ninety male outbred Swiss CD-1 mice pups (mean body weight = 8.35 g; SD = 0.893) born of 15 dams purchased from Charles River Italia (Calco, Italy) were used. Females were inspected twice daily at 9:00 a.m.

and 8:00 p.m. for delivery (PND 1). All litters were reduced at birth to six males, and housed in acrylic maternity/litter cages with sawdust placed on the floor. Litters were kept in an air conditioned room at $21^{\circ} \pm 1^{\circ}$ and $60 \pm 10\%$ relative humidity with a 12-12 red light/white light cycle (red lights on at 9:30 a.m.). Pellet food (enriched standard diet purchased from Piccioni di Brescia, Italy) and tap water were continually available to the dams through a stainless steel wire cage lid, except when the litter was removed to the testing room.

The ultrasound vocalization stimulus used in this experiment was recorded from a CD-1 mouse, 8 days old, isolated from the dam and litter mates and placed in a constant temperature environment of 25°C . A Brüel and Kjaer microphone (Model 4135), connected to a preamplifier (Brüel and Kjaer, Model 2633), was suspended 1 cm above the level of the mouse. The audio signal was filtered (tunable band-pass Kron-Hite 3500 Filter set at 35-85 kHz), amplified (Brüel and Kjaer, Model 2633), and recorded with a Racal Store 4DS tape recorder using a direct mode recording procedure on Ampex (797-15DWII) tapes. The same instrumentation arrangement was used for recording subjects' ultrasound vocalizations, except that the microphone was fixed at the center of the top of the ramp at about 5 cm above the ramp itself (for further details, see Cagiano et al. 1986, Santucci et al. 1994). Ultrasonic vocalization stimuli were replayed by an amplifier (Ultrasound Advice, Model 5515) and an ultrasonic speaker (Model 5614). During replay, the acoustic signals were observed on high resolution analyzer (Brüel and Kjaer, Model 2033) in order to verify whether the sound parameters (frequency and intensity) of the recorded ultrasound vocalizations reproduced the characteristic of the natural ones.

The procedure for testing orientation extended that proposed by Ehret (1976). Subjects were tested individually for orientation to counterbalanced presentations of the three intensities of recorded ultrasounds at PNDs 12 and 13, when each subject was placed individually on the center of a 50- x 12-cm wire grid (each grid was 0.9 cm square) inclined at 45° . Testing occurred in a sound-attenuating chamber (Amplisilence, Robassomero, Italy), with a dim, 15 w bulb suspended 60 cm above the center of the grid, and the room temperature was kept at $22 \pm 2^{\circ}$. Each subjects was placed at the center of the inclined plane and allowed an acclimation period of 15 min during which it could move freely along the inclined surface. Subjects rarely climb the boarder of the surface, but if they did, a piece of plexiglass was placed to block fur-

ther advance. Three young subjects had such rapid movements that they fell off the plane surface. These subjects were replaced with naive animals. At the conclusion of the acclimation period, subjects were placed in a holding cage for 5 min. The testing period consisted of placing the subject at the center of the plane surface, and one of the three intensities of the recorded ultrasound was presented for a duration of 5 s. Head movements, cessation of motor activity, and pups' vocalizations to replayed ultrasound stimuli were videotaped. The pups were returned to the holding cage for 5 min, then placed on the surface and the next recorded intensity was presented. At the conclusion of the three presentations, subjects were returned to the litter. The frequencies of each response were scored independently by two observers subsequently viewing the videotapes. After brief training with trial tapes and resolution of differences, the total disparity in the frequency of observations from the tapes was less than 2% on any of the three measures of orientation.

For statistical tests in this experiment as well as for Experiments 2 and 3, analyses of variance (ANOVA) were performed (Winer 1971, Chiarotti et al. 1987) on orientation response data and on latency data. Post-hoc comparisons were made by Duncan Tests.

Results and discussion

Observations of head movements did not reveal any systematic effects from ultrasound onset or offset. The remaining observations of cessation of motor activity and detection of pups' vocalization associated with onset of the ultrasound probe stimuli were collated to form a composite measure of orientation. Figure 1 shows the mean number of composite orientation responses from the subjects tested on both days to each ultrasound probe intensity level for the stimulus frequency range from 55- to 75-kHz. A clear intensity dependency emerged in the young mice across testing Days 1 (open bars) and 2 (slashed bars). None of the animals on either day seemed to detect the 20 dB stimuli indicating that the weakest intensity was subthreshold for this age level. An improvement in detection emerged from testing at PNDs 12 to 13. Overall analysis of a composite measure of responses to ultrasound vocalizations revealed a marginally significant effect of testing days ($F_{1,106} = 3.82$, $P=0.0533$), a highly significant effect of ultrasound intensity ($F_{2,212} = 41.81$, $P<.0001$, and a marginal effect of their interaction ($F_{2,212} = 2.99$, $P=0.0583$). Neither the main nor the in-

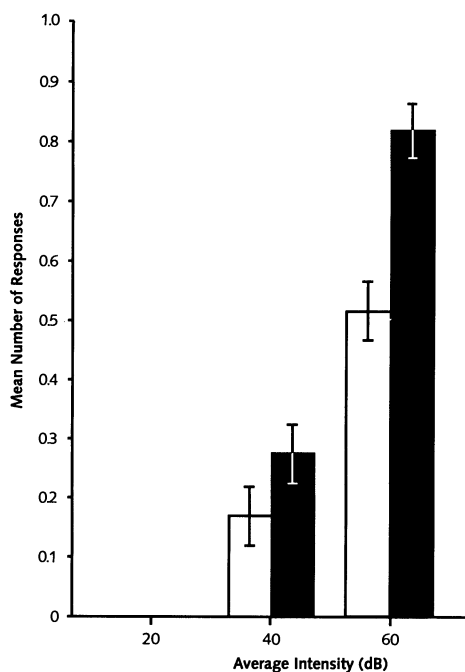


Fig. 1. Mean number and associated SEM of detections of ultrasound stimuli of each intensity in Experiment 1. Open bars indicate responses on Day 1 (PND 12); closed bars on Day 2 (PND 13).

teractive effects with the litter of subjects' origin proved significant.

The results of Experiment 1 indicated that subjects could detect, through behavioral responding, ultrasound vocalizations at an age when the auditory system is only beginning to acquire neurophysiological maturity to support hearing. In addition, mice of PNDs 12 and 13 can discriminate ultrasound stimuli along the intensity dimension.

EXPERIMENT 2. ULTRASOUND ASSOCIATION WITH AVERSION

The purpose of the second experiment was to determine whether conditioned associations with ultrasounds and mild aversion would transfer to a passive avoidance task later in ontogeny, using a paradigm similar to that employed by Hyson and Rudy (1984).

Methods

SUBJECTS

A total of 138 CD-1 pups distributed among age levels of PNDs 12, 14, or 16 at the time of pre-exposure treat-

ment were born of dams, as described in Experiment 1. Except where noted below under the procedures, rearing and housing conditions were the same as described for Experiment 1. At the time of pre-training, subjects weighed as follows: PND 12, $M = 9.1$ g, $SD = 0.74$; PND 14, $M = 10.3$ g, $SD = 0.94$; PND 16, $M = 13.8$ g, $SD = 0.85$.

APPARATUS

Pre-exposure treatments were done in a grey acrylic chamber, measuring 25 x 15 x 25 cm, with a floor composed of 2 mm steel grids spaced 0.5 cm apart and a clear acrylic lid, on which a speaker (Ultrasound Advice, Model 5641) was centered. Scrambled shock pulses of 0.75 mA were generated from a Colbourn Instruments Shock Generator (Model E13-08) to the grid floor. Passive avoidance training was conducted in a black-white, acrylic double compartment chamber, with each compartment measuring 18 x 9.5 x 16 cm. The compartments were separated by a guillotine door, and the floor of the chamber consisted of steel grids of the same width and spacing as the grey, pretreatment chamber. Sound stimuli were presented through a speaker (Ultrasound Advice, Model 5641) mounted 18 cm above the chamber.

PROCEDURE

During the pretreatment phase of the experiment, subjects were exposed to either contingent or noncontingent pairings of taped ultrasonic vocalizations of 55-75 kHz, with an average intensity of 40 dB SPL, and mild inescapable footshock of 0.75 mA, or taped vocalizations or footshock only, beginning at PND 12, 14, or 16. The total duration of preexposure treatment lasted 5 min. At each age level, subjects were pre-exposed to one of the following six treatments (12 days, $n = 6$ /group; 14 days, $n = 8$ /group; 16, $n = 9$ /group):

1. Contingent presentations of 5, 0.5 s footshocks of 0.75 mA were each associated with a 5 s presentation of recorded ultrasound vocalizations ranging from 55- to 75-kHz and averaging 40 dB in intensity. The actual presentation of the footshock pulse was counterbalanced over both the 5 ultrasound presentations and the subjects to occur during each second of the vocalization recording. Each subject was initially placed in the grey acrylic chamber for 1 min without any stimuli, followed by the 5 ultrasound presentations within a 3-min period during which the vocalization recordings were separated by

35-40 s of silence. At the conclusion of the presentations an additional minute of no stimulation occurred, for a total of 5 min of pre-exposure treatment.

2. Noncontingent arrangement of the 5 footshock presentations was randomly distributed within the 3 min period in which the 5, 5 s vocalization recordings were presented as in the contingent treatment. The total pre-exposure duration, including 1 min intervals before and after the 3 min of stimulation, involved a total of 5 min.

3. Ultrasounds only were presented 5 times for 5 s each during the 3 min period, without footshock exposure. As in the other treatments, pre-exposure involved a total of 5 min.

4. Footshock pulses only, without ultrasounds, were presented 5 times during the middle 3 min period of the 5 min pre-exposure period.

5. Apparatus exposure was given for 5 min, without ultrasound or footshock presentations.

6. Non-handled subjects were untouched during the pre-exposure period.

On PND 18, all subjects were tested for passive avoidance, without additional footshock presentations. Subjects were placed in the white compartment of the black-white chamber, and were exposed to the taped ultrasound stimulus only upon entry to the black compartment. Test trials lasted up to 118 s, and subjects were tested for at least 8 trials, which were separated by variable intervals in a holding cage averaging 45 s. The session was discontinued if the subject attained 2 consecutive trials of 118 s, and the number of trials to this criterion as well as trial latencies were recorded.

Results and discussion

Neither the total number of test trials, the number of trials to first avoidance or to 118 s, nor the number of entries to the dark side proved sensitive to pretreatment or to age at pretreatment. Indeed, all subjects, regardless of pretreatment, showed increasing latencies across trials, so that testing trials were terminated after the elapse of 8 trials in all subjects, since they reached an asymptotic level of avoidance. Statistical analyses of the latency data using both repeated measures as well as nested ANOVAs indicated that both of the main effects of age and pretreatment were nonsignificant, and only the obviously pronounced effect of progressive trials ($F_{7,840} = 74.59$, $P < 0.0001$) and the interaction of trials \times age at pretreatment ($F_{14,840} = 2.41$, $P < 0.003$) were significant. This interaction is illustrated in Fig. 2, which depicts the

increasing trial latencies across test trials for subject groups distinguished by their age at pretreatment exposure. Duncan *post hoc* tests revealed that significant group effects were found on the first trial between the fastest latencies from subjects pretreated on PND 12 and slowest from subjects pretreated on PND 16 ($P < 0.05$). On the fourth trial the groups pretreated on PND 14 differed from the other age levels ($P_s < 0.05$), which in turn were not different from each other.

Various combinations of subset groups - e.g., Contingent, Noncontingent, Nonhandled - were examined separately to determine whether the most obvious pretreatment extremes might reflect effects masked by the totality of six group pretreatments. The selected pretreatment group effects were analyzed for the latency measure and the trials measures of the number to the first latency of 118 s as well as the number of entries. However, none of these selections produced any significant effects from pretreatment, and only age at pretreatment and progressive trial effects exerted a marked effect on latencies. For example, examination of trial latencies among only the three groups mentioned above revealed a significant effect of progressive trials ($F_{7,420} = 38.69$, $P < 0.0001$); none of the main effects of Age or Pretreat-

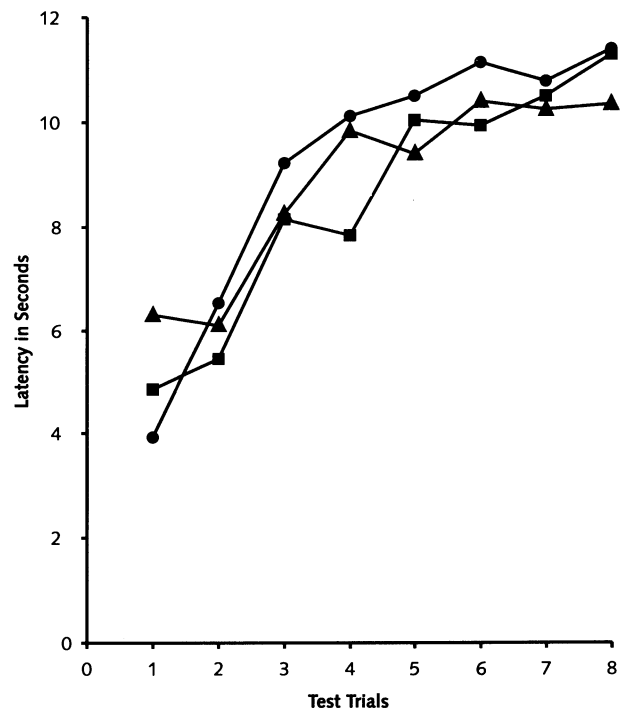


Fig. 2. Mean time to enter the black compartment during the passive avoidance test trials in Experiment 2. Circles indicate pretreatment at postnatal Day 12; squares, Day 14; triangles, Day 16. All passive avoidance testing was conducted on Day 18.

ment or any of the interactions attained significance. Since the pretreatment groups from PND 14 seemed somewhat more variable than the other groups (perhaps due to eye opening on PNDs 12 or 13 producing differences from visual effects), this age level was eliminated, and the other groups compared under a similar rationale. An example of these analyses indicated that when the PND 14 groups were eliminated, analysis of trials latencies produced a significant effect of progressive trials ($F_{3,234} = 37.23$, $P < 0.0001$) and the interaction of trials \times age at pretreatment ($F_{3,234} = 3.78$, $P < 0.011$), a conclusion similar to the overall analysis.

The results of Experiment 2 suggested that ultrasound stimuli, at moderate sound pressure levels, do not exert specific effects in passive avoidance performance for preweanling subjects, but provide considerable signal value, consistent with prior studies (Kent and Grossman 1968, Dokla et al. 1989). While nonspecific and general pretreatment at earlier ages seems to have exerted an effect when tested at PND 18, that effect cannot necessarily be attributed to the ultrasound component of the pretreatment and may be ascribed to handling or other nonspecific contextual factors of the pretreatment procedures. Presentations of ultrasound stimuli alone, or either contingently or noncontingently with shock, did not produce any differential effect; only the nondifferential overriding effect of pretreatment at a particular age exerted any systematic influence.

EXPERIMENT 3. THE AVERSIVE PROPERTY OF ULTRASOUND STIMULI

A final experiment attempted to extend the study of ultrasound stimuli in passive avoidance conditioning by examining more systematically their possible effects on behavior at PND 15. Based upon the results of Experiment 2, it appears that subjects are certainly capable of detecting recorded ultrasounds as early as PND 15, while they also have the motor ability to perform passive avoidance requirements. Two intensities of ultrasound recordings were used to assess whether subjects would avoid their occurrence as an unconditioned stimulus.

Methods

A total of 36 CD-1 pups, born and raised under conditions similar to those described for Experiments 1 and 2, were used in this experiment. At the time of passive

avoidance training, subjects were 15 days of age with a mean weight of 13.87 g (SD = 1.21)

All training was completed within a single session and followed the passive avoidance training procedure outlined for Experiment 2. Individual subjects were placed in the white compartment of the same black-white chamber used in Experiment 2. Entry to the black side produced onset of the taped ultrasound stimulus at the 40 dB value ($n = 12$) or the 85 dB level ($n = 12$). The remaining 12 subjects did not receive any ultrasound stimulation. Test trials lasted up to 120 s, and subjects were tested for at least 8 trials.

Results and discussion

As was the case with Experiment 2, none of the measures of the number of entries to the black compartment or the number of trials to first 120 s latency trial proved systematic. Figure 3 depicts the mean latency over eight training trials for each intensity level group of subjects. Analysis of the latency data (2-way ANOVA with repeated measures) revealed main effects of intensity ($F_{2,33} = 3.60$, $P < 0.039$) and progressive trials ($F_{7,231} = 26.54$, $P < 0.0001$), while the interaction was not significant.

The results of Experiment 3 offer some qualified support that ultrasounds possess inherent properties that can affect behavioral responses in young mice. That is, the detection of ultrasound vocalizations may function in a way that is equivalent to expectations based upon aversive stimuli, such as footshock. It is interesting to observe that, while both ultrasound groups tended to have somewhat disparate response levels on the first trial when they were exposed to the ultrasounds for the first time, they performed at a comparable level on the second trial. Differences attributed to the ultrasound onset seemed to emerge from Trial 3. A confounding influence is apparent with later trials, strongly suggested by the control subjects, -- namely an effect of repeated handling, so that by Trial 5 all groups, including the control, tended to remain in the white compartment, which was probably an expression of freezing behavior. To examine this possibility, separate 1-way ANOVAs were performed on the latencies from Trial 3 and from Trial 4. For Trial 3, the effect of intensity attained acceptable significance ($F_{2,33} = 3.38$, $P < 0.050$) and for Trial 4, the effect reached only a marginal level ($F_{2,33} = 2.11$, $P < 0.10$).

Given the trend in Fig. 3 that shows the control group moving into the black compartment faster than the other

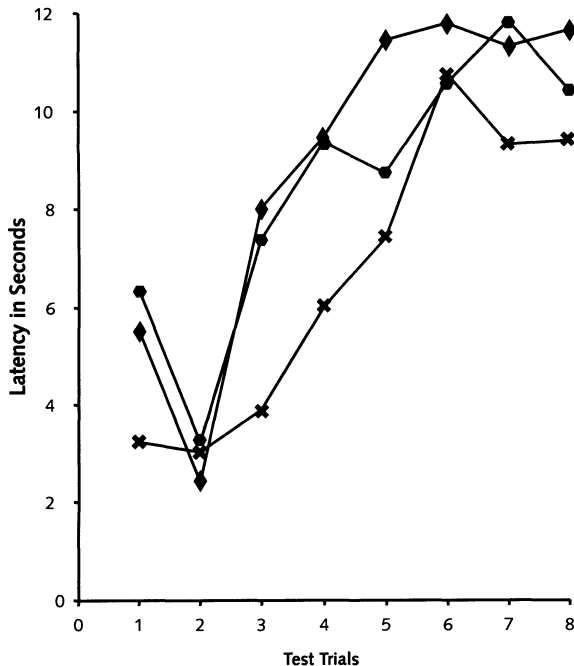


Fig. 3. Mean passive avoidance time to enter the black compartment across test trials in Experiment 3. Crosses indicate the 0 dB control condition; hexagons the 40 dB presentations of ultrasound recordings; diamonds, 85 dB.

groups exposed to ultrasound onset, the results of testing to that point suggest effects from ultrasounds that accounts for the similarity of both ultrasound intensities. That this effect may be aversive supports the conclusion of a brief study with adult rats (Kent and Grossman 1968) and more recent observation reported by Brudzynski and Chiu (1995). The possibility that ultrasound emissions may act as an "alarming" signal for littermates may be taken into account in order to attribute an adaptive value to the reported aversive effect of pup ultrasonic calls.

GENERAL DISCUSSION

This study confirmed and extended several findings from earlier studies of the functional role of ultrasonic vocalizations during pre-weaning ontogeny. First, as shown in Experiment 1, subjects detect ultrasounds in the environment at an age when the auditory system is only beginning to become neurophysiologically functional, and detection is expressed behaviorally. Moreover, an intensity relationship can be discriminated in ultrasonic stimulation in mice as young as PND 12. Secondly, in terms of the qualitative significance of environ-

mental ultrasounds for the perceiving pup, both Experiments 2 and 3 offer qualified support for the signaling value of ultrasounds as sensitive to associative conditioning. Within the simple leaning context of passive avoidance training, a somewhat weak effect emerge, but the data are inconclusive with respect to inherent aversive value of the signal or whether the stimulation is detected as prepotent in the sense of attentional value. The lack of clear evidence of pretreatment effects, predicted from associative conditioning expectations, suggests a more complicated role of environmental ultrasound stimuli. While detected and attended to, further functional value as an inherent property of the signal was not found.

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