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Original Research

Ultrasonic Sound as an Indicator of Acute Pain in Laboratory Mice

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In response to pain, mice may vocalize at frequencies above the range of human hearing (greater than 20 kHz). To determine whether an ultrasonic recording system is a reliable tool for assessing acute pain, we measured audible and ultrasonic vocalization in mice subjected to either nonpainful or potentially painful procedures performed routinely in animal facilities. Data were collected from 109 weanling mice (*Mus musculus*; B6, 129S6-Stab 5b) scheduled for 2 potentially painful procedures: DNA testing by tail snip and identification by ear notching. The mice each were assigned randomly to 1 of 4 groups: 1) actual tail snip, 2) sham tail snip, 3) actual ear notch, or 4) sham ear notch. Vocalizations during the treatments were recorded with an ultrasonic recorder. Most mice (65%; n = 55) demonstrated no vocal response to the potentially painful procedures. More mice that received actual tail snips produced audible sounds (11 of 29 mice) than did those that underwent sham tail snips (0 of 30 mice). In addition, audible vocalizations occurred more frequently during ear notch procedures (8 of 26 mice) than during sham ear-notch manipulations (2 of 24 mice). For all 20 of the mice that produced ultrasonic vocalizations, these calls were accompanied by simultaneous audible components. We conclude that ultrasonic vocalizations do not provide any more information than do audible vocalizations for assessing responses to potentially painful procedures. In addition, because many mice made no sound at all after a potentially painful stimulus, vocalizations generally are not good metrics of acute pain in laboratory mice. Alternatively, the lack of vocalizations in many of the mice may suggest that tail snipping and ear notching are not particularly painful procedures for most of these mice.

In biomedical research, mice routinely undergo potentially painful procedures such as tail snips for DNA collection and ear punching for animal identification. Many also experience some level of discomfort as a direct result of research manipulations or from spontaneous health issues. The assessment of pain in these animals can be difficult because unlike humans, animals cannot convey verbally that they are experiencing pain. Few noninvasive tools are available to provide quantitative measures of pain, especially in small animals such as laboratory rodents. Furthermore, because mice are common prey to a broad range of animals, they may mask the expression of pain[7] and thus complicate accurate assessment of their distress. Research and animal care personnel often must assess rodent discomfort only by observing animal behavior and appearance.[2] Because these assessments may be inaccurate, novel means of quantitative pain evaluation in rodents would be helpful.

Various species of animals, including rodents [1,14] and humans, vocalize in response to pain, and audible vocalizations often are used as an indicator of pain in rodents that are exposed to acute noxious stimuli.[9,10] However, many of the sounds emitted by rodents are beyond the range of human hearing,[2] so further information about pain might be available if observers monitored ultrasonic frequencies in addition to audible ones. In this study, we tested whether ultrasonic vocalization of rodents might function as a noninvasive tool for the assessment of pain.

Ultrasonic frequencies are defined as frequencies above the threshold of human hearing,[3] 20 kHz, and previous studies have shown that rodents emit a variety of social vocalizations at ultrasonic frequencies.[4,8,10,15] For example, vocalizations in the ultrasonic range are made by mouse pups when cold or distressed,[5] male mice in response to the presence of females or their pheromones,[4] and mice during anticipation of punishment or avoidance of a painful stimulus.[12] Ultrasonic vocalizations in response to both acute and chronic pain have been studied in the laboratory rat,[6,9-11] but despite the wide use of mice as laboratory animals, little work has addressed ultrasonic vocalizations in mice.

We recorded the audible and ultrasonic vocalizations of weanling mice during 2 routine laboratory research procedures that we presume cause acute discomfort, tail snipping and ear notching. We asked (a) whether mice make more audible noise when exposed to a potentially painful acute stimulus than during a sham treatment of handling only and (b) whether mice experiencing pain make ultrasonic sounds that are not associated with audible vocalizations and thus are imperceptible to humans.

Materials and Methods

Animals. Data were collected from 109 specific pathogen-free (SPF) laboratory mice (59 male and 50 female; *Mus musculus*, B6;129S6-Stat 5b) of weaning age (21 to 28 d). Mice were bred in the Cornell University, Transgenic Mouse Core Facility (Ithaca, NY). Serology, bacteriology, and parasitology evaluations were performed quarterly, at each cage change for sentinel animals exposed to bedding of the subject mice. These mice were part of an Institutional Animal Care and Use Committee-approved research protocol in which ear punches and tail snips were

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performed; the principal investigator of that study gave written consent to collect auditory data while those procedures were done. We obtained further approval to perform the ultrasonic recording procedures.

Housing. Mice were housed at an AAALAC-accredited institution, in polycarbonate individually ventilated cages (7 × 11 × 5 in.), on autoclaved 1/8-in. corncob grit (1040; Harlan Teklad, Fredrick, MD). Cages were enriched with nestlets (Ancare, Bellmore, NY) and PVC tubing and placed on a rack (Micro-FLO/Micro-VENT Environmental Rack System, Allentown Caging Equipment Company, Allentown, NJ). Mice were maintained on a 14:10-h light:dark cycle, with free access to water through an automated watering system (Edstrom, Waterford, WI) and food (LM 485 Irradiated rat/mouse diet 7912, Harlan Teklad).

Procedures. Recordings were taken in an empty cage-changing station (NU-612 Cage Changing Station, NuAire, Plymouth, MN). We used an ultrasonic microphone to record vocal emissions made by mice during ear notching and tail snipping. A different mouse was used in each trial and underwent only 1 of the 2 treatments (ear punch or tail snip). Each mouse was assigned randomly to actual or sham treatment. Vocalizations of pain subjects were recorded during actual ear notch or tail snip procedures. In compliance with the IACUC-approved protocol, these mice were neither anesthetized nor given analgesia for these procedures. Vocalizations of mice that underwent sham ear punch procedures were recorded while a thumb punch ear notch instrument was punched close to, but not touching, the ear. Similarly, sham tail snips were performed by snipping with a pair of sharp iris scissors beside the tail, without touching it. The random assignment of mice to actual or sham treatments resulted in 59 tail-snip recordings (29 actual, 30 sham) and 50 ear-punch recordings (26 actual, 24 sham). Manipulations were performed by the same animal handler for all subjects. Mice were restrained for a minimum of 3 s prior to performance of the procedure, allowing enough time for the animal to cease any vocalizations that occurred in response to restraint.

Sound recording. During data collection, a high-quality condenser microphone (USG 116 to 200 UltraSoundGate Kit, Avisosft, Berlin, Germany) was pointed directly at the head of the subject at a distance of 10 cm. Recordings were analyzed by using software provided by the manufacturer (SASLab Pro, version 4.3, Avisosft) that was sensitive to frequencies as high as 62.5 kHz. A spectrogram analysis was performed on each recording to determine visually whether any ultrasonic sounds were emitted during a trial, regardless of whether audible sound had been heard.

Statistics. For each trial, we scored the presence or absence of ultrasonic emissions immediately after the procedure was performed, and where ultrasonic calls were present, we scored the presence or absence of audible components to the call. Loudness, duration, and other call parameters were ignored; only presence or absence of sound emission of any frequency was used for analysis. Using χ^2 tests for both comparisons,[17] we compared the presence of audible calls in pain and sham groups in the tail-snip procedure separately from the ear-punch procedure. We tested for the effect of sex on whether an audible call was made by using χ^2 analysis of all 109 trials, regardless of treatment. An alpha level of 0.05 was used to define significance.

Results

All mouse vocalizations (n = 21) were broadband, consisting of several harmonics (Figure 1), and all included components of sufficiently low frequency that the observer could hear the call without specialized equipment. In other words, all calls were audible. All mouse vocalizations also included ultrasonic components, except for the call of 1 female mouse that underwent an actual tail snip. In that 1 trial, no components above 20 kHz accompanied the audible call.

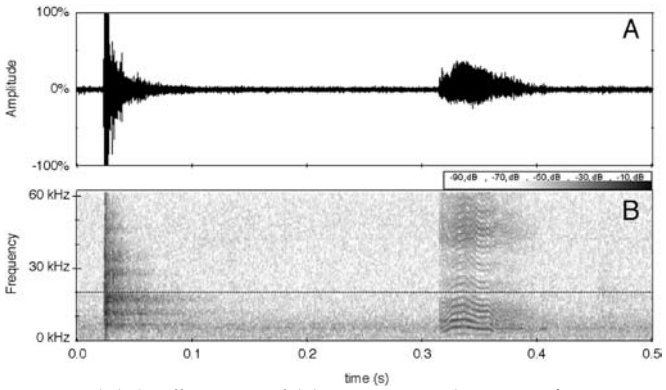

Figure 1. (A) Oscillogram and (B) spectrogram (256-point fast Fourier transform, Hanning window) of a typical mouse call in response to acute pain. First, the scissors made a sound as they closed, removing part of the mouse's tail. Then, approximately 0.3 s later, the mouse made a broadband call with audible (below the dashed line at 20 kHz) and ultrasonic (above the dashed line) components. Although the call contained ultrasonic frequencies, they are accompanied by sounds well within the hearing range typical of humans.

Mice did not reliably vocalize (at any frequency) when treated with actual potentially painful procedures, but audible vocalization was recorded more from mice in the group that underwent actual potentially painful procedures. Only 19 of the 55 mice (34.5%) that received actual tail snips or ear punches vocalized compared with 2 of the 54 animals (3.7%) that underwent sham procedures.

Mice made audible noise significantly ($P < 0.05$) more often during actual tail snipping and ear notching than during sham procedures (Table 1). Of the 21 animals that vocalized, 10 were female and 11 were male. There was no sex-associated difference in vocalization in response to pain ($\chi^2 = 0.44$, $P = 0.50$).

Discussion

The results of this study suggest that neither audible nor ultrasonic vocalizations are a reliable tool for the assessment of acute pain in laboratory mice. Although our data showed that mice vocalize slightly more frequently in response to potentially painful manipulations than to sham procedures, vocalization occurred in fewer than half of the potentially painful trials, and some procedures, including ear notching, caused no more frequent vocalizations than occurred during normal handling. This interpretation of our results presumes that ear notching and tail snipping cause acute pain in laboratory mice. Piercing the pinna or amputation of the distal portion of the tail seems likely to induce pain, but verification of pain would require comparative assessment in the presence of analgesics. Although vocalizations happened more frequently during both treatments than in control animals, a possible interpretation is that the procedures are not particularly painful in most mice.

Although ultrasonic emissions by laboratory rodents are common,[4,8,10,15] our results suggest that monitoring ultrasonic frequencies provides no added benefit to the assessment of acute pain compared with listening for audible calls. These results are similar to those for rats,[11] which showed no direct relationship between any component of ultrasonic vocalization and the presence or absence of chronic pain. Previous studies have demonstrated that compared with males, female rodents

Table 1. Vocalization of mice in response to tail snip, ear punch, and sham procedures

	Actual procedure		Sham procedure		Difference between values for actual and sham procedures	
	No. of mice evaluated	No. (%) that vocalized	No. of mice evaluated	No. (%) that vocalized	χ^2	P
Tail snip	29	11 (37.9%)	30	0 (0%)	13.99	0.0002
Ear punch	26	8 (30.8%)	24	2 (8.3%)	3.93	0.048

For both procedures, the actual procedure resulted in vocalization more frequently than did the sham procedure, but most mice made no sound, regardless of the presence or absence of a potentially painful procedure.

have lower levels of stress-induced analgesia and are more sensitive to noxious stimuli.[16] However, we did not find any sex-associated difference in ultrasonic vocalization of mice in response to pain. Genetic background may influence the presence or perception of pain.[13] All ultrasonic recordings in the current study were collected from the same strain of mice to minimize strain-associated variability in pain perception. However, results may differ for other rodent species or mouse strains. Because our result for mice mirror those found for rats,[11] we recommend against using vocalizations to assess acute pain in laboratory rodents.

The greater number of mice that vocalized in response to tail snips compared with ear notches might reflect the degree of pain caused by the stimulus. Mice vocalized more often in response to actual painful procedures than to sham manipulations and more often in response to tail snips than ear punches; therefore one might infer that tail clipping is a more painful procedure than is ear punching. This information could be useful for researchers and animal care personnel in selecting a method for DNA collection, for example. However, because pain cannot be isolated from other experimental differences between the 2 procedures, our results cannot validly be interpreted in this way. For example, an ear punch is likely to create a greater auditory stimulus than does a tail snip, and this difference might influence resulting vocalization as much as does the experience of pain. An experiment using pain as the sole independent variable, with presence or absence of analgesia, would be necessary to determine how call rate changes with pain. Indeed, animals are not likely to respond in a simple linear fashion, with increasing numbers of calls in response to increasing pain stimulus. More likely is that a threshold exists above which call rate does not increase or may even decrease with increased pain.

To summarize, we found no reliable benefit of ultrasonic recording for the assessment of pain in laboratory rodents. More mice vocalized when they experienced potentially painful procedures, but many mice did not vocalize in response to the same treatments. Therefore, vocalizations might be a clue to the presence of or reaction to pain or the associated manipulations, yet silence may not indicate absence of pain or distress.

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