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Physiologic Stress of Ear Punch Identification Compared with Restraint Only in Mice

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Social housing of laboratory rodents is recommended whenever possible to encourage natural behavior and social dynamics. Several identification methods are used to distinguish rodents from one another. One of the most common means of identifying mice is ear punching. The effect of ear punching for identification or genotyping on the welfare of mice remains a concern, because this method negatively affects welfare in other species. To assess the influence of ear punching on the welfare of mice, we implanted telemetry units in 6 female Swiss–Webster mice and monitored heart rate, body temperature, and activity after various routine procedures. The physiologic and behavioral responses to restraint (by scruffing) only, restraint and ear punching, and routine handling for husbandry were evaluated. The mean heart rate of mice after receiving an ear punch was significantly higher than baseline values at 30 min after the procedure, and the mean body temperature was significantly increased over baseline for at least 1 h. The heart rate, body temperature, and activity levels of mice after scruffing only and routine handling did not differ from baseline values. The proportion of time mice spent head grooming, a potentially nocifensive behavior, was increased immediately after ear punching and began to decline by 60 min. We show that the physiologic stress of mice receiving an ear punch was greater than that from restraint (scruffing) alone, whereas behavioral indices of pain were unchanged, suggesting that ear punching causes a transient response in mice.

Abbreviations: RH, routine handling

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The housing of laboratory mice in harmonious social groups is suggested when possible, because of its ethologic relevance[13] and biobehavioral benefits.[15] Frequently mice that are group-housed need to be identified individually. Means of identifying mice include tattooing, toe clipping, and ear punching. As a means of individual identification, ear punching has been a common practice in laboratory animal science for the better part of a century,[1] is easy to perform and interpret, and can be used for numbering animals from 1 to 999. In both the United States and Europe, ear punching is the most common means of identification of socially housed rodents.[6] In addition to being used for mouse identification, ear punching may also be used to collect samples for genotyping.[2] In calves, ear notching has been shown to induce stress behaviors and increase cortisol levels, likely indicating acute pain as a result of the procedure.[19] Similarly, ear punching in rats increased heart rate and mean arterial pressure, relative to ear tattooing and microtatooing.[16]

Concern exists regarding the effect that ear punching may have on the welfare of mice and the procedure's potential to cause pain and distress. Ensuring optimal animal welfare requires the minimization of pain, and poor welfare practices have the potential to compromise the validity of a physiologic model system.[25] The optimization of animal welfare, in effect, increases model validity, and efforts are underway to minimize potential stressors with laboratory rodents.[11] In calves, the use of analgesics, such as a topical vapocoolant spray, has proven an effective means of decreasing procedural pain associated with ear notching.[19] Because some animals have an increased stress response to ear punching, it has been argued that we should assume the procedure is painful in rodents, until there is evidence to suggest otherwise.[20] Analgesics are not often administered for ear notching of rodents, which is generally considered less invasive than alternative individual identification methods (for example, toe clipping and tail docking). In mice, increases in heart rate and body temperature have been associated with ear punching, but the physiologic changes resulting from ear punching were equivalent to those associated with simple restraint, or handling by the base of tail, moving to the top of the cage, and then scruffing the neck.[4]

Common behavioral assessments of pain in laboratory rodents include grimace scales, burrowing assays, weight bearing, and gait analysis, and the use of behavioral instruments, such as accelerometers and more advanced behavioral spectrometers.[7,8,14,17,18] Because of the somewhat subjective nature of behavioral assays, it is helpful to measure relevant physiologic parameters simultaneously when behavioral spectrometers are not in use. Pain in rodents is associated with an increase in grooming behaviors, at the expense of locomotion,[3] as well as a marked decrease in burrowing behaviors.[6] The Mouse Grimace Scale has recently been used to assess potential pain associated with ear punching in laboratory mice but did not reveal significant differences after ear punching procedures.[20] Previous studies in other rodent models have shown that the most pronounced behavioral indicators of stress or pain occur within 1 h after ear punching.[16] At present, it is unclear whether ear punching is more stressful or painful than restraint by scruffing alone, and here we attempt to characterize the physiologic and behavioral response to ear punching compared with restraint only and routine handling in female mice.

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Materials and Methods

Mice. As a part of a parent study on environmental enrichment, 8 outbred female Swiss–Webster mice (SW-F, Tac:SW) from Taconic Biosciences (Rensselaer, NY) were implanted with radiotelemetry transmitters (ETA-F10, Data Sciences International, St Paul, MN). Briefly, mice were anesthetized and aseptically prepared for surgery. A 1.5- to 2.0-cm midline skin incision was made, and the device was placed on top of the intestines parallel to the long axis of the body. Leads were passed through the abdominal wall and tunneled subcutaneously. The positive lead was sutured adjacent to the xyphoid process, and the negative lead was sutured to the right pectoral muscle. The incision was closed using 5-0 nonabsorbable sutures and wound clips, and mice treated with buprenorphine postoperatively. Before shipment, telemeter-equipped mice were identified with tail tattoos so that they could be distinguished from future cage mates and held for a postsurgery recovery period of 7 d prior to shipment. Mice were pathogen free for Sendai virus, mouse hepatitis virus, minute mouse virus, mouse parvovirus, mouse norovirus, Theiler murine encephalitis virus, rotavirus, *Mycoplasma pulmonis*, pinworms, and ectoparasites. Mice were housed in open-top cages (7.7 in. × 12.2 in. ×5.8 in.; Small Mouse II Cage model no. 9, Thoren Caging Systems, Hazelton, PA). Cages were clear to allow observer visibility, and all behavioral observations for this study took place during the lighted portion of the day. Mice had free access to irradiated feed (Teklad 2918, Envigo, Madison, WI). Cages were bedded with Teklad Sani-chips (Envigo), which were replaced on a biweekly basis. Water was filter-sterilized. Mice were maintained on a 12:12-h light:dark cycle at a temperature of 21 to 24 °C. All experimental procedures were IACUC-approved.

Study design. Mice were housed in groups of 3, with one mouse telemeter-equipped; the 2 other females were used for the parent study but not this study. Mice were allowed to acclimate for 1 wk prior to initiating the studies and were 9 wk old at the time that the study began. Telemetry collection began during the acclimation period, and electrocardiograms were collected continuously as 1-min averages of the heart rate. In addition, core body temperature and activity levels were recorded through telemetry for analysis. Preprocedural (baseline) telemetry values were collected on the most recent day prior to each handling manipulation. A telemetry failure occurred in 2 of the mice, reducing the total number of mice to 6 mice with telemetry.

All handling manipulations occurred during the midpoint of the photoperiod and began with the handler removing the mouse from its home cage by grasping the base of the tail with one hand and supporting the mouse's body with the other hand. Mice were returned to their group cage. The first manipulation, on experimental day 1, involved removal from home cage and placement on top of the cage for restraint by scruffing for 3 s; this interval was chosen as the estimated time that mice are similarly restrained prior to receiving an ear punch. On experimental day 2, mice were restrained by scruffing for 3 s, during which time either their right or left ear was punched once by using a standard ear punch (Kent Scientific, Torrington, CT). A 24-h washout period was deemed appropriate between restraint only and ear punching, because previous studies have seen return to baseline heart rate values within 1 h.[4,16] On experimental day 10, mice were handled for routine husbandry, which included transport to a small container where mice were weighed, and fecal pellets were collected. Care was taken to ensure that mice were handled similarly during all manipulations.

Behavior. The telemeter that we used in this study included a 3-axis accelerometer, which provided real-time information regarding the orientation and acceleration of the implant along the x, y, and z axes. Values from each of the axes are used to calculate total activity in counts per minute. Total activity was measured as 30-min, 1-h, and 24-h averages for all manipulations.

Focal grooming assays (5 min each) were performed on each of the 6 mice immediately after the ear punching procedure and were repeated 1 h later. Mice were observed cageside, the amount of time spent on focal grooming was recorded over a 5-min period, and the proportion of focal grooming was determined. This observation was done to test whether the proportion of the 5-min sample that was spent engaged in focal grooming decreased or extinguished during that interval. Grooming was the only behavior considered in this assay. Because other manipulations did not involve a potentially painful mechanical stimulus, the focal grooming assay was performed for the ear punching procedure only.

Statistical analysis. We performed 2-way repeated measures ANOVA to analyze potential differences in heart rate, body temperature, and activity level as a consequence of treatment compared with the prior day's baseline values. To examine the length of a potential spike in heart rate, body temperature and activity, mean values were collected and analyzed at 3 time points on the day of manipulation: 30 min, 1 h, and 24 h. Data was normally distributed as determined by normal Q–Q plot analysis. Focal grooming assays were analyzed by using a one-way repeated-measures ANOVA. A P value less than 0.05 was considered statistically significant. Statistical analysis was performed by using R Studio (R Studio, Boston, MA). Posthoc observed power between-subject effects were 0.78 for treatment and 0.75 for condition.

Results

Ear punching caused transient changes in both the heart rate and body temperature of mice (Table 1). After ear punching, mice showed a 26.9% increased heart rate relative to baseline for 30 min ($P = 0.004$), but heart rate returned to baseline at the 1-h time point ($P = 0.13$). Although the mean heart rates of the restraint only and routine handling groups were numerically higher than baseline at the 30-min and 1-h time points (by 12.2% and 0.1%, respectively), the differences were not statistically significant. Heart rate returned to baseline by 24 h after ear punching, restraint only, and routine handling.

Body temperature in ear-punched mice was elevated over baseline at the 30-min ($P = 0.0005$) and 1-h ($P = 0.0022$) time points. No statistical relationship between body temperature and either restraint only or routine handling was found at any time. At 24 h, body temperature did not differ from baseline in any group.

Activity measurements over the first hour did not follow a reliable trend after either ear punching or routine handling. At 30 min, mice that experienced restraint only showed a trend toward decreased activity ($P = 0.065$); this difference became significant ($P = 0.03$) at the 1-h time point (Table 1). Activity after ear punching or routine handling did not differ from baseline at either 30 min or 1 h. The activity of mice that were restrained returned to baseline at 24 h, as did the ear punching and routine handling groups. The proportion of time that mice spent grooming their heads did not differ ($P = 0.45$) between immediately after ear punching and 1 h afterward (Table 2).

Discussion

The 3 Rs—replacement, reduction, and refinement—as delineated by Russell and Burch have become guiding principles in the use of laboratory animals.[21,23] At present, many important

Table 1. Physiologic responses to ear punching and restraint in laboratory mice

	Ear punching				Restraint				Routine handling			
	Baseline	Post	% change	*P*	Baseline	Post	% change	*P*	Baseline	Post	% change	*P*
Heart rate (bpm)												
0–30 min	499.5 (75.7)	633.8 (57.3)	26.9	0.004[b]	574.5 (70)	644.8 (58.8)	12.2	0.28	511 (82.6)	511.5 (99.8)	0.1	1
0–60 min	510.7 (55.5)	582.5 (61.3)	14.1	0.13	594.7 (69.4)	618 (51.7)	3.9	0.95	497.5 (66.7)	529.3 (98.3)	6.4	0.85
24 h	544.2 (40.8)	543 (34.2)	0.2	1	544.2 (40.8)	553.8 (24.5)	1.8	0.99	530.2 (58.5)	532.5 (44.3)	0.4	1
Body temperature (° C)												
0–30 min	37.4 (0.3)	38.3 (0.3)	ND	5×10^{-4}[c]	37.7 (0.3)	37.9 (0.3)	ND	0.96	37.5 (0.43)	37.1 (0.4)	ND	0.16
0–60 min	37.4 (0.3)	38.1 (0.3)	ND	0.002[b]	37.8 (0.2)	38.1 (0.5)	ND	0.78	37.3 (0.47)	37.1 (0.3)	ND	0.85
24 h	37.8 (0.2)	37.9 (0.2)	ND	0.16	37.7 (0.11)	37.8 (0.2)	ND	0.87	37.7 (0.18)	37.7 (0.3)	ND	1
Activity (no. of counts per minute)												
0–30 min	0.015 (0.03)	0.042 (0.06)	ND	0.99	0.24 (0.17)	0.01 (0.07)	ND	0.063	0.01 (0.02)	0 (0)	ND	1
0–60 min	0.045 (0.04)	0.03 (0.03)	ND	1	0.21 (0.14)	0.093 (0.07)	ND	0.03[a]	0.005 (0.01)	0 (0)	ND	1
24 h	0.1 (0.05)	0.12 (0.04)	ND	0.91	0.13 (0.09)	0.1 (0.04)	ND	0.52	0.115 (0.07)	0.1 (0.05)	ND	0.94

ND, not done; Post, after procedure
Data are shown as mean (1 SD; $n = 6$ mice per group).
[a] $P < 0.05$
[b] $P < 0.005$
[c] $P < 0.0005$

Table 2. Frequency of focal grooming in mice that underwent ear punching

	Proportion of time spent in focal grooming	
Mouse	Immediately after ear punching	1 h after ear punching
1	0.47	0
2	0.19	0.06
3	0.02	0.43
4	0.18	0.07
5	0.12	0
6	0.03	0

Each assessment lasted 5 min. The reported values reflect the proportion of time each mouse engaged in focal grooming behavior.

biomedical questions still require the use of rodents as models; therefore, following the refinement principle, researchers are obligated to identify potentially stressful/painful stimuli and minimize their frequency. Pain in laboratory animals is often defined in human terms, such that if a procedure is considered painful in humans, then it is considered painful in animals.[12] Children (age, 5 to 12 y) undergoing ear piercing rated the procedure as moderately painful (50 on a scale of 100), according to a visual analog scale;[22] however duration was not assessed. In comparison, children who received a needle prick for vaccinations rated the pain associated with the procedure as 3 to 4 on a scale of 10.[10] For laboratory animals, a published severity index of procedures rated the influence of the procedure on the welfare of the animals according to a 44-point scale.[24] Intraperitoneal injection, intradermal injection, tail venous puncture, and tail tipping had relatively low severity indexes of 4, 7, 5 and 9 respectively. The authors do not severity index for ear punching,[24] but when we applied the criteria for scoring components of severity, we achieved a severity index in the range of 10 to 15. In the current study, we found that ear punching causes a transient but significant physiologic response in mice that is greater than restraint by scruffing and routine handling only. Elevated heart rate values occur during the first 30 min after the ear punching procedure, but the effect is diminished by 1 h. We also found that the body temperature values remain elevated compared with baseline for at least 1 h after ear punching.

Our findings are similar to the transient physiologic responses seen by others, with a few differences. Whereas we found a significant difference in heart rate and body temperature after ear punching and restraint only, one study comparing several genotyping methods identified a transient increase in heart rate, body temperature, and activity that returned to baseline by 60 min after restraint only and ear punching, with no difference in response.[4] When the welfare of the mice was followed for 3 d after the procedure, there were no indications that the ear punching negatively affected mouse welfare.[4] These previous findings from mice are similar to those from a study in rats that compared ear punching with tattooing: there was an immediate increase in heart rate that had declined by 1 h after ear punching.[16] The differences in other species may reflect the thickness of the ear cartilage and the amount of pressure required to achieve a full-thickness cut. Ear pinnae in mice are 1 to 2 mm thick, whereas those in cattle and humans are several centimeters in thickness; the increased pressure needed to produce a full-thickness cut is likely the cause of the increased discomfort in these species.

Although we did not evaluate the physiologic effects of other routine procedures, such as blood collection and injections, the potential effects of these procedures on the welfare of mice have previously been reported[5,9] and offer a useful comparison to assess the effect of ear punching, particularly in light of how children perceive this pain and the severity index of procedures in laboratory animals. As a proxy for routine procedures, blood was collected from the tail vein for blood glucose assessments and rectal temperatures were taken. After blood collection, mice had an immediate increase in heart rate and arterial pressures, which lasted as long as 50 min before returning to baseline, and body temperature was found elevated for 15 to 60 min.[5,9] A similar increase in heart rate and body temperature was seen after intraperitoneal injection.[9] Elevations in body temperature and activity were likewise seen in mice after 15 s of restraint,[4] and restraint for rectal temperature found to cause a transient increase of body temperature for 60 min.[26] Although the physiologic response in our study was more pronounced in the mice that underwent ear punching than restraint only or routine handling, the transient nature was similar to the responses in the previous ear punching study[9] and to other routine procedures[4,8,26] that typically are regarded to result in slight or momentary pain.

Our physiologic findings are more informative than our behavioral data. Total activity in the current study did not show the slight increasing trend seen in other studies.[4] Reduced ambulation has been reported as a behavioral sign of distress in rodents,[8] perhaps partially explaining why we saw a decrease in activity for the restraint-only group. However, the lack of a similar response in the ear punching trial, in particular, is unexpected, given that mice are scruffed for the same amount of time (3 s) during this procedure. At 1h after ear punching, we noted a small decrease in activity, but the 30-min level was increased compared with baseline, and neither difference was statistically significant. The inconsistency of activity in response to procedures suggests that activity itself is not the most informative metric of postprocedural distress.

Rather than using the Mouse Grimace Scale, we chose to analyze focal grooming behavior, because previous behavioral assessments using this scale have proven ineffective in evaluating pain after ear punching in laboratory mice.[20] Although not specifically evaluated, we did not note any facial grimace. All but 1 of the 6 subjects showed a decrease in focal grooming at 1 h after ear punching compared with immediately after ear punching. One of the 6 mice (no. 3) had a marked increase in focal grooming at 1 h, from 2% of observed time immediately after the ear punching to 43% of observed time. With the removal of this outlier from the analysis, the proportion of time spent in focal grooming shows a decreasing trend ($P = 0.07$) at 1 h compared with immediately after ear punching. Why this one animal had increased focal grooming is unclear. The decreased incidence of focal grooming at 1 h after ear punching suggests that there is discomfort or pain associated with the procedure, consistent with our previous study evaluating indicators of pain in mice.[17] In those studies, we found wound licking, comparable to facial grooming, of the surgical site to be a reliable indicator of pain that could easily be assessed in a timely manner. Although there was a trend toward decreased focal grooming at 1 h, we need to be cautious about interpreting these findings to be a result of the ear punching procedure or scruffing, because we did not evaluate focal grooming prior to the procedure. Future exploration into the extinguishment of focal grooming after ear punching may benefit from the incorporation of longer focal sampling ethograms and of baseline values to correct for normal levels of head grooming with and without handling.

Compared with restraint (scruffing) only or routine handling, ear punching appears to cause a transient increase in physiologic responses, suggesting this procedure causes slight to momentary pain or distress in mice. However, the degree of pain or distress associated with ear punching does not seem to differ from that associated with ear piercing or injection pain as identified by children, the severity index of procedures in laboratory animals, or previously published physiologic responses to routine procedures. Although each IACUC must assess whether analgesics are needed for ear punching, our findings do not support their use because the procedure causes no more than slight to momentary pain or distress in mice.

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