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Effects of Home Cage Tunnels on Within-cage Behaviors of Mice with Cranial Implants

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Keeping tunnels in the home cages of mice used in research appears to both reduce handling-related stress and provide environmental enrichment. However, for mice that have surgical implants that extend beyond their body, having tunnels in the home cages could engender concerns for their welfare, including the possibility of them becoming stuck in the tunnel. The goal of this study was to determine how mice with different sizes of cranial implants interacted with a tunnel in their home cage. We used male and female mice with a C57BL/6J background in this study. The mice underwent a either a craniotomy in which they received either no implant (sham), an indwelling cannula used for drug delivery, or a ferrule-type implant. The number of mouse interactions with tunnels was recorded over a 30-min period while the mouse was in its home cage with its tunnel. We found that sham mice interacted significantly more with the tunnels than did mice with either cannulae or ferrule implants. On average sham mice interacted more with the tunnel by walking through or over it whereas mice with either type of implant rarely even touched the tunnel with their heads. Our results indicate that mice with implants do not enter in the tunnels, and thus the tunnel reduces accessible cage-space rather than providing enrichment benefits. These results raise the question of whether tunnels should be routinely available for mice with cranial implants.

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Introduction

Animal welfare can be promoted in many ways, including through using safe handling methods and provision of environmental enrichment. In research mice, environmental enrichment can support both the physical and mental health of the animal compared with standard housing.[1,10] Likewise, safe handling practices reduce stress that is inherent for mice being handled and may reduce the risk of injury during handling.[5]

A growing body of literature[9,16] suggests that handling mice by using tunnels can accomplish both of these goals–providing enrichment and promoting safe handling. Using tunnels to handle mice during cage changing is considered ideal for mice that may not respond well to traditional handling methods (i.e., by the tail or with forceps), or when a study would be confounded by traditional forms of handling.[4, 12] Capturing a mouse in a tunnel may also reduce the risk that a mouse could injure itself while trying to escape from traditional handling methods. Handling mice via tunnels could also reduce experimental variation.[13] In addition to the handling benefits of tunnels, the tunnels can also provide enrichment benefits, as mice can interact with the tunnel and even rest or nest in it. One report indicates that handling by using tunnels reduces anxiety and stress in mice as compared with lifting them using forceps or the tail.[9] This reduction in anxiety and stress may also benefit some study endpoints. For example, compared to mice handled with traditional methods, mice handled and housed with tunnels display increases in consuming sweet solutions in behavioral tests, indicating anhedonia.[3] Tunnel enrichment/handling also changes some physiologic variables in mice, including adult-born neurogenesis in the brain.[16] In addition, tunnel handling can have positive effects on the breeding capacity of mice because mice housed and handled with tunnels display higher litter production and fewer litter losses.[8]

Although tunnel handling and enrichment are associated with an array of benefits, they may be unsuitable for mice with surgical implants that extend beyond their bodies. Cranial implants are commonly used in research mice, and many of these implants extend well beyond the head and can be large in relation to the mouse cranium depending on their purpose.

A historically common implant is the indwelling cannula, which is widely used for intracerebral drug delivery and for sampling interstitial fluid (for example, microdialysis).[2] Another type of implant that is becoming increasingly common is the cranial implantation of a fiber optic ferrule that can be used to deliver for optogenetic light into the brain (to stimulate modified neurons with light) or gathering excited light from within the brain (fiber photometry of biosensors).[11] Both of the latter implant types are commonly used in pharmacological and neuroscience research. In addition, both of these implant types can be quite tall relative to the head of the mouse, with many commercially available ferrules measuring approximately 12-mm-long, thus resulting in the implant extending 12-mm above the head. Tunnel enrichment/handling may be problematic in mice with these types of cranial implants.

We hypothesized that mice with cranial implants would avoid interactions with tunnels as compared with mice that did not have cranial implants that extended above the head. Data to support this hypothesis would call into question the value of tunnels for these mice. In contrast, if implanted and unimplanted mice interacted equally with tunnels, the use of these tunnels would be supported. Therefore, in this study we monitored the interactions of 3 groups of that underwent different types of cranial surgeries for use in other ongoing studies. These 3 groups received either: 1) an intracranial injection after a skin incision and craniotomy (sham), 2) implantation of an indwelling cannula, or 3) implantation of a fiber optic ferrule. After surgical recovery, the number of interactions with the

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tunnel was recorded in a single 30-min behavioral monitoring session. Our results indicate that mice with implants interact with enrichment/handling tunnels significantly less than do mice without implants. The results raise the questions of whether tunnels should be routinely available to mice with cranial implants and whether they improve the welfare of these mice.

Materials and Methods

Animals. All animal care was conducted at the animal research program of the University of Florida, which is AAALAC accredited and operates in accordance with the Public Health Service Policy and the *Guide for the Care and use of Laboratory Animals*.[15] All animal use was approved by the University of Florida Institutional Animal Care and Use Committee.

A total of 60 mice were monitored in this study. Both male and female mice were used. All mice were on a C57BL/6J background and ranged between 2 to 6 mo of age. All were bred inhouse in a University of Florida vivarium, with breeder stock originating from either the UC Davis Mutant Mouse Regional Resource Center (MMRRC; Davis, CA or the Jackson Laboratory (Bar Harbor, ME). Strains included C57BL/6J mice (Jackson labs strain #000664), *drd1*-Cre mice (MMRRC strain #30989EY262Gsat), and *drd2*-Cre mice (MMRRC strain #032108ER44).[6]

All mice were raised housed in groups of 2 to 5 and then housed individually after surgery. Food (Envigo Teklad Global 18% rodent diet irradiated pellet, catalog # 2018; Indianapolis, IN) and reverse osmosis water were available ad libitum except during behavioral observation sessions. Mice were housed in standard cages (Allentown Jag 75 microvent system; Allentown, PA; L: 29.2 cm W: 18.5 cm H: 12.7 cm) on an IVC rack. Each cage contained sterilized corncob bedding and a cotton square (Ancare; Bellmore, NY). Tunnels are part of our standard enrichment and handling program for mice at the University of Florida. Tunnels were available to mice during their rearing and prior to our behavioral observations (see below). Mice were housed on a 0600 to 1800 h light cycle with lights on during the daytime. Temperature in the room averaged 70 ± 2 °F, with 30 to 70% humidity and 10 to 15 room air changes per hour. The housing room was SPF, as monitored by testing of sentinel animals maintained on the same ventilated rack as the experimental animals. Observations occurred from 1300 to 1600 h.

Surgical procedures. All mice in this study had already been assigned to future sensory-driven studies on motivated behavior and required surgery. No mice were manipulated in any way specifically for conducting this study. As part of the study, some mice received cranial implants and some were injected with Cre-dependent adeno-associated viruses (AAV) to introduce proteins (including fluorophores, opsins, or biosensors; all obtained from Addgene [Watertown, MA]) into specific neurons in the brain. None of these AAV-driven proteins, when expressed, are known to influence mouse behavior without administration of additional reagents/stimuli.

Mice underwent one of 3 different craniotomy-based surgeries. The basic preoperative and postoperative procedures were identical for all procedures, and all surgeries were conducted under aseptic conditions. Mice were anesthetized in an induction chamber with 2% to 4% isoflurane (IsoFlo, Patterson Veterinary, Loveland, CO) in oxygen at 1 L/min and then transferred to a stereotaxic apparatus and maintained under isoflurane anesthesia. Their body temperatures were maintained at 38 °C by using a water-filled circulating heating pad. Preoperatively, the local anesthetic bupivacaine hydrochloride (Marcaine, Patterson Veterinary; 5 mg/kg) was injected subcutaneously into the site of the future wound margin, and meloxicam was injected subcutaneously for analgesia (5 mg/kg, Patterson Veterinary). After a surgical plane of anesthesia had been achieved, a midline incision was made on the scalp and the skin was retracted. Each mouse received 1 or 2 craniotomies (approximately 0.5 to 1-mm-diameter; as described in more detail below depending upon the implant type) over their forebrain and then received a small intracranial injection of AAV (< 200 nl/hemisphere, 2 nl/sec) with a pulled glass capillary tube; the tube was removed from the brain after infusion.

The appearance and relative size of the cranial implants are shown in Figure 1. Some mice were implanted bilaterally with stainless steel indwelling guide cannulae (26 GA, P1 Technologies, Roanoke, VA) (Figure 1B). The total weight of the mplant including dental cement was 0.2 g and its height was 5 mm above the skin. Another group of mice (Figure 1C) received a fiber optic implant ('fiber optic ferrule' group; 400um diameter). The total weight of the implant including dental cement was 0.83 g and its height was 12 mm above the skin. Both of these types of implants were secured to the skull with dental cement (Teets cold cure, Cooralite Dental; Diamond Springs, CA). A third group of mice ('sham') simply had an incision made and closed following just a craniotomy. All incisions were closed with Vetbond (3M; St. Paul, MN). All mice received injections of the analgesic meloxicam daily (dosed as above) for 3 d after surgery.

In total, 20 sham mice (n = 11 male, 9 female), 15 mice with indwelling cannulae (n = 9 male, 6 female), and 25 mice with fiber optic ferrules (n = 14 male, 11 female) were used in this study. Mice were selected based on the surgery performed and on surgical records, and no mice were excluded from the study once selected. The unequal number of mice in each group was due to the use of convenience samples associated with other ongoing studies.

Behavioral monitoring. All mice were observed for their tunnel interactions at least 5 d after surgery but prior to their intended experimental use. Mice were housed individually after surgery. Observations occurred between 1- and 6-wk after surgery and were performed in the home cages with cage lids in place. Cages were placed on a designated lab bench; the same lab bench was used for all behavioral observation sessions and, before each individual observation session, the bench was wiped down with 70% ethanol to ensure cleanliness. Prior to placing the cage on the bench, all mice had been acclimated to the room for over 1 hr and water and food had been removed so that the only object remaining in the cages, other than the bedding, was the clear plastic handling/enrichment tunnel (Petro Extrusion Technologies; Middlesex, NJ; L: 8.89 cm W: 5.08 cm H: 5.08 cm) which was open on both ends (Figure 2). This gave the mice the opportunity for unobstructed approach and interaction

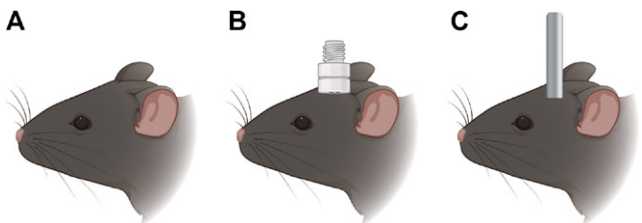

Figure 1. Illustrations of the 3 cranial implant groups. The groups were (A) sham implanted mice that had a midline incision and craniotomy but did not have a cranial implant nor anything that extended beyond the head, (B) mice implanted with an indwelling intracranial cannula that had a connector port extending outside of the head, and (C) mice implanted with an indwelling optical fiber connected to an optical ferrule that extended outside of the head.

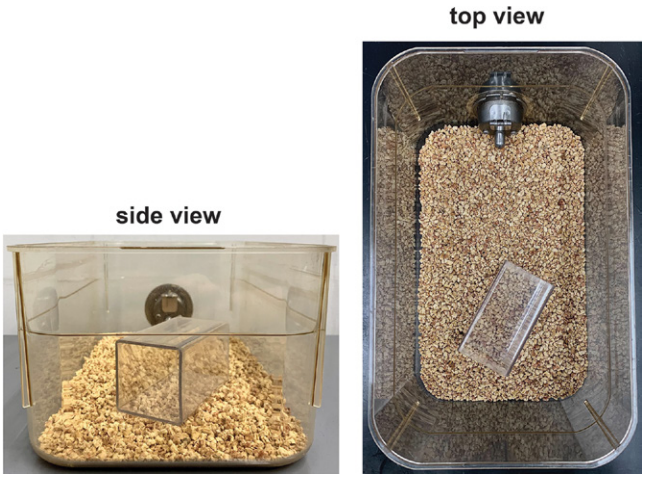

Figure 2. Side view (left) and top view images (right) of a tunnel in a cage. This picture shows the same type of tunnel (L: 8.89 cm W: 5.08 cm H: 5.08 cm), home cage (L: 29.2 cm W: 18.5 cm H: 12.7 cm), and bedding used in monitoring mice in this study.

with the tunnels. Before and during observations, the room was well lit with wide-spectrum visible fluorescent light, and room temperature was maintained at 20 to 22 °C.

Observation occurred from 1300 to 1600 h. Interactions with the tunnel were recorded continuously over a 30 min period. Table 1 shows the defined tunnel interactions that were monitored for each mouse. For repeated behaviors, any new occurrence of a behavior was scored as a separate instance. The set of defined interactions was developed based on pilot observations that indicated the scope of mouse interactions with the tunnels. All monitoring was performed visually in real-time by a single trained experimenter (M.C.).

Statistics and data analysis. Raw data were recorded manually onto testing log sheets. After testing, the data were entered into Microsoft Excel (Seattle, WA) independent of groups and then organized. An independent observer sorted the data by group to prevent bias in data handling and also cross-checked a subset of the manually entered data for basic quality control. The number of interactions of each type by group and the order of interactions were calculated in Excel. Statistical analyses included 2-tailed *t* tests and 2-way ANOVAs with corrections for repeated measures when applicable, with a significance level of $P < 0.05$. All summary data are presented as mean ± SEM.

Table 1. Definitions of recorded tunnel interactions

Interaction abbreviation	Description
O	Mouse walks over the tunnel.
W	Mouse walks through the tunnel.
H	Mouse inserts head into the tunnel.
C	Mouse comes within 1 cm of tunnel entrance*.
T	Cranial implant taps into / makes contact with the tunnel once and the mouse stays near tunnel.
L	Cranial implant taps into / makes contact with the tunnel once and the mouse walks away from tunnel.
R	Mouse remains inside the tunnel for 1 min.
M	Mouse remains inside the tunnel for ≥5 min.
S	Mouse gets stuck in the tunnel.

*1 cm was approximated by the experimenter during testing.
**While monitored for, R and S interactions were never observed.

Results

Effect of head implants on the total number of tunnel interactions. Our analysis revealed a main effect of group on the mean total number of tunnel interactions (F (2,33)18.30, $P < 0.0001$)) (Figure 3). As shown in Figure 3, sham mice interacted with the tunnel significantly more often than either cannula (t (33,4.185), $P = 0.0002$) or ferrule (t (43,4.664), $P < 0.0001$)) implanted mice. The mean number of interactions between cannula and ferrule implanted mice was not significantly different (t (38,1.193), $P = 0.240$). These results indicate that mice with physical implants that extend beyond the head show fewer interactions with handling/enrichment tunnels than do mice that received a craniotomy alone.

Effect of head implants on the number of specific interactions with the tunnel. By examining how the implants affected specific tunnel interactions as defined in Table 1, we found that sham mice showed significantly more interactions than did implanted mice in two types of interactions. These were walking over the tunnel [sham compared with cannula, t (33,4.076), $P = 0.0003$; sham compared with ferrule, t (43,4.789), $P < 0.0001$] and walking through the tunnel [sham compared with cannula, t (33,3.253), $P = 0.0026$, sham compared with ferrule, t (43,3.602), $P = 0.0008$)] (Figure 4).

We found no differences between sham and implanted mice for poking the head into the tunnel, coming close to the tunnel, placing the head in the tunnel once before entering, and placing the head in tunnel and then leaving. We found no significant differences between cannula and ferrule mice in walking over [t (38,1.295), $P = 0.2030$] or through the tunnel [t (38,0.714), $P = 0.4798$] (Figure 4).

Effects of head implants on the patterns of behaviors mice display around tunnels. Some behaviors by definition require another behavior to precede it. For example, for a mouse to tap

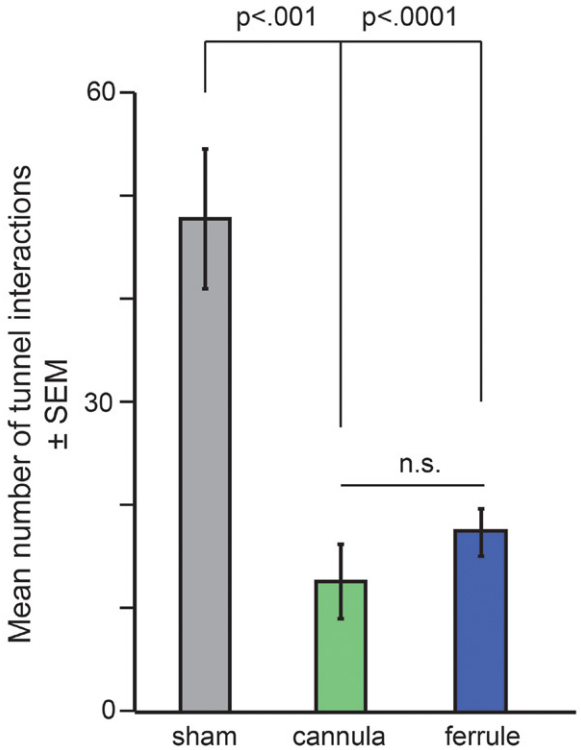

Figure 3. Effects of cranial implants on interactions with tunnels. Figure shows the mean number of home cage tunnel interactions for each mouse averaged across all mice in the group. n.s. = not significant. P values are derived from 2-tailed t tests.

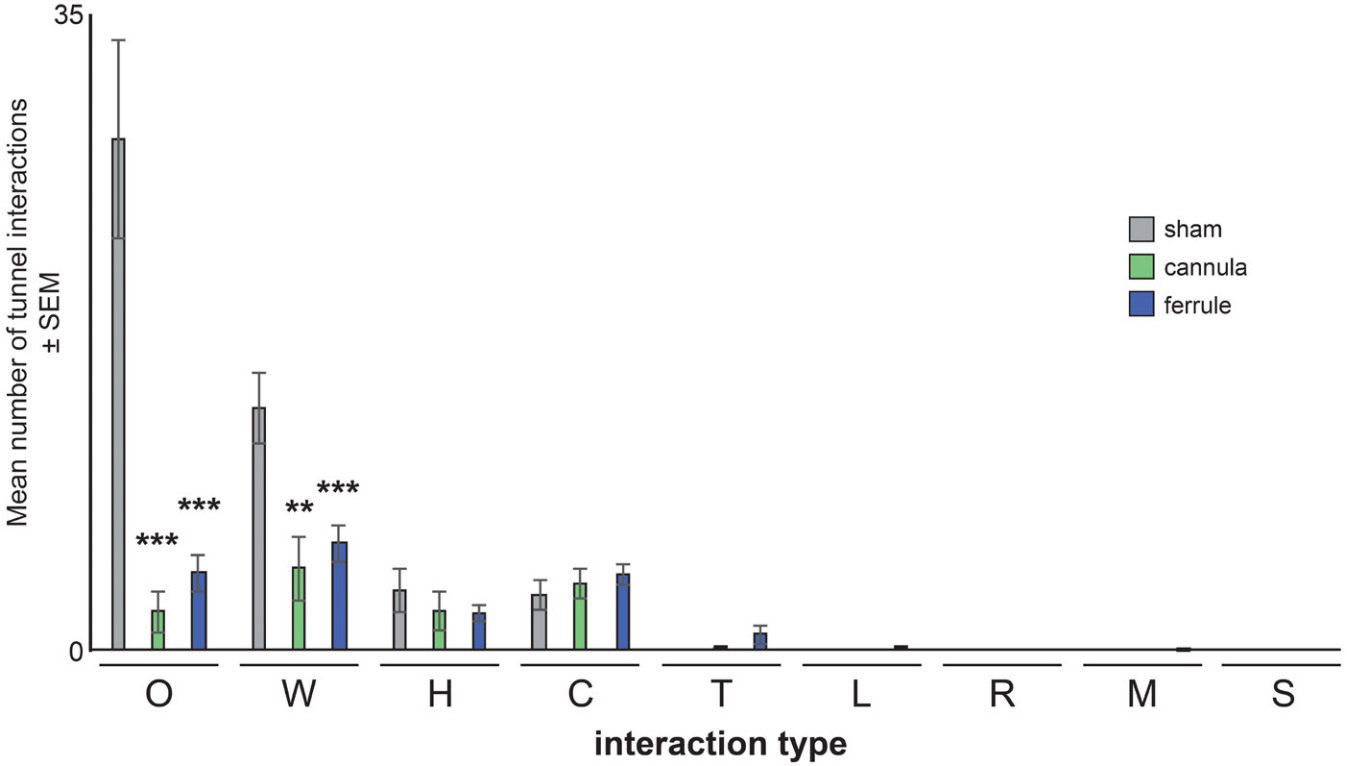

Figure 4. Effects of head implants on the number of specific interactions with the tunnel. Data are mean numbers of each type of tunnel interaction, averaged across mice within groups. Please see Table 1 for an explanation of interaction abbreviations. n.s. = not significant. ***$P < 0.0001$, **$P < 0.001$, 2-tailed t tests. All comparisons were n.s. unless indicated in the figure.

their head on the tunnel entrance, they must first approach and come within a centimeter of the tunnel. We therefore evaluated whether the order of behavioral events or sequences was altered in mice with externally extending head implants by quantifying the number of times a given behavior followed another (Figure 5).

We found that indeed, animals with externally-extending head implants showed differences in their behavioral sequences. For instance, compared to sham implanted animals, animals with head implants displayed significantly fewer O→O sequences (X^2 (1, N = 431) = 103.765, $P < 0.0001$), sham compared with cannula; (X^2 (1, N = 471) = 74.174, $P < 0.0001$), sham compared with ferrule). They also displayed significantly fewer O→W interaction sequences (X^2 (1, N = 180) = 23.802, $P < 0.0001$), sham versus cannula; (X^2 (1, N = 216) = 17.580, $P < 0.0001$), sham compared with ferrule). Implanted mice seemed to display more C→C interactions (Figure 5). In other words, mice with head implants would come up to the tunnel entrance but then would disengage tunnel interaction, only to subsequently re-approach the tunnel entrance. In contrast, Sham mice often would follow through with putting their head inside or even walking through the tunnel. This change in behavioral sequences as head implanted mice interacted with the tunnels suggests that head implants alter the fundamental ethology of mice in the context of their home cages.

Discussion

In this paper we report that mice with either of 2 types of cranial implants interacted less with tunnels than did sham-operated mice that had no implants. These differences were due to a higher frequency of sham mice walking over or through the tunnel as compared with implanted mice. This work suggests that tunnel enrichment may not be beneficial for mice with surgical head implant.

The goal of environmental enrichment is to provide sensory and motor stimulation specific to each animal, thereby increasing the animal's sense of control over the environment and improving its ability to cope with stress.[16] Some common types of environmental enrichment for mice are chew toys, nesting materials, running wheels, and larger living spaces.[18] A tunnel perhaps provides environmental enrichment for mice, which in their natural habitat live underground and can use tunnels for escape or nesting purposes. Tunnels also allow less stressful handling of mice during routine cage changes. Tunnels can also be used to move mice to a new area, as opposed to moving them either manually either by their tails or with forceps. The handling method itself can induce fear and anxiety that is induces in mice by human contact.[9] Mice that are handled with tunnels appear to be less resistant to human contact and interact more readily with the handler.[7] Chronic stress induced by tail-handling methods can even lead to depressive-like states in mice.[17] The stress and anxiety associated with tail-based handling methods present a potentially confounding variable that could influence experimental results.[7,13] Therefore, handling/enrichment tunnels are now recommended to improve animal welfare and perhaps the quality of data collected. A recent report indicates that tunnel handling promotes higher yield of litters among breeding mice.[8]

A potential concern with using tunnels is that they reduce the available cage floor space if they are not used. Our study showed that mice with implants use the tunnels significantly less than do nonimplanted mice. The *Guide for the Care and Use of Laboratory Animals*[15] states that floor space occupied by food or water containers should not be considered floor space because they

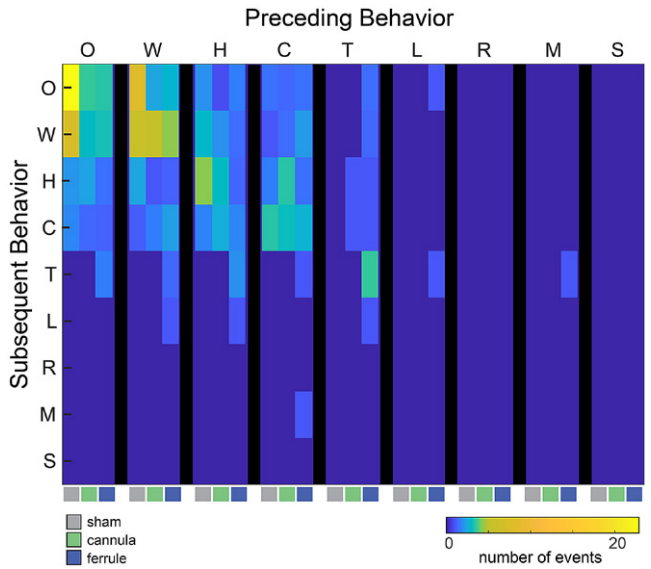

Figure 5. Effects of head implants on the behavioral patterns that mice display around tunnels. Bars indicate the likelihood that a given behavior will follow a preceding behavior. Columns are organized by interaction types, which are further divided by cranial implant group. Please see Table 1 for a list of interaction abbreviations.

reduce the usable cage space. Accordingly, although tunnels may improve animal welfare for normal mice, they could have a negative effect on mice with implants. The *Guidelines for the Care and use of Mammals in Neuroscience and Behavioral Research* recognizes the need for creativity in husbandry procedures and the possible need to consider special/modified housing for animals with implanted devices.[14] Based on our results, such housing adaptations should include omitting handling/enrichment tunnels for mice with cranial implants.

Several reasons could explain why mice with head implants did not interact with the tunnels to the same extent as mice without implants. One likely reason could simply be that the physical size of the implant reduces the mobility or overall balance of the mouse as it approaches and/or subsequently interacts with the tunnel. We found that mice with both types of cranial implants displayed less walking into or over the tunnels. Although the cranial implants the mice received do penetrate the brain and span some brain regions that are important for motor control, work from our lab and many others indicates that mice with similar implants have no overt motor impairments. Furthermore, mice in the present study were observed at least 5 d after implantation, reducing the possibility that postoperative discomfort or stress was a factor in changing tunnel interactions. Inclusion of our sham group allowed for valid comparison among groups and for results to be more likely attributed to the implant size than simply consequences of a surgical procedure. Thus, we conclude that the physical size of the implants likely impedes their interactions with the tunnels. Although the 2 implants studied were not greatly different in size (< 8 mm in height different), future experiments to compare tunnel interactions in mice with greatly different sized implants or tunnels could resolve this. This is important since some implants maybe over 2.5 cm in height, which is nearly half the height of the tunnels used herein (5.08 cm).

One study documented that mice with cranial implants can have special enrichment needs; recent work showed adverse effects of nesting materials on mice with head implants.[19] In that study, the authors compared different types of shredded paper and discussed its safety for use with mice with exteriorized devices, specifically head bars used for temporary head-fixation. They reported that some nesting material were associated with less risk of entanglement of the head bars than were other nesting materials. They concluded that housing exceptions should be made for mice with head implants. Our data and the cited study indicate that mice with cranial implants warrant special consideration with regard to standard housing and enrichment protocols.

Our study has some important limitations. First, we only monitored behaviors the mice for a single 30-min period, and tunnel interactions could change over time. For example, perhaps mice with cranial implants are prone to neophobia that would manifest to a lesser extent after more prolonged time with the tunnel. Lighting conditions could also affect the outcomes; our study was performed in during the light phase of the circadian cycle, but mice are normally most active during the dark phase. Another limitation is that larger tunnels are available and could have been used in our study. We used tunnels that we routinely provide in mouse cages in our vivarium. A potential future direction would be to determine how mice with implants would interact with larger tunnels. An additional limitation was that while all mice in the present study had a C57BL/6J background, some mice expressed Cre recombinase under control of several promotors. While each implant group had some mixed genotypes, these differences could have added variability to the present results. Further, several surgeons had performed the surgeries, which also may have added variability to the data. Also related to surgeries, our sham control mice had received the 'sham' surgery of a cranial incision and craniotomy but did not have a head implant. This comparison group was selected to make manipulations as comparable as possible across groups, except for the implant extending above their head. Finally, we did not monitor any physiologic indicators of stress or anxiety that could have affected the data. Future studies could examine how stress and anxiety are affected by the presence of a handling/enrichment tunnel in mice with cranial implants.

In summary, by monitoring the behaviors of mice with head implants, we found that the presence of a head implant significantly reduces mouse interactions with handling/enrichment tunnels. This finding suggests that handling/enrichment tunnels might not be suitable for mice with cranial implants. Consideration of alternative means of enrichment and handling for mice with implants is warranted.

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Conflict of Interest

The authors have no conflicts of interest to declare.

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