Taming anxiety in laboratory mice

Jane L Hurst & Rebecca S West

Routine laboratory animal handling has profound effects on their anxiety and stress responses, but little is known about the impact of handling method. We found that picking up mice by the tail induced aversion and high anxiety, whereas use of tunnels or open hand led to voluntary approach, low anxiety and acceptance of physical restraint. Using the latter methods, one can minimize a widespread source of anxiety in laboratory mice.

Routine handling of laboratory animals is an essential but frequently ignored component of animal experiments that has considerable potential to influence anxiety and aversion to human approach and contact. Laboratory mice seek to avoid capture and restraint unless they have learned that handling is not harmful. Prior handling experience (which includes routine maintenance) can have positive or negative effects on stress responses that influence experiments, depending on the animal's experience during handling[1–5]. We show here that handling method itself is critical as it can induce fear and anxiety responses to human contact.

The most common method used to capture and handle laboratory mice is to pick up and restrain the mouse by its tail, a method usually specified in standardized protocols[6–8]. We compared this with two alternatives (**Supplementary Movies 1–3**). Mice handled by 'tunnel' walked into a clear acrylic home cage tunnel brought toward them (present in all cages regardless of treatment) and were lifted without direct contact. Mice that handlers 'cupped' were scooped up and allowed to walk freely over the handler's open gloved hands without direct physical restraint; as mice unfamiliar with this technique immediately jump away, handlers closed their hands loosely around the mouse for up to 30 s on first experience, until the mouse's attempts to escape declined. Subsequently the handlers could pick up the mouse on open hands.

Unconditioned exploration models of fear and anxiety assess caution or avoidance in potentially threatening situations[2,9]; urination and defecation are measures of emotionality associated with anxiety or stress[10]. To assess anxiety-related behavior in anticipation of handling (similar to the use of human threat to assess anxiety in nonhuman primates[11]), we assessed voluntary approach and interaction with the handler immediately before and after daily handling using the same handling method, as well as urination and defecation during the handling procedure itself. We also compared anxiety in an elevated plus maze when mice were familiar with one of the three handling methods (Online Methods); in this test, anxiety is evident from reduced entry into two open arms without walls to provide physical protection, and an increased frequency of protected stretched attend postures in which the mouse stretched forward into an open arm but then retracted back into the protected center or closed arms of the maze.

As strains and sexes can differ substantially in anxiety and stress responsiveness[12,13], we assessed responses of both sexes for two common inbred mouse strains, BALB/c and C57BL/6, and the outbred strain ICR(CD-1). Mice of all strains developed a consistent response to the three handling methods over nine daily 60-s handling sessions (**Fig. 1** and **Supplementary Tables 1** and **2**). Those handled by the tail (mouse body weight was supported on the hand or arm) showed the lowest voluntary interaction with the handler. By contrast, voluntary interaction was prolonged among all tunnel-handled mice and among cupped ICR(CD-1) mice and cupped BALB/c females; other cupped mice showed intermediate responses. Prolonged interaction reflected the willingness of many tunnel- or cup-handled mice to voluntarily enter tunnels or climb on the hand and arm of

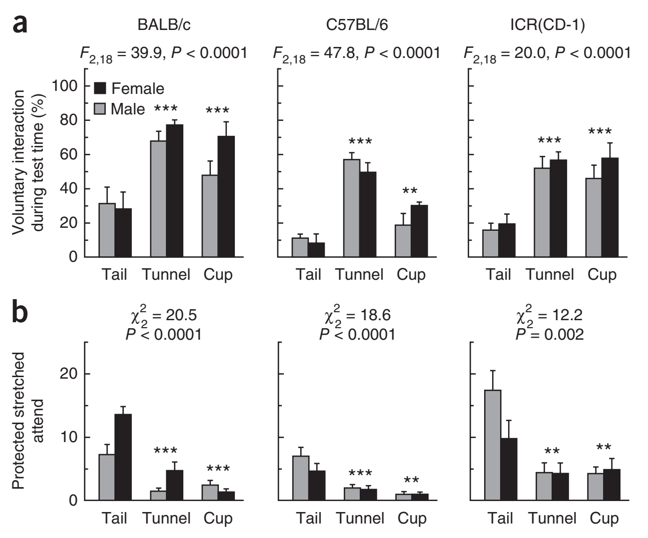

Figure 1 | Effect of handling method on voluntary interaction with the handler and behavior in an elevated plus maze, tested during the dark phase of the diurnal cycle. (**a**) Voluntary interaction in the ninth handling session immediately after handling by the tail, home cage tunnel or cupped on open hands among male and female BALB/c (left), C57BL/6 (middle) and ICR(CD-1) (right) mice. Error bars, s.e.m., n = 4 cages. (**b**) Mean frequency of protected stretched attend postures during a 5-min elevated plus maze test after nine handling sessions. Error bars, s.e.m., n = 8 mice. The overall effect of handling method is shown for parametric (F) or nonparametric (χ^2) ANOVAs; planned contrasts to tail method: *P < 0.05, **P < 0.01, ***P < 0.005.

Mammalian Behaviour and Evolution Group, Institute of Integrative Biology, University of Liverpool, Leahurst Campus, Neston, UK. Correspondence should be addressed to J.L.H. (jane.hurst@liv.ac.uk).

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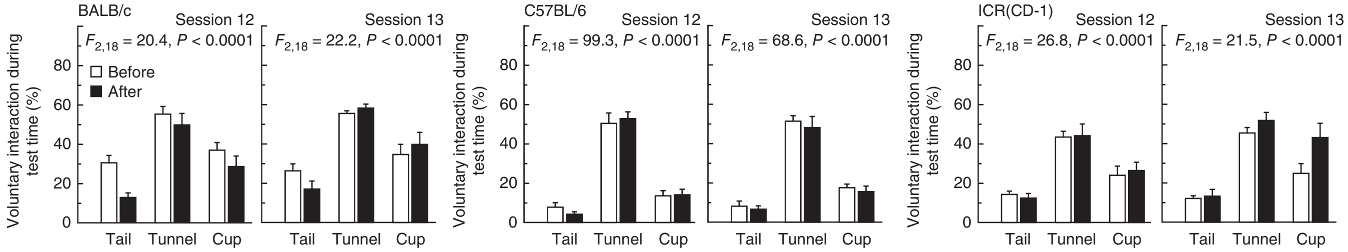

Figure 2 | Voluntary interaction with the handler after restraint by the scruff for mice experienced with different handling methods. After 11 previous handling sessions by tail, home cage tunnel or cupping on the open hand during the dark period, each mouse was picked up by its familiar method, restrained by the scruff and held on its back in the palm of the hand for 10 s (session 12). Voluntary interaction was assessed before and after scruff handling (session 12) and before and after normal handling the following day (session 13). Error bars, s.e.m., n = 8 cages (4 for each sex) for BALB/c (left), C57BL/6 (middle) and ICR(CD-1) (right) mice. The overall effect of handling method is shown for repeated measure ANOVAs.

the handler by session 9; this response developed quickly toward familiar home cage tunnels and more slowly to cupping on the open hand (**Supplementary Fig. 1**). This contrasted strongly with brief and cautious approaches among mice handled by the tail (**Supplementary Movies 4–6**). Compared to tunnel or cup handling, tail handling also induced greater urination and defecation during handling (**Supplementary Fig. 2**), together with a higher frequency of protected stretched attend postures (**Fig. 1**) and fewer open arm entries on the elevated plus maze (**Supplementary Fig. 3**). Responses were very similar in the light and dark phases of the diurnal cycle and were not influenced by individual differences or prior experience of handlers, and any interactions between sex and handling method were only minor (**Supplementary Figs. 1–5** and **Supplementary Tables 1–3**). However, handling tunnels provide an alternative means to accustom mice to being picked up. Mice previously experienced with tunnel handling (16 sessions) responded as positively to their first experience of being cupped on the hand (BALB/c mean ± s.e.m., 42.5 ± 5.2% of test time interacting with the handler) as those preconditioned to cupping (32.7 ± 6.0%; t-test: t_{12} = 1.24, P = 0.24). Mice readily sat on the hand without jumping after five sessions of tunnel handling (**Supplementary Movie 7**), providing a convenient combined handling method that reduced anxiety in the elevated plus maze and in response to the handler (**Supplementary Fig. 4** and **Supplementary Movie 8**).

Restraining and lifting mice by the tail for abdominal inspection was not aversive if we first picked up the mice and placed them on the hand by tunnel or cupping (**Supplementary Fig. 6**). Aversion to tail handling thus was induced by being captured and picked up by the tail, probably stemming from a naturally selected ancestral imperative to avoid capture when fleeing from predators. For many procedures, mice need to be restrained more securely by the scruff of the neck. Scruff restraint did not reverse the taming effects of being handled by tunnel or by cupping as this did not stimulate increased avoidance of the handler (**Fig. 2** and **Supplementary Table 4**), suggesting that anxiety in anticipation of handling was not increased, whereas tail-handled mice continued to avoid handler interaction.

The notable difference in response induced by these alternative routine handling methods has not previously been recognized to our knowledge, maybe because picking up mice by the tail is so widely used in laboratories that the aversive and anxious response is perceived as 'normal'. Consistent use of methods that do not induce strong anxiety responses will minimize confounding responses owing to routine handling before and during experiments, reducing the need to standardize handling experience and timing. Conversely, when a strong anxiety response is required for specific research purposes, it may be important to ensure that mice are handled only by the tail and are not tamed to human contact accidentally during routine husbandry procedures. In addition to providing more robust scientific outcomes, appropriate choice of handling method could enhance the welfare of the many millions of mice that are housed and handled in laboratories worldwide.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemethods/.

Note: Supplementary information is available on the Nature Methods website.

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AUTHOR CONTRIBUTIONS

J.L.H. gained the funding and designed the project with contributions from R.S.W.; J.L.H. and R.S.W. collected data; and J.L.H. supervised all aspects of the work, analyzed the data and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Subject animals. A total of five separate batches of mice were tested. Batches 1–4 were obtained from an approved supplier (Harlan; mice aged 3–4 weeks); batch 5 mice were bred in-house from mice obtained from Harlan (parents handled by the standard tail method). On arrival or at weaning, mice were housed in single sex groups of two to six mice in 45 × 28 × 13 cm polycarbonate cages (MB1; North Kent Plastics) on Corn Cob Absorb 10/14 bedding with shredded paper nest material, *ad libitum* food (Lab Diet 5002 Certified Rodent Diet; Purina Mills) and water. Before the experiment, mice were handled using the standard tail method to transfer them between cages during cage cleaning. Mice were marked for individual identification on the shoulders or rump using hair dye (Clairol Nice 'n' Easy Natural Black for BALB/c and ICR(CD-1), Clairol Nice 'n' Easy Brilliant Blond for C57BL/6; Bristol-Myers Co. Ltd.) and split into single sex dyads in 43 × 11.5 × 12 cm cages (M3, North Kent Plastics), each containing a clear acrylic tunnel (50 mm diameter, 200 mm long) 3 d before the start of testing (mice aged 8–10 weeks). Pilot tests confirmed that the marking procedure did not influence the response to different handling methods. Batches 1 and 2 each consisted of eight cages of two BALB/c females assigned randomly to each of three handling methods (48 females per batch). To assess the generality of response to the three handling methods across sexes and strains, four cages of two mice per sex and strain were assigned randomly to each of the three methods for outbred ICR(CD-1) mice (batch 3, 48 mice) and inbred BALB/c or C57BL/6 mice (batch 4, 48 mice per strain). Batches 1–4 were all handled and tested during the dark phase under red lighting so that mice would be naturally active during testing. Mice in batch 5 were handled and tested during the light phase, when many laboratory animals are normally handled, with three to four cages of two mice per sex and strain assigned randomly to each of three (BALB/c, 42 mice) or four handling methods (C57BL/6, 64 mice). In each batch and strain, cages handled by each method were arranged in a balanced design on the cage rack. Throughout, mice were maintained under a reversed 12-h light–12 h dark schedule (white lights on 21:00–09:00) or a nonreversed light schedule (09:00–21:00, batch 5 only). All procedures were noninvasive and involved standard husbandry practices, so did not require any specific licences (University of Liverpool Animal Welfare Committee).

Handling methods. From the start of testing, mice were handled only by their designated method. Before handling, nest material and the home cage tunnel were removed from the cage. For tail handling, the base of the tail was grasped between thumb and forefinger and the mouse gently lifted onto the opposite gloved hand or laboratory coat sleeve and held there by the tail for 30 s before release back into the cage. For tunnel handling, the mouse was guided into the home cage tunnel (soiled with familiar scent) as it was brought toward it, and the tunnel lifted above the cage and held for 30 s. The handler's hands were sometimes loosely cupped over the tunnel ends to prevent escape on the first 1 or 2 d before mice became accustomed to tunnel handling. A cupped mouse was scooped up on one or both open hands and allowed to sit or walk over the hands for 30 s without other physical restraint. To prevent mice leaping from the hands, for the first experience of cupping the hands were closed loosely around the mouse for a maximum of 30 s until attempts to escape declined, after which the hands were opened. This was not necessary after the first handling session. All batches compared responses to these three different methods. A fourth handling method, which combined tunnel and cup handling, was tested using C57BL/6 mice (batch 5) to establish an alternative way to prevent mice initially leaping from the hands during cup handling. Relatively inexperienced handlers may, at first, find cup handling of untamed mice quite difficult for some strain and age combinations that are more likely to jump from the hands, or handlers may be concerned about being bitten when confining untamed mice between the hands (though no cupped mice attempted to bite in this study). For the first five handling sessions, mice were picked up in their home cage tunnel; on the sixth session they were picked up in the tunnel then tipped backwards onto the open hand for 30 s, after which they were picked up directly by cupping on the open hand. **Supplementary Movies 1–3** and **7** demonstrate each handling method.

In each handling session, the handler picked up each mouse in turn by the designated method and held it for 30 s. After moving away from the open cage for 60 s, the mice were handled again so that each mouse was picked up twice and held for a total of 60 s. Handlers wore laboratory coats contaminated with mouse scent from previous handling sessions and clean close-fitting nitrile surgical gloves that were rubbed in bedding soiled by the same strain and sex of mice before the start of each handling session. During the first nine handling sessions, the occurrence of any urination or defecation each time a mouse was picked up and held was recorded (maximum score per measure for each mouse = 18).

Handling and test schedule. The first batch of BALB/c females was handled by a single handler (n = 8 cages per handling method) for 16 separate handling sessions. To assess variability in response across handlers, the second batch of BALB/c females was handled by eight different handlers that varied widely in their prior experience of handling rodents (each individual handled three cages of mice using one cage per handling method; cages were assigned randomly in a balanced design). Two handlers had no prior experience, three handlers were researchers with 3–4 years of experience working with rodents, three handlers were full-time animal care staff and a researcher with substantial rodent handling experience. Handlers were given a practice session using stock mice before the start of the experiment after demonstration of each method and test procedure. Response toward the handler was assessed over nine daily handling sessions using all batches of mice, with responses tested during handling sessions 1 (day 1), 5 (day 5) and 9 (day 11 or 12). Experienced batch 1 mice were also used to test response after 11 d without handling, an open field test of anxiety, the effect of mouse-soiled protective clothing on response toward the handler (data not shown) and the response to cup handling among mice previously handled by tail or tunnel. Experienced batch 2 mice were used to test anxiety in an elevated plus maze, response to an unfamiliar handler and response to tail manipulation for abdominal inspection by the unfamiliar handler. Batch 3 outbred mice were handled by one of two handlers (one experienced and one inexperienced; data were combined as handler had no effects), and batches 4–5 were handled by a single experienced handler. Details of the handling sessions and tests conducted on each batch of mice, and their timing are summarized in **Supplementary Table 5**. In each batch, order of testing cages assigned to the same method was randomized, and the order was balanced across the three different methods throughout. Gloves were changed between cages housing mice of the opposite sex. Cage cleaning was carried out fortnightly at the end of a week to avoid interference with testing, and mice were transferred to a clean cage by their familiar handling method.

Response to the handler. To assess behavior in anticipation of handling, we assessed voluntary approach and interaction with the handler before and after handling on specified test days. After removal of the cage lid, nest material and home cage tunnel, the handler stood unmoving for 60 s directly in front of the cage. A gloved hand (tail and cup methods) or gloved hand holding the home cage tunnel (tunnel method) was then held resting on the substrate in the front half of the cage without moving for an additional 60 s to assess voluntary interaction. Both mice were then handled by the designated method as described above, the handler stood back from the cage for 60 s, and then repeated the two 60-s tests. Trials were recorded on video or DVD for transcription by a single observer using an event timing program (blinded with respect to test on the second batch of mice). For each test, we measured the time spent in the front half of the cage by each mouse and time spent interacting at close contact with the introduced hand or hand and tunnel (time spent sniffing the handling device, paws on, climbing on, inside tunnel or chewing the glove were summed for analysis).

Anxiety tests. Mice were tested using an elevated plus maze after seven or nine handling sessions (**Supplementary Table 5**). Mice were delivered by their familiar handler and handling method to the center of the maze (arms 30 × 5 cm with side walls 15 cm high on the two closed arms, elevated 57 cm above the ground), facing an open arm, for a 5 min test. Mice handled by the combined tunnel and cup method were delivered to the maze in their home cage tunnel. We scored the number of entries and total time when all four feet were in each arm of the maze (summed for open or closed arms), the frequencies of protected stretched attend postures into the open arms from the central hub or closed arms, and unprotected stretched attends on the open arms[2]. At the end of the test, mice were returned to their home cage using their familiar method of handling and the arena cleaned with 70% ethanol and dried with a paper towel. Mice from the same cage were tested successively on the same day.

Tail manipulation for abdominal inspection. To assess response to lifting the mouse by its tail to examine its ventral surface and anogenital area, BALB/c females (batch 2) and outbred ICR(CD-1) mice of both sexes (batch 3) were captured using their familiar method (following 13 or 10 previous handling sessions, respectively) and placed on the handler's open gloved hand (tunnel mice were tipped backwards out of the tunnel on to the hand). The tail base was then grasped between the thumb and forefinger of the other hand and the back legs of the mouse lifted off the hand to expose the ventrum and held for 10 s. Response to the handler was assessed immediately before and after handling.

Scruff handling. To assess response to the experience of being restrained by the loose skin of the scruff, ICR(CD-1), BALB/c and C57BL/6 mice in batches 3 and 4 were captured using their familiar method after 11 previous handling sessions and placed on the bars of a clean cage top. The loose skin of the scruff was grasped between thumb and forefinger to immobilise the animal in the hand, and the mouse held on its back above the home cage for 10 s before being released back into the cage. Response to the handler was assessed immediately before and after scruff handling, and before and after handling by their normal method the following day to assess how quickly mice recovered from scruff handling.

Response to cup handling. The final test of BALB/c batch 1 females assessed response to the familiar handler before and after all mice were handled using the cupping method. This was designed to test how mice previously handled indirectly using a tunnel would respond to direct physical contact with the hand. Response to the handler was assessed before and after cup handling by inserting a gloved hand only into the cage for all mice, tested on day 45 after 15 prior handling sessions with the familiar handling method.

Data analysis. For tests in which all cages were handled by a single handler, data were averaged for the two mice in each cage; repeated measures ANOVAs examined response immediately before and after handling as a within-subjects effect and handling method and sex as between-subjects effects. To assess response to the handler using nine different handlers, data were averaged across all mice handled by each method for each handler (that is, two mice per cage for eight handlers in batch 2, two mice times eight cages for one handler in batch 1); repeated measures ANOVAs examined handling method and response immediately before and after handling as within-subject effects per handler. For elevated plus maze tests where mice were removed from their home cage and tested individually, the effect of handling method was examined using parametric or nonparametric[14] ANOVAs, with sex as an additional factor. In these tests, data from each mouse were treated as independent to follow normal convention. However, confirmatory repeated measures ANOVAs of mean response per cage found similar statistical significance between handling methods (for example, for the data shown in **Figure 1** and **Supplementary Figure 3**, mean response per cage for protected stretch attend: BALB/c, $\chi^2 = 14.2$, $P = 0.001$; C57BL/6, $\chi^2 = 12.3$, $P = 0.002$; ICR, $\chi^2 = 10.5$, $P = 0.005$; open arm entries: BALB/c, $F_{2,18} = 0.29$, $P = 0.76$; C57BL/6, $F_{2,18} = 6.19$, $P = 0.009$; ICR, $\chi^2 = 7.0$, $P = 0.030$). Within each ANOVA, planned contrasts tested whether the response to each alternative handling method differed significantly from the standard tail handling method (Mann-Whitney U tests were used for nonparametric analyses). Nonparametric tests[14] were used when graphical inspection of data and Kolmogorov-Smirnov and Shapiro-Wilk tests indicated that data did not approximate normality and data could not be transformed to meet assumptions of parametric analyses.

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