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An improved conflict avoidance assay reveals modality-specific differences in pain hypersensitivity across sexes

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Abstract

Abnormal encoding of somatosensory modalities (ie, mechanical, cold, and heat) are a critical part of pathological pain states. Detailed phenotyping of patients' responses to these modalities have raised hopes that analgesic treatments could one day be tailored to a patient's phenotype. Such precise treatment would require a profound understanding of the underlying mechanisms of specific pain phenotypes at molecular, cellular, and circuitry levels. Although preclinical pain models have helped in that regard, the lack of a unified assay quantifying detailed mechanical, cold, and heat pain responses on the same scale precludes comparing how analgesic compounds act on different sensory phenotypes. The conflict avoidance assay is promising in that regard, but testing conditions require validation for its use with multiple modalities. In this study, we improve upon the conflict avoidance assay to provide a validated and detailed assessment of all 3 modalities within the same animal, in mice. We first optimized testing conditions to minimize the necessary amount of training and to reduce sex differences in performances. We then tested what range of stimuli produce dynamic stimulus–response relationships for different outcome measures in naive mice. We finally used this assay to show that nerve injury produces modality-specific sex differences in pain behavior. Our improved assay opens new avenues to study the basis of modality-specific abnormalities in pain behavior.

Keywords: Operant pain behavior, Conflict avoidance, Neuropathic pain, Cuff, Preclinical pain models, Sex difference, Male, Female, Modality, Heat, Cold, Mechanical, Thermal, Translation, Preclinical, Mouse, c57bl/6

1. Introduction

The somatosensory system has functionally and anatomically distinct pathways conveying specific sensory modalities from the periphery to the brain.[24,33,39,59] In a healthy state, somatosensory information (eg, touch, cold, and warm) drives approach and avoidance behaviors. However, pathological states can lead to abnormal sensory encoding of certain sensory modalities and, ultimately, to painful states.[21,58,61] Patients with neuropathic pain display distinct sensory gain-of-function and/or loss-of-function profiles regardless of etiology, suggesting that there might be common mechanisms underlying the same sensory profile even from different pathologies.[4,26,78] Clinical researchers have since turned to modality-specific assessment in peripheral painful neuropathies to reveal interactions between sensory phenotypes and the response to specific analgesics.[2,7,16,17,25,42] Still, few links are known with specific neurobiological mechanisms, making targeted development of analgesics difficult.

By applying stimuli of different modalities (ie, mechanical, heat, and cold) in different transgenic lines, preclinical studies have made significant headway into describing circuitries linked to modality-specific nociception in normal and experimental models of pathological conditions.[33,49] Approximately 70% of articles using animal models rely on reflexive assays, such as von Frey and Hargreaves tests.[66] Although these methods can reliably detect whether certain nociceptive modalities are affected by a particular injury, they rely on entirely different physical quantities (ie, grams vs latency) that do not allow for direct comparison of modality-specific nociceptive behavior. Thus, one can hardly make conclusions about the relative importance of certain modalities in specific pathological conditions or the relative effect of certain analgesics on a specific modality.

Although these assays measure spinal reflexes, they do not consider the cognitive and emotional dimensions of pain. In the past decade, there has been a rise in the use of operant tests to measure voluntary pain behavior.[32,66] Such assays use painful stimuli to alter reinforced behavior (ie, reward seeking or punishment avoidance) and assess the motivation to avoid certain stimuli.[53,54,63] They are often limited to a single modality and rewards need to be carefully selected to avoid introducing sexually dimorphic preference/avoidance that could bias results.[27,46,63] The conflict avoidance test was previously developed to obtain

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a detailed, stimulus–response assessment of pressure-evoked pain using light as an avoidance factor to cross pointed probes.[23,28,70,71] Its use for modalities other than mechanical sensitivity has not been explored. A unified assay measuring mechanical, cold, and heat stimulus–response relationships on the same quantitative scale would offer new insights in mechanisms linked to certain phenotypes across pain models.

Our objective was to optimize and validate an operant and quantitative method based on the conflict avoidance paradigm to assess, using comparable parameters, modality-specific sensory behavior in mice. The dynamic range of stimulus–response relationships were determined for various outcome measures with mechanical, cold, and heat stimuli in naive animals. These measures were then used to estimate response profiles in nerve-injured animals and reveal differences in hypersensitivity across modalities and between the sexes.

2. Material and methods

2.1. Animals

All experimental procedures have been performed in accordance with guidelines from the Canadian Council on Animal Care and approved by the committee for animal protection of Université Laval (CPAUL; authorization number: 2018-027-3). Experiments were performed on adult (8-14 week old) male and female C57Bl/6J (JAX #000664; Jackson Laboratory or Crl : COBS.CD-1(ICR)BR colony (CD-1 mice, Charles River). Mice were housed in standard plastic cages in groups of 3 to 4 animals in ventilated racks under a 12-hours light–dark cycle starting at 7:00 AM. Mice were allowed at least 1-week acclimatization to the facility after delivery before starting experiments. They were tested during light cycle, between 9:00 AM and 6:00 PM. Testing was done in climate-controlled rooms (22-23°C, 30%-50% humidity). Behavioral assessment was conducted by Samuel Ferland (male experimenter).

2.2. Nerve injury

A mixture of isoflurane and oxygen was used during anesthesia (4% induction, 2% maintenance). In the cuff model, nerve injury was induced by placing a polyethylene cuff (0.38 mm ID, 1.09 mm OD, BD Intramedic PE20) around the sciatic nerve as previously described.[5] In the spared nerve injury model (SNI), the tibial and common peroneal nerves were ligated using 6-0 silk sutures, then cut to leave the sural nerve intact.[14] Baseline measurements for sensory tests were taken the week before surgery. Testing started 4 weeks after surgery.

2.3. Conflict avoidance assay

2.3.1. Apparatus

The apparatus (Coy MCS, Noldus, the Netherlands) consisted of a dark and a lit chamber linked by a corridor that we modified to deliver mechanical, cold, and heat stimuli in different sets of experiments (**Fig. 1A**). The dark and light chambers each measured 16.5 cm wide × 21.5 cm deep × 15.25 cm high. A mouse-adapted corridor (4.2-cm large, 4.2-cm high, and 39.5-cm long) linked the 2 chambers. The lighting in the light chamber was composed of a white 6.5 W LED bulb giving approximately 18,400 lux when on. Light intensities in the corridor and the dark chamber with the light on were of approximately 55.0 and 8.0 lux, respectively. Mice were acclimatized to the room for 60 minutes before tests, and all tests were done under red lighting in the room. A piece of white vinyl was put on the wall behind the corridor to improve the quality of video tracking. Testing order was randomized for each testing day by assigning a random number to each animal using the RAND() function in Microsoft Excel.

2.3.2. Tracking

Trials were recorded using a USB webcam (Microsoft LifeCam Cinema). Experiments were planned, recorded, and run using Anymaze (Stoelting Co, Wood Dale, IL, version 6.34), but tracking had to be done *post hoc* using DeepLabCut (Mathis lab; version 2.2.0.3).[44,52] DeepLabCut uses deep neural networks to estimate animal poses, thus providing better performances than traditional software on the videos with a nonuniform background. To train the model, we followed DeepLabCut recommendations and, specifically, we labeled the corners of the apparatus, the door, and the mouse center in 2904 images and used a ResNet50-based neural network for 5 training iterations (150-200 k iterations each, final test error = 2.77 pixels, train error = 2.20 pixels). Another model was trained to detect CD-1 with similar performances (test error = 3.29 pixels, train error = 3.07 pixels). These models were then used to extract positions from each video, and a modification of a preexisting code (https://github.com/FedeClaudi/DLCutils/blob/master/time_in_each_roi.py) was used to measure latency to escape from the light chamber, the latency to return from the dark chamber and the traversing speed (centimeters per second) for each trial.

2.3.3. Validation protocol

To validate the aversive nature of the light chamber, mice were allowed to explore the apparatus for 15 minutes with the lights off followed by another 15 minutes with the lights on. Time spent in the light chamber was measured with lights on and off and with or without a white background in the chamber.

2.3.4. Training protocol

To establish how training affected behavior, one mouse cohort underwent a 4-day training protocol without the presence of mechanical, cold, or heat stimuli. On the first day, they were acclimatized to the apparatus for 10 minutes (5 minutes lights off, 5 minutes lights on). In the following sessions (one session per day), they were placed in the dark chamber for 60 seconds, then in the light chamber for 60 seconds before the light was turned on and the doors were opened. Mice were allowed to escape to the dark chamber and return to the light chamber once. Trials were ended if animals stayed more than 60 seconds in any chamber without crossing. This was repeated for a total of 4 trials separated by at least 30 minutes per each session (**Fig. 1B**). Training was tested with and without white background in 2 separate cohorts of mice.

2.3.5. Testing protocol

A different cohort of mice was tested with the presence of stimuli in the corridor. The testing protocol consisted of 5 days: acclimatization, training (1 session; see Results 3.1), and 3 testing days. Acclimatization and training were conducted as described above. On testing days, mice were presented with mechanical, cold, and heat stimuli. Two baseline trials (0 mm for mechanical, 30°C for thermal) were taken before pursuing with 3 stimuli of increasing intensity (raw baseline values are reported in Supplementary Fig. 2, available at http://links.lww.com/PAIN/B971). The cutoff point was increased to 120 seconds to allow for a wider range of behavior as previously used by others in mice.[23,71] Mechanical stimuli consisted of a custom-made array of probes with adjustable height (0, 2, 3, 4

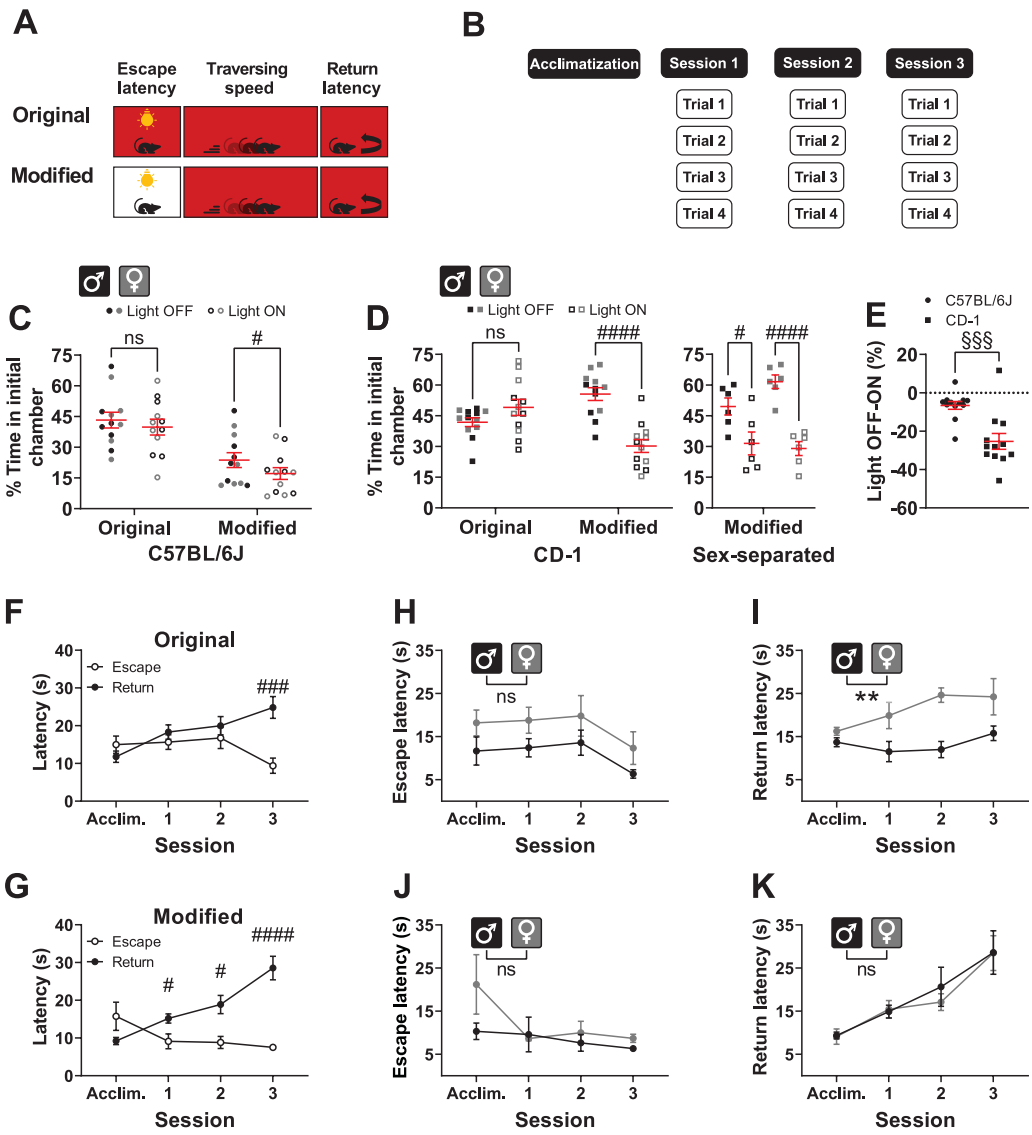

Figure 1. Validation of the effect of aversion to the initial compartment on training performances. (A) Diagram of the original and modified apparatus with measures of interest. (B) Diagram of the training protocol. (C and D) Percentage of the time spent in the initial compartment in the original and modified apparatus (n = 12 each), with and without light for (C) C57BL/6J mice (compartment $F_{1,22} = 20.11$, $P = 0.0002$, RM 2-way ANOVA) and (D) CD-1 mice (Compartment x light, $F_{1,22} = 32.30$, $P < 0.0001$, RM 2-way ANOVA). Sex-separated values for CD1 are provided (D, right panel). Male mice are represented in black, and female mice in grey. (E) Difference between light off and on conditions for the modified compartment in both strains tested. (F and G) Comparison of escape and return latencies over the training procedure in the original (F), and modified (G) apparatus. Escape (H) and return (I) latencies of male and female mice trained in the original apparatus (Escape $F_{1,18} = 3.739$, $P = 0.07$; Return $F_{1,18} = 10.73$, $P = 0.004$, RM 2-way ANOVA). (J and K) Similar comparison for the modified apparatus (Escape $F_{1,14} = 2.713$, $P = 0.1$; Return $F_{1,14} = 0.07963$, $P = 0.8$, RM 2-way ANOVA). Data are shown as mean ± SEM. Differences between the groups are represented as asterisks (*), results from *post hoc* tests are represented as hashes (#), and results from the Mann–Whitney test are represented as a section sign (§). $^{**}P < 0.01$, $^{\#}P < 0.05$, $^{§§§}P < 0.001$, $^{\#\#\#\#}P < 0.0001$, $^{§§§}P < 0.001$, $^{§§§§}P < 0.0001$, ns, not significant. ANOVA, analysis of variance; RM, repeated measures.

mm) separated by 5 mm. Thermal stimuli consisted of an aluminum plate placed on top of 2 hot–cold plates (Bioseb, France). Temperature was adjusted on the hot–cold plates so that the temperature of the aluminum plate reached wanted values (hot = 40°C, 45°C, 50°C; cold = 25°C, 20°C, 15°C). A piece of black electrical tape was affixed on the plate to measure temperature using an IR thermometer (Lasergrip 774, Etekcity). During test sessions, escape and return latencies, as well as traversing speed, were measured and expressed (in seconds and centimeters per second, respectively) as the difference from the group average measure of the 2 baselines taken on the same day. Because not all the tested animals escaped the light chamber or returned from the dark chamber within the cutoff time, the percentage of animals successfully crossing the corridor was also considered. Animals that crossed the midpoint of the corridor without reaching the dark chamber were excluded from the analysis of return successes.

2.4. Reflexive tests

Animals were acclimatized to the testing room for 60 minutes and to the apparatus for another 60 minutes before testing. Mechanical threshold was tested using the simplified up–down method[6] with von Frey filaments #1 to #9.

2.5. Statistics

Statistical analysis was performed with Graphpad Prism 9 (Graphpad Software). An unpaired Mann–Whitney test was used to compare effect size after light exposure between C57BL/6J and

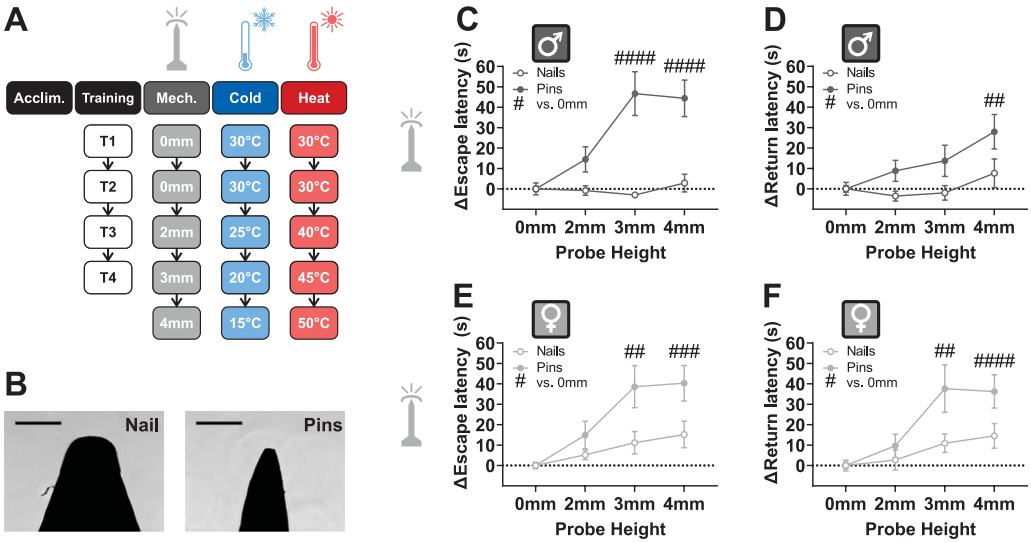

Figure 2. Aversive response to mechanical probes of different sizes in naive male and female mice. (A) Diagram of the testing protocol for all modalities. (B) Microscopic images of nail and pin tips (left and right panel, respectively; scale bar = 500 μm). (C) Escape latency of male mice exposed to nails (open circles) or pins (full circles) (stimulus × probe height $F_{3,130} = 8.105$, $P < 0.0001$, 2-way ANOVA). (D) Return latency of male mice exposed to nails or pins (probe height $F_{2.242,79.23} = 6.662$, $P = 0.001$; stimulus $F_{1,45} = 5.307$, $P = 0.03$, 2-way ANOVA). (E) Escape latency of female mice exposed to nails or pins (stimulus $F_{1,48} = 3.735$, $P = 0.06$; Probe Height $F_{2.470,\ 112.0} = 10.89$, $P < 0.0001$). (F) Return latency of female mice exposed to nails or pins (stimulus × probe height $F_{3,\ 114} = 3.532$, $P = 0.02$, 2-way ANOVA). Data are shown as mean ± SEM. Differences from baseline for pins are represented as hashes (#). Scale bar = 500um. $^{\#\#}P < 0.05$, $^{\#\#\#}P < 0.001$, $^{\#\#\#\#}P < 0.0001$. ANOVA, analysis of variance.

CD-1 mice and results from the von Frey test. Three-way analyses of variances (ANOVAs) were used to compare the effect of sex, light, and compartment color on the time spent in the light compartment. Two-way ANOVAs were used to compare time spent in the light compartment, escape latency, return latency, and traversing speed between and within the groups. Repeated-measures (RM) 1-way ANOVAs followed by Bonferroni *post hoc* tests were used instead when only one variable was compared. Differences within and between the groups were analyzed using repeated-measures 2-way ANOVA followed by Bonferroni *post hoc* tests when they were appropriate. Within-group measures include light status, training session, or stimulus intensity. Between-group measures include the type of mechanical probes, sex, or surgery. Mixed-effects analysis was used instead when values were missing, such as when animals failed to escape the room and traverse the corridor. Geisser–Greenhouse correction was applied to correct for the violation of sphericity. The lowest *P* value between the main effect and the interaction was reported when results did not reach significance. Statistical differences in the number of successful vs unsuccessful escapes and returns from the light and dark rooms were assessed in a contingency table with Fisher exact test. $P < 0.05$ was considered statistically significant. The data are presented as mean ± SEM, with *n* representing the number of animals.

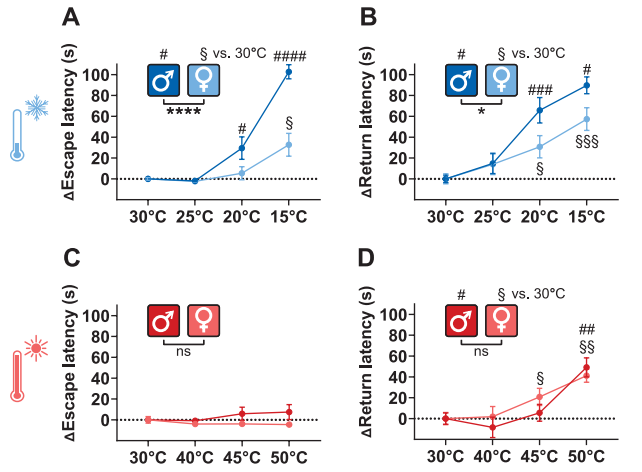

Figure 3. Aversive response to various temperatures in naive male and female mice. (A) Escape and (B) return latency of male (blue) and female (light blue) mice in response to cold temperatures (escape sex × temperature $F_{3,\ 96} = 15.45$, $P < 0.0001$; Return Sex × Temperature $F_{3,\ 78} = 2.974$, $P = 0.04$, RM 2-way ANOVA and 2-way ANOVA). (C) Escape and (D) return latency of males (red) and females (pink) in response to hot temperatures (Return Temperature $F_{2.674,\ 75.76} = 18.50$, $P < 0.0001$, RM 2-way ANOVA; Return Sex $F_{1,\ 29} = 0.3699$, $P = 0.6$, RM 2-way ANOVA). Data are shown as mean ± SEM. Differences from baseline are represented as hashes (#) for male mice and section signs (§) for female mice. $^{*}P < 0.05$, $^{****}P < 0.0001$, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, $^{\#\#\#\#}P < 0.0001$, $^{§}P < 0.05$, $^{§§}P < 0.01$, $^{§§§}P < 0.001$, ns, not significant. ANOVA, analysis of variance, RM, repeated measures.

3. Results

3.1. Testing light aversiveness in the conflict avoidance apparatus

The conflict avoidance test uses a bright light to motivate animals to escape a chamber, traverse a corridor with noxious probes, and reach a dark chamber (**Fig. 1A**). The escape latency was previously shown to increase in the presence of mechanical probes in the

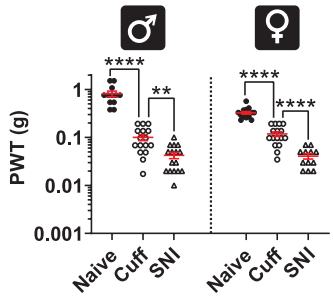

Figure 4. Validation of hypersensitivity following surgery using the von Frey test, a classical reflexive method. Paw withdrawal threshold of male and female naive, cuff, and SNI mice. $^{**}P < 0.01$, $^{****}P < 0.0001$. PWT, paw withdrawal threshold; SNI, spared nerve injury.

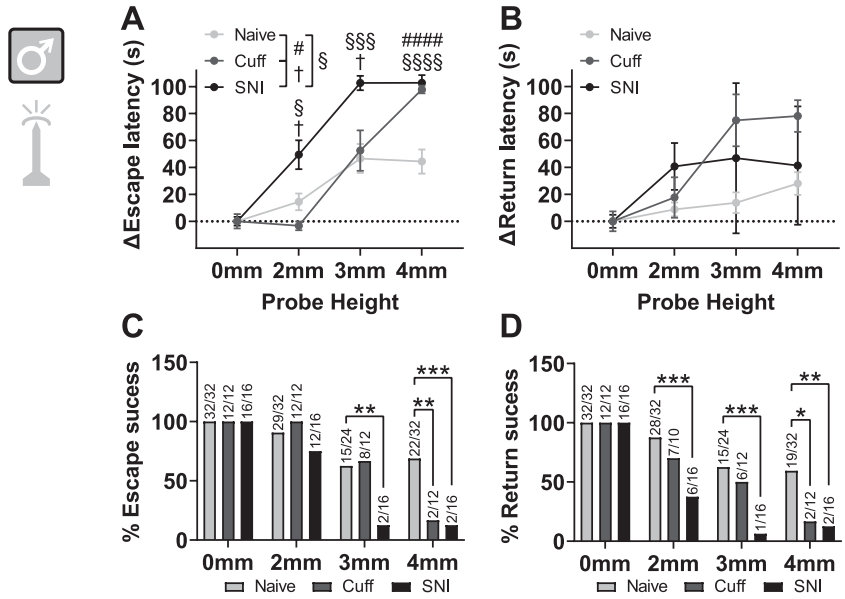

Figure 5. Aversive response of male mice to mechanical probes following nerve injury. (A and B) Escape and return latency of nerve-injured male mice in comparison to naive male mice (Escape Injury × Probe height $F_{6, 163} = 10.09$, $P < 0.0001$, 2-way ANOVA; Return Injury × Probe height $F_{6, 83} = 4.296$, $P = 0.0008$, 2-way ANOVA). (C and D) Percentage of escape and return success of naive and nerve-injured male mice in the presence of mechanical probes. Data are shown as mean ± SEM. Results from *post hoc* tests comparing naive vs cuffs (#), naive vs SNI (§), and cuff vs SNI (†). Results from Fisher exact test are represented by asterisks (*). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{\#\#\#\#}P < 0.0001$, $^{\S}P < 0.05$, $^{\S\S\S}P < 0.001$, $^{\S\S\S\S}P < 0.0001$, $^{\dagger}P < 0.05$. ANOVA, analysis of variance; SNI, spared nerve injury.

corridor, and this increased further in nerve-injured rats.[28] The aversive effectiveness of light in the initial chamber is therefore a key factor in driving the animals across the corridor, but light aversion was not investigated in the initial article. Therefore, we set out to investigate the aversiveness of the starting chamber, and how light affects performances after a 3-day training protocol (**Fig. 1B**). Surprisingly, we found that light was unable to induce strong aversive behavior in the original chamber, whose plexiglass walls were dark and transparent (**Fig. 1C**). Therefore, we decided to modify the light chamber by covering the walls with white vinyl stickers. C57BL/6J mice spent significantly less time overall in the modified chamber than the original one (**Fig. 1C**, $P = 0.0002$, n = 12 per group, RM 2-way ANOVA). *Post hoc* comparisons showed that light further decreased the time spent in the modified chamber ($P = 0.04$, Bonferroni *post hoc* test) but not the original one ($P = 0.4$). Conversely, the time in the dark compartment did not increase significantly after light exposure (Supplementary Fig. 1A, available at http://links.lww.com/PAIN/B971, $P = 0.09$). Because no sex-dependent effect on the time spent in the light chamber was observed (n = 6 per sex, $P = 0.8$, 3-way ANOVA), data from male and female mice have been combined. To test whether aversiveness was strain dependent, we also tested CD-1 mice in the original and modified apparatus (**Fig. 1D**). Unlike C57BL/6J, chamber configuration alone did not decrease the time spent in the initial chamber (**Fig. 1D**, $P = 0.5$, n = 12 per group, RM 2-way ANOVA), although light decreased it depending on the configuration used ($P < 0.0001$, eta-squared = 0.34). *Post hoc* comparisons showed that light significantly decreased the time spent in the compartment when it was delivered in the modified chamber ($P < 0.0001$). Comparison of the light aversion between C57BL/6J and CD-1 mice revealed that light had a bigger effect in CD-1 (**Fig. 1E**, $P = 0.0004$, Mann–Whitney test). Also, time in the dark compartment was significantly increased after light exposure (Supplementary Fig. 1B, available at http://links.lww.com/PAIN/B971, $P = 0.0004$) with a stronger effect for CD-1 than for C57BL/6J (Supplementary Fig. 1C, available at http://links.lww.com/PAIN/B971, $P = 0.002$, Mann–Whitney test). Sex had a small but significant effect on light aversion in the modified chamber (**Fig. 1D**, $P = 0.03$, eta-squared = 0.05, 3-way ANOVA), which can be explained by a higher preference of CD-1 female mice for the white chamber when the light is off. Still, both male and female mice decreased their time spent in the modified compartment when the light was turned on (males $P = 0.01$, females $P < 0.0001$, Bonferroni *post hoc* test).

Subsequently, we addressed training performances with both modified and original backgrounds for both escape and return latencies in C57BL/6J. Training was deemed successful when animals showed avoidance for the lit chamber (ie, shorter escape latency than return latency). The original chamber required more training sessions because escape latencies were shorter than return latencies only after the third training session (**Fig. 1F**, $P = 0.0001$, RM 2-way ANOVA with Bonferroni *post hoc* test). Conversely, using the modified chamber, escape latencies were shorter than return latencies after a single training session (**Fig. 1G**, $P = 0.02$). In these experiments, we also addressed differences because of the animal sex. Interestingly, sex differences were only observed with the original chamber, where returning times were longer in female mice than in male mice (**Figs. 1H** and **I**, $P = 0.004$, RM 2-way ANOVA). Instead, the modified chamber produced similar escape and return latencies in both sexes (**Figs. 1J** and **K**, escape $P = 0.1$, return $P = 0.8$). These data indicate that using a modified chamber with white walls provides stronger aversion, quicker training, and responses that are not affected by sex. Therefore, the modified chamber with a single training session was chosen for subsequent tests.

3.2. Testing mechanical sensitivity in mice with the conflict avoidance apparatus

Mice were tested for mechanical, cold, and heat sensitivity over a 5-day protocol (**Fig. 2A**). Mechanical sensitivity was originally tested by others in rats by measuring the avoidance induced by mechanical probes in the corridor against the aversiveness

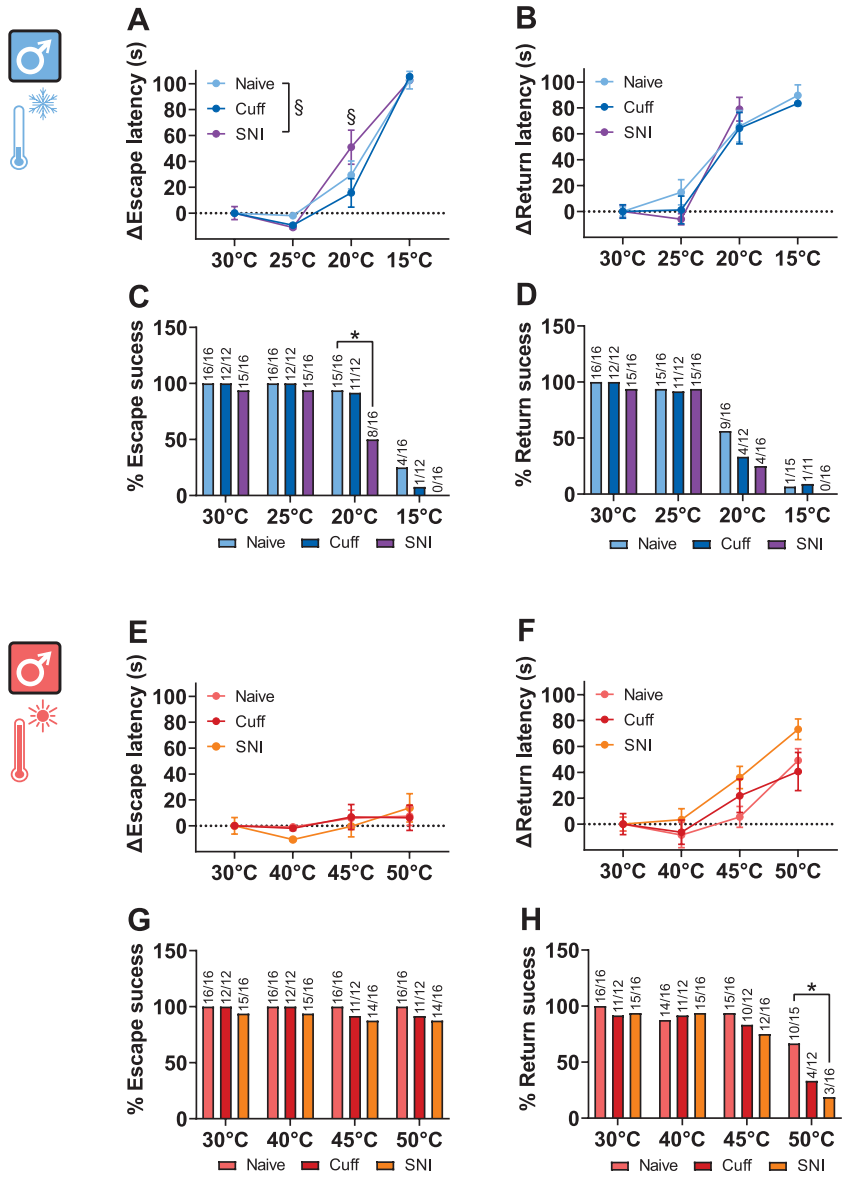

Figure 6. Aversive response of male mice to thermal stimuli following nerve injury. (A and B) Escape and return latency of naive and nerve-injured male mice in response to cold temperatures (Escape Temperature × Injury $F_{6, 120} = 2.273$, $P = 0.04$, RM 2-way ANOVA; Return Injury $F_{2, 40} = 0.2559$, $P = 0.8$, 2-way ANOVA). (C and D) Percentage of escape and return success of naive and nerve-injured male mice in response to cold temperatures. (E and F) Escape and return latency of naive and nerve-injured male mice in response to hot temperatures (Escape Injury $F_{2,40} = 0.09868$, $P = 0.9$, RM 2-way ANOVA; Return Injury $F_{2,38} = 2.109$, $P = 0.1$, 2-way ANOVA). (G and H) Percentage of escape and return success of naive and nerve-injured male mice in response to hot temperatures. Data are shown as mean ± SEM. Results from *post hoc* tests comparing naive vs SNI are represented as section signs (§). Results from Fisher exact test are represented by asterisks (*). $^{*}P < 0.05$, $^{§}P < 0.05$. ANOVA, analysis of variance; RM, repeated measures; SNI, spared nerve injury.

induced by light. Briefly, increasing the probe height was shown to increase the latency of the animal to escape from the light chamber.[28] However, we noticed that although the original interprobe distance (1 cm) was well suited for the rats' paws, it was not adequate for mice. Therefore, to develop an apparatus suitable for mice, we designed 2 custom-made arrays of probes separated by 0.5 cm, one with nails and one with map pins (**Fig. 2B**, nail diameter = 500 μm, pin diameter = 125 μm). Then, escape and return latencies and traversing speed were compared in naive mice of both sexes exposed to either nails or pins with different heights. To facilitate comparisons between stimuli intensities, data are expressed as the difference between each measurement and the corresponding average baseline. Raw baseline values are reported in Supplementary Fig. 2A-D (available at http://links.lww.com/PAIN/B971).

In male mice, pins produced a larger aversive effect as compared with nails for both escape latencies (**Fig. 2C**, $P < 0.0001$, n = 16 for nails and n = 24-32 for pins, 2-way ANOVA) and return latencies (**Fig. 2D**, $P = 0.03$, 2-way ANOVA). *Post hoc* comparisons revealed a significantly longer escape latency for pins set at 3 mm ($P < 0.0001$, Bonferroni *post hoc* test) and 4 mm ($P < 0.0001$) when compared with baseline (0 mm), which indicates an intensity-dependent aversive effect (**Fig. 2C**). Conversely, return latencies for pins were only significantly longer than baseline at 4 mm (**Fig. 2D**, $P = 0.003$). No differences from baseline were found for either escape or return latencies when animals were exposed to nails (**Figs. 2C** and **D**).

Also, in female mice, a stronger aversive effect was observed with pins rather than nails, although it was not significant in escape latency (**Fig. 2E**, $P = 0.06$, n = 18 for nails and n = 24-32

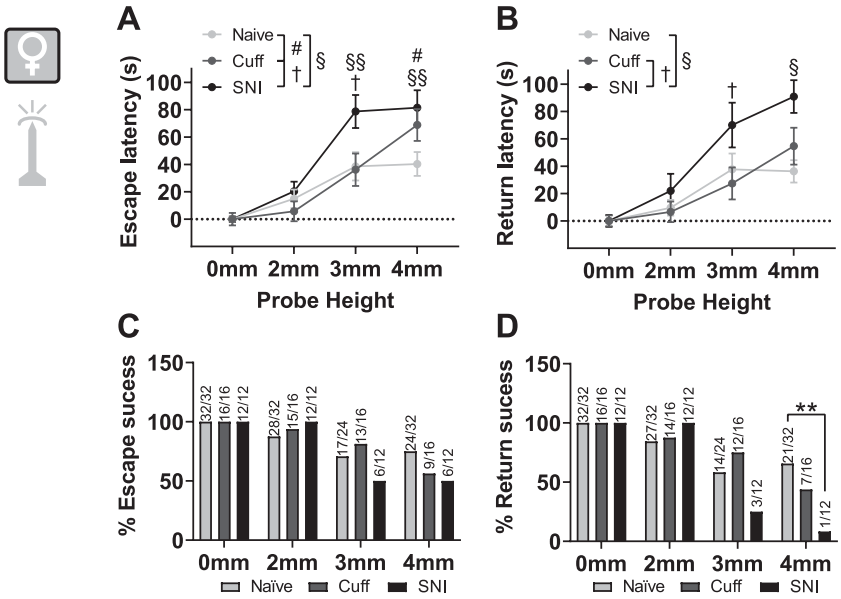

Figure 7. Aversive response of female mice to mechanical probes following nerve injury. (A and B) Escape and return latency of female cuffs and SNIs in comparison to naive female mice (Escape Injury x Probe height $F_{6, 163} = 3.837$, $P = 0.001$, 2-way ANOVA; Return Injury × Probe Height $F_{6, 123} = 2.158$, $P = 0.05$, 2-way ANOVA). (C and D) Percentage of escape and return success of naive and nerve-injured female mice in response to mechanical probes. Data are shown as mean ± SEM. Results from *post hoc* tests comparing naive vs cuffs (#), naive vs SNI (§), and cuff vs SNI (†). Results from Fisher exact test are represented by asterisks (*). $^{**}P < 0.01$, $^{\#}P < 0.05$, $^{\S}P < 0.05$, $^{\S\S}P < 0.01$, $^{\dagger}P < 0.05$. ANOVA, analysis of variance; SNI, spared nerve injury.

for pins, 2-way ANOVA) but only for return latency ($P = 0.02$). Indeed, probe height increased escape latency for both stimuli (**Fig. 2E**, $P < 0.0001$, n = 18 for nails, n = 24-32 for pins, 2-way ANOVA). Conversely, pins had a significantly stronger effect than nails on return latency depending on probe height (**Fig. 2F**, $P = 0.02$). *Post hoc* comparisons revealed that escape latency was longer with pins set at 3 mm ($P = 0.003$, Bonferroni *post hoc* test) and 4 mm ($P = 0.0002$) when compared with baseline (0 mm). Similar results were also found for return latencies with pins set at 3 mm ($P = 0.003$) and 4 mm ($P < 0.0001$). As for male mice, *post hoc* comparisons failed to detect significant differences with nails when compared with baseline.

When comparing aversion with mechanical stimuli between the sexes, we observed that female mice exposed to nails displayed longer escape latencies than male mice ($P = 0.03$, n = 16 for male mice, n = 18 for female mice, RM 2-way ANOVA), whereas no effect was observed on return latencies ($P = 0.1$). Contrastingly, both sexes had similar escape and return latencies when exposed to pins (escape, $P = 0.7$, return $P = 0.2$, n = 24-32 for each, 2-way ANOVA).

In addition to escape and return latency, we also measured the traversing speed on the first crossing (from light to dark chamber) and the second crossing (from dark chamber to light chamber). No differences were found in traversing speed between either stimulus for male or female mice. However, increasing probe height significantly decreased traversing speed for male and female mice (Supplementary Fig. 3, available at http://links.lww.com/PAIN/B971, $P < 0.0001$ for first and second crosses, RM 2-way ANOVA).

Altogether, pins were found to be more aversive than nails in both sexes and capable to induce a steeper stimulus–response relationship. Moreover, female mice appear more sensitive to mechanical stimuli than male mice in the conflict avoidance apparatus.

3.3. Testing thermal sensitivity in mice with the conflict avoidance apparatus

To allow testing thermal sensitivity, the floor of the corridor was equipped with a single aluminum plate placed upon 2 hot–cold plates for temperature adjustments. We then tested responses of male and female mice to temperatures ranging from innocuous to noxious cold and heat.

Cold stimuli induced a progressive increase in both escape and return latencies along with the lowering of the temperatures (**Figs. 3A** and **B**, $P < 0.0001$ for both, n = 16 for male mice, n = 18 for female mice, RM 2-way ANOVA and 2-way ANOVA). In male mice, both escape and return latencies were significantly longer at 20°C (escape: $P = 0.04$, return $P = 0.0001$; Bonferroni *post hoc* test) and 15°C (escape $P < 0.0001$, return $P = 0.03$; Bonferroni *post hoc* test) compared with baseline temperature. In female mice, longer escape latencies were only found at 15°C ($P = 0.02$), whereas return latencies were significantly increased at both 20°C ($P = 0.01$) and 15°C ($P = 0.0002$). The differences in escaping and returning behavior between the sexes were statistically significant (Escape $P < 0.0001$, Return $P = 0.04$, RM 2-way ANOVA and 2-way ANOVA). No relevant correlations with temperature and/or sex were found for the traversing speed on the first and second crossing (Supplementary Fig. 4A and B, available at http://links.lww.com/PAIN/B971).

Heat stimuli significantly increased return latencies (**Figs. 3C** and **D**, $P < 0.0001$, n = 16 male mice and 18 female mice, RM 2-way ANOVA) but had no effect on escape latencies ($P = 0.6$, RM 2-way ANOVA). Pairwise comparisons indicated that return latencies were significantly longer than baseline at 45°C for female mice only (female mice $P = 0.05$, male mice $P = 1$, Bonferroni *post hoc* test) and 50°C for both sexes (females $P = 0.0009$, males $P = 0.002$). No effect of sex on return latency was found ($P = 0.4$). Heat also significantly reduced traversing speed on the first ($P = 0.05$, RM 2-way ANOVA) and second ($P = 0.0006$, 2-way ANOVA) crossing (Supplementary Fig. 4C and D, available at http://links.lww.com/PAIN/B971). None of the above measurements highlighted relevant sex differences in aversive behavior to thermal stimuli. A separate cohort of male mice was tested to determine the effect of even higher temperature on escape latency. Heat was only found to increase escape latency

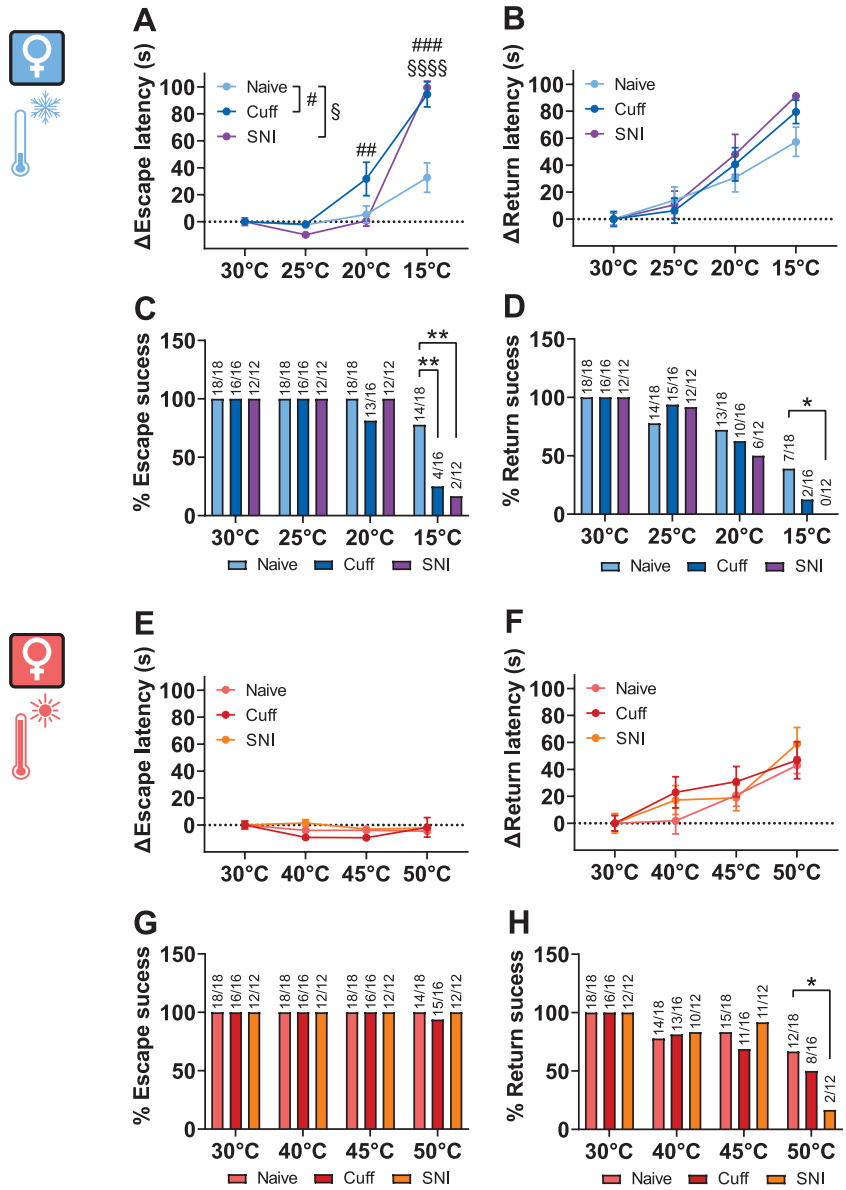

Figure 8. Aversive response of female mice to thermal stimuli following nerve injury. (A and B) Escape and return latency of naive, cuff and SNI female mice in response to cold temperatures (Escape Injury × Temperature $F_{6, 129} = 10.03$, $P < 0.0001$, RM 2-way ANOVA; Return Injury × Temperature $F_{6, 100} = 1.209$, $P = 0.3$, 2-way ANOVA). (C and D) Percentage of escape and return success of naive and nerve-injured females in response to cold temperatures. (E and F) Escape and return latency of naive and nerve-injured female mice in response to hot temperatures (Escape Injury × Temperature $F_{6, 129} = 1.205$, $P = 0.3$, RM 2-way ANOVA; Return Injury $F_{2, 39} = 0.5396$, $P = 0.6$, 2-way ANOVA). (G and H) Percentage of escape and return success of naive, cuff and SNI female mice in response to hot temperatures. Data are shown as mean ± SEM. Results from *post hoc* tests comparing naive vs cuffs (#) and naive vs SNI (§). Results from Fisher exact test are represented by asterisks (*). $^{*}P < 0.05$ $^{**}P < 0.01$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, $^{\S\S\S\S}P < 0.0001$. ANOVA, analysis of variance; RM, repeated measures; SNI, spared nerve injury.

when set at 55°C (Supplementary Fig. 5, available at http://links.lww.com/PAIN/B971, $P = 0.02$, 1-way ANOVA with Bonferroni *post hoc* test).

Only return latency represented reliable parameters to measure heat sensitivity, although both escape and return latency could be used for cold. Although male mice appeared more sensitive to cold stimuli than female mice, no sex differences were observed for heat stimuli.

3.4. Sex-specific hypersensitivity profiles in nerve-injured animals detected by the conflict avoidance apparatus

We next addressed whether the improved conflict avoidance assay can detect changes in aversive behavior to different types of stimuli in 2 different animal models of nerve injury: a chronic injury model based on the sciatic nerve constriction by cuff (cuff model) and the SNI. The development of mechanical allodynia, a classical hallmark of neuropathic pain,[12] was firstly assessed by the von Frey test in both male and female mice (**Fig. 4**). Male and female mice that underwent cuff surgery had decreased mechanical thresholds when compared with naive animals (**Fig. 4**, $P < 0.0001$, n = 16 for both sexes and both groups, Mann–Whitney test), and mechanical thresholds were even lower after SNI (**Fig. 4**, male mice $P = 0.006$, n = 16; female mice $P < 0.0001$ n = 12).

We then proceeded to test them in our conflict avoidance apparatus. Data are reported as difference with the average baseline values to facilitate comparisons between the models;

however, raw baseline data are shown in Supplementary Fig. 2E-H, available at http://links.lww.com/PAIN/B971. Both cuff and SNI male mice displayed significantly increased escape latencies in response to pins compared with naive animals (**Fig. 5A**, Escape $P < 0.0001$, n = 24-32 for naives, n = 12 for cuffs, n = 16 for SNIs, 2-way ANOVA). Pairwise comparisons of escape latencies revealed longer escape latencies in cuff animals when pins were set at 4 mm ($P < 0.0001$, Bonferroni *post hoc* test), and SNI animals when pins were set at 2 mm ($P = 0.03$), 3 mm ($P = 0.0001$), and 4 mm ($P < 0.0001$). In addition, SNI animals had longer escape latencies than cuffs at 2 mm ($P = 0.0006$) and 3 mm ($P = 0.02$). Return latencies were also significantly longer after nerve injury (**Fig. 5B**, Return $P = 0.0008$, n = 24-32 for naive, n = 12 for cuffs, n = 16 for SNIs, 2-way ANOVA), although the low number of animals that failed to reach the dark chamber precluded any pairwise comparison (**Fig. 5C**). Indeed, we observed that for some nerve-injured mice, mechanical stimuli were so aversive that they failed to either escape or return within the cutoff time. In these cases, mice either remained at the corridor entrance, explored the pins with their front paws and whiskers, or even went back again after venturing out shortly. By comparing the number of mice that left the light or dark chambers vs those that did not in a contingency table, we found that the number of failed escapes and returns was significantly greater in nerve-injured mice than naive mice when presented with 3-mm pins for SNI only (**Figs. 5C** and **D**, Escape $P = 0.003$, Return = 0.0007, Fisher exact test) and with 4-mm pins for both cuff and SNI mice (**Figs. 5C** and **D**, Cuff Escape $P = 0.005$, Return $P = 0.02$; SNI Escape $P = 0.0005$, Return $P = 0.002$). In addition, less SNI mice successfully returned from 2-mm pins (**Fig. 5D**, $P = 0.0006$).

Cold temperatures only mildly affected escape latencies in nerve-injured male mice (**Fig. 6A**, n = 16 for naives, n = 12 for cuffs, n = 15 for SNIs, $P = 0.03$, RM 2-way ANOVA). Pairwise comparison revealed that escape latencies increased at 20˚C in SNI but not in cuffs (SNI $P = 0.04$, Cuff $P = 0.3$, Bonferonni *post hoc* test). No effect on return latencies was observed (**Fig. 6B**, $P = 0.8$, 2-way ANOVA). Similarly, a larger number of SNI mice failed to escape at 20˚C (**Fig. 6C**, $P = 0.02$, Fisher exact test), although no differences were observed for return successes (**Fig. 6D**). No effect of nerve injury on heat responses were observed (**Figs. 6E** and **F**, Escape $P = 0.9$, Return $P = 0.1$, n = 16 for naives, n = 12 for cuffs, n = 15 for SNIs, RM 2-way ANOVA and 2-way ANOVA). Conversely, the number of SNI mice that returned at 50˚C was significantly lower than the other groups (**Figs. 6G**and H, $P = 0.01$, Fisher exact test). These data show that male mice develop injury-dependent hypersensitivity profiles, with prominent mechanical hypersensitivity in both models and some thermal hypersensitivity detectable in SNI only.

Nerve-injured female mice also showed longer escape latency when exposed to mechanical stimuli as compared with naive mice (**Fig. 7A**, $P = 0.001$, n = 24-32 for naives, n = 16 for cuffs, n = 12 for SNIs, 2-way ANOVA). Pairwise comparison revealed that escape latency was increased in SNI exposed to 3-mm ($P = 0.005$) and 4-mm pins ($P = 0.004$, Bonferonni *post hoc* test). This was only significant in cuffs at 4 mm ($P = 0.04$). Comparing escape latencies between cuff and SNI female mice revealed a significant difference between them at 3 mm ($P = 0.02$). An interaction was also found between injury and return latency (**Fig. 7B**, $P = 0.05$, 2-way ANOVA). Pairwise comparison indicated significant differences between SNI and naive at 4 mm ($P = 0.01$). In addition, SNI had longer return latencies than cuffs at 3 mm ($P = 0.05$). Moreover, pins did not discourage cuff female mice to leave the light or dark chamber even at 4 mm (**Figs. 7C** and **D**, Escape $P = 0.2$, Return $P = 0.2$), although it did discourage SNI female mice to return at 4 mm (**Figs. 7C** and **D** $P = 0.006$, Fisher exact test). Contrary to male mice, both cuff and SNI female mice showed a marked increase in escape latencies for cold stimuli (**Fig. 8A**, $P < 0.0001$, n = 18 for naives, n = 16 for cuffs, n = 12 for SNIs RM 2-way ANOVA). Pairwise comparison indicated longer escape latencies for both cuff and SNI at 15˚C ($P < 0.0001$ for both, Bonferroni *post hoc* test) and at 20˚C for cuffs only (cuff, $P = 0.007$, SNI $P = 1$), although no effect was observed on return latencies (**Fig. 8B**, $P = 0.3$, 2-way ANOVA). These observations were further supported by the higher number of nerve-injured female mice reluctant to escape the light chamber when presented with 15˚C stimuli in the corridor (**Fig. 8C**, Escape $P = 0.005$ for cuffs, $P = 0.002$ for SNI, Fisher exact test). Female SNI also returned less than naive at 15˚C (**Fig. 8D**, $P = 0.1$ for cuffs, $P = 0.02$ for SNI). No changes were observed in escape or return latency in response to heat (**Figs. 8E** and **F**, Escape $P = 0.3$, Return $P = 0.6$, n = 18 for naives, n = 16 for cuffs, n = 12 for SNIs, RM 2-way ANOVA and 2-way ANOVA). Although heat did not change the number of female mice escaping or not, a larger number of SNI failed to return when exposed at 50˚C (**Figs. 8G** and **H**, $P = 0.01$, Fisher exact test). This effect was not observed in cuffs ($P = 0.5$). In conclusion, nerve-injured female mice display aversive behavior for both mechanical and cold stimuli across both models tested, with modest injury-specific aversion to heat stimuli.

Collectively, our approach shows that each sex displays distinct hypersensitivity profiles across modalities for each nerve injury model. Aversion for mechanical stimuli appears to be a more robust parameter across sexes and models to test for nerve injury–induced hypersensitivity.

4. Discussion

In this study, we have developed a novel conflict avoidance approach to measure the voluntary response to thermal and mechanical stimuli. This method allows comparing sensory modalities with the same outcome measure, contrary to classical reflexive tests. The application of our method in nerve-injured mice has led to the identification of new sex differences in neuropathic animals.

4.1. Improvement of existing conflict avoidance method

Reflexive responses to mechanical, thermal, and cold stimuli have been used to study pain-related hypersensitivity in rodents for decades. They typically quantify paw withdrawal responses to various stimulus intensities as a measure of pain. These responses are conserved in decerebrated animals and are consequently purely dependent on spinal reflexive circuits.[80] By contrast, threshold and suprathreshold responses in humans are usually quantified through voluntary actions, such as the use of a button to stop an increasingly intense stimulus or by verbally rating the stimulus intensity.[65] Importantly, brain areas activated by painful stimuli, including the anterior cingulate cortex, the insula, the prefrontal cortex, and the somatosensory cortex,[18] are involved in the motivational, cognitive, and sensory discriminative aspects of acute pain.[45] The lack of most of these components in classical reflexive tests has sparked interest in conflict-based assays to study the voluntary escape or avoidance of painful stimuli in animal pain models. These paradigms typically involve associating a reinforced behavior (eg, light avoidance, food or reward seeking) with touch, punctate, or thermal stimuli[10,29,34,54] and quantifying whether animals still engage in the reinforced

behavior despite the stimulus. Interestingly, stimulus-induced aversion is abolished by lesioning the anterior cingulate cortex, a brain region involved in emotional processing of pain in rodents and humans.[22,36] However, these methods are physically demanding for the experimenters, requiring manual stimulation of the animals every 10 to 15 seconds. In addition, stimuli are often limited to a single intensity per session, limiting the ability of researchers to draw complete multimodal stimulus–response profiles in various pathological pain models.[29,34] Methods that do not require manual stimulus delivery exist, but they are limited to orofacial pain or temperature assessment.[53,54,63]

To develop a novel operant behavior assay allowing simple and direct comparison of multiple pain modalities, we set to modify and improve an existing conflict avoidance assay. Indeed, it has already been shown to provide an objective, detailed stimulus-response relationship to mechanical stimuli by measuring the latency of animals to escape a brightly lit chamber into a corridor equipped with pointed probes.[23,55,71] Conversely, the system was never adapted to measure aversion to thermal stimuli.

In the process of validating the system for both mechanical and thermal modalities, we found that properly setting the aversiveness of the light compartment is a necessary preliminary step to achieve optimal baseline conditions and to minimize the training required to achieve stable performances. Surprisingly, we found that factory-set light conditions were actually insufficient to produce aversion and that a consistent light aversion in both sexes can only be achieved by changing the light chamber background from dark to white. Indeed, light aversion is driven by nonimage-forming irradiance detection by retinal ganglion cells.[73] Mice were eventually successfully trained over multiple sessions in the original conditions, hinting that aversion to the lit chamber overcomes the exploratory drive. Because contrast against background is a major risk factor for predation in nature,[77] it is likely that the use of a lighter background would drive aversion in black-coated mice even in lowly lit conditions. Indeed, albino CD-1 mice, unlike black C57BL/6J, exhibited a preference for the white background with the light off. Interestingly, illumination in the modified chamber was aversive for both strains, although C57BL/6J showed a weaker light sensitivity compared with CD-1, as observed in other inbred albino strains.[35] Similarly, Sprague Dawley rats used in the original study display stronger aversion to light than nonalbino Long Evans rats.[28,75] The increased aversiveness of light in combination with white walls is presumably due to the increased irradiance in comparison to the original dark transparent wall. Although this reconciles our work with the original method, it also stresses the importance of validating the aversiveness of the light compartment before using different strains, species, or sexes. The improvements made in light aversiveness also reduced the training required to achieve stable aversive responses to light. Indeed, although the original study described a training and testing protocol lasting multiple days,[28] we found that a single training session is sufficient, consistently with more recent reports using a white background.[23,70,71]

4.2. Testing sensory modalities with a conflict avoidance method

Different mechanical probes and temperature ranges were applied to identify the best experimental design to test avoidance against specific sensory modalities. Interestingly, smaller diameter mechanical probes produced stronger aversion, thus indicating that aversive behavior is associated with pressure and skin deformation. Increasing mechanical pressure on the skin is known to increase firing of spinothalamic neurons, with a shift of the stimulus–response curve to lower thresholds in normally nociceptive-specific neurons in neuropathic conditions.[37] In thermal modalities, we found aversive effects for temperature above 45°C or below 20°C, which is consistent with the aversion shown in different temperature-based assays.[38,60,68] This also coincides with response of cold and heat transducer channels TRPM8 and TRPV1 to temperatures below 25°C and above 45°C, respectively, and the activation of thermal nociceptors[60,62,72] and human pain thresholds.[41,64] Surprisingly, only intense heat (55°C) could deter mice from escaping, although mild cold stimuli were sufficient to do so. Conversely, a clear effect on return latency was seen in both contexts. Heating responses are known to be slower than cooling responses and provide a weaker learning stimulus, thus a prolonged contact is likely necessary to produce aversion to heat.[57,76] Such contact only occurs as the animals traverse the corridor, thus return latencies provide complementary information to escape latencies depending on the modality tested.

4.3. Sex differences in sensory profiles of naive and neuropathic mice

The main advantage of the conflict avoidance assay is that it can be used to compare modality-specific responses between multiple conditions. Given that sex is a major factor in pain mechanisms,[48] one of our main objectives was to compare how sexes interact with the responses to different sensory modalities. We noticed that naive male mice exhibited weaker aversion to mechanical probes. This is consistent with a general consensus indicating that male rodents are less sensitive than female rodents to mechanical stimuli,[48] although this is the first time that it was reported in an operant assay to our knowledge. In our hands, male mice also displayed stronger aversion to cold stimuli than female mice. This is consistent with previous observation that male rats avoid temperatures of 10°C more readily than female rats.[9] A recent study has identified a male-specific role of brain-derived neurotrophic factor (BDNF) in spinal cord excitability.[15] Interestingly, the loss of one BDNF allele decreases cold sensitivity (withdrawal threshold from 18.5°C to 12.9°C) in male rats,[15,67] suggesting that sex-specific differences in cold sensitivity may be BDNF dependent.

Nerve-injury deeply alters conflict avoidance behavior in both constriction and transection models. However, avoidance is consistently greater across sex and sensory modalities in SNI mice, where nerve transection leads to a more severe nerve injury and subsequent loss of both myelinated and unmyelinated fibers.[51]

Nerve-injured mice also display dramatic sex-related differences in their respective sensory profiles. Indeed, although male mice developed a strong aversion to mechanical stimuli, but only mild changes to thermal ones, nerve-injured female mice displayed robust avoidance to cold hypersensitivity and moderate avoidance to mechanical stimuli. The divergence of mechanical and thermal sensory profiles in male mice after nerve injury is consistent with the cardinal role played by mechanical allodynia in clinical neuropathic pain.[12,81] Indeed, previous studies have evidenced that during the first days after surgery, both mechanical allodynia and thermal (heat) hyperalgesia are expressed; however, the former lasts for months, while the latter for a few weeks only.[5,20,47,69,82] This may indicate that thermal hyperalgesia is likely a main consequence of the transitory inflammatory response after surgery. However, the development of moderate heat avoidance in male SNI mice, but not in cuff, suggests that also the type of nerve injury may have

consequences in shaping the sensory profile by differentially affecting different types of afferent fibers. For instance, mechanical hypersensitivity is greater with a moderate loss of sensory afferents, and thermal hypersensitivity develops as uninjured nociceptors invade denervated territories.[11,19] Finally, the onset of mechanical allodynia, unlike thermal hyperalgesia, is often associated with central sensitization mechanisms involving spinal neurons.[21,74,79]

Female mice developed significant aversion to mechanical stimuli with lesser heat aversion, which confirms that mechanical hypersensitivity shares a common mechanism in both sexes, probably relying on spinal cord disinhibition.[21,43] However, unlike male mice, we observed a strong aversion to cold temperatures in female mice. Cold hypersensitivity is reported in inflammatory-, nerve injury–, and chemotherapy-induced pain models.[40] Although present in both sexes, cold hypersensitivity is often reported as stronger in female mice and also lasts longer.[8,50] In addition, pharmacologic or optogenetic silencing of calcitonin gene-related peptide α (CGRPα) + nociceptors partially reduces cold allodynia induced by nerve injury.[13,31] Interestingly, we have recently shown that CGRP plays a greater role in female mice in causing spinal disinhibition and pain hypersensitivity.[56] Importantly, our results on female mice are consistent with finding in human volunteers that women exhibited higher menthol-induced cold hyperalgesia.[1]

5. Conclusion

Our method opens new possibilities for testing pain-induced avoidance to multiple sensory modalities and offers a more ecological approach for a direct comparison of animal response to different types of stimuli. This is a necessary step to start clustering and stratifying pain behaviors in preclinical models according to specific sensory profiles, which can vary according to the type of pain, as well as the animal's sex, strain, or species. Noteworthy, the identification of sensory profiles is now largely adopted in human studies to contain the variability in the output of clinical trials and to propose better-tailored therapeutic strategies.[3] The German Neuropathic Pain Research Network have identified 3 main clusters of patients with neuropathic pain displaying sensory loss (with little or no alterations in the response to evoked pain), thermal hyperalgesia, or mechanical hyperalgesia.[4] Each profile can be associated with a specific pattern of alterations in myelinated and unmyelinated fibers functions.[4,30] A limitation of our method is that it is not suitable to measure loss-of-function profiles in animal models because the method is selectively designed to measure hypersensitivity, hence gain of function. Addressing mechanism-based differences underlying evoked pain submodalities in heterogeneous animal models is a necessary step to improve clinical translation. This is what the proposed assay enables.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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S.F. designed experiments, collected, and analyzed the behavioral data and wrote the manuscript. F.W. critically reviewed the manuscript. F.F. designed experiments, wrote, and reviewed the manuscript and provided ideas for the analysis of the results. Y.D.K. designed experiments, reviewed the manuscript, provided ideas for the analysis of the results, and provided supervision through the realization of the study.

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Code and data are available upon reasonable request.

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