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Comparison of Thermal and Mechanical Pain Testing Modalities in Sprague Dawley and Fischer 344 Rats (*Rattus norvegicus*)

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While rodents are used extensively for studying pain, there is a lack of reported direct comparisons of thermal and mechanical pain testing methods in rats of different genetic backgrounds. Understanding the range of interindividual variability of withdrawal thresholds and thermal latencies based on these testing methods and/or genetic background is important for appropriate experimental design. Testing was performed in two common rat genetic backgrounds: outbred Sprague–Dawley (SD) and inbred Fischer 344 (F344). Male and female, 10- to 14-wk-old F344 and SD rats were used to assess withdrawal thresholds in 3 different modalities: the Randall-Selitto test (RST), Hargreaves test (HT), and tail flick test (TFT). The RST was performed by using an operator-controlled handheld instrument to generate a noxious pressure stimulus to the left hind paw. The HT and the TFT used an electronically controlled light source to deliver a noxious thermal stimulus to the left hind paw or tail tip, respectively. Rats of each sex and genetic background underwent one type of test on day 0 and day 7. Withdrawal thresholds and thermal latencies were compared among tests. No significant differences were observed. Our findings can serve as a guide for researchers considering these nociceptive tests for their experiments.

Abbreviations and Acronyms: F344, Fischer 344; HT, Hargreaves test; RST, Randall-Selitto test; SD, Sprague–Dawley; TFT, tail flick test

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Introduction

Rodents have been used extensively to study pain pathways, and the knowledge gained has directed research to improve the identification and management of pain. Various methods to measure pain have been developed in mice and rats through the assessment of physiologic parameters, spontaneous behaviors, and use of specialized equipment often referred to as "pain tests."[2,31] This equipment is commercially available and commonly used for studies specifically evaluating pharmacological agents. Most commonly, these tests infer pain by measuring the latency of limb or tail withdrawal following a noxious stimulus. However, these pain testing modalities most accurately measure nociception, as compared with pain.

Pain is defined by the International Association for the Study of Pain as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, whereas nociception is the neural processing of a noxious stimulus.[17] Therefore, equipment that measures limb or tail withdrawals assesses nociceptive responses. Behavioral assessments such as ethograms and electroencephalogram analysis are a means to measure pain in animals.[18] Therefore, the modalities that we are testing are more accurately described as nociceptive tests, rather than pain tests. Increases and decreases in nociceptive thresholds, hyperesthesia and hypoesthesia, respectively, can also be measured with nociceptive testing equipment.

Nociceptive testing methods have been described using several noxious stimuli, such as thermal, mechanical, chemical, and electrical stimulation.[11] Many factors related to how these tests are performed can influence the outcomes and impact reproducibility. For example, all these methods require the animal to be removed from the home cage and, in some circumstances, restrained. Stress from handling can cause physiologic variations that impact the results of nociceptive testing methods.[1,7] Other factors, such as operator sex, time of day, and social housing, are known to affect responses to painful stimuli.[5,6,25,30]

Another factor that can influence variability is genetic background. In mice, strain-related differences in response to pain have been described for different types of stimuli.[20-22,29,35] For example, BALB/c mice have a longer thermal latency to thermal stimulus via the Hargreaves test (HT) compared with C57BL/6 mice. Conversely, C57BL/6 mice exhibit a higher withdrawal threshold to mechanical stimulus with the Von Fray filament test compared with BALB/c mice.[21] While fewer data are available for rats, strain and sex differences have been reported for withdrawal thresholds and thermal latencies using a nerve injury model.[4,15,34] In addition, vendor origin is a factor for nociceptive differences in outbred Sprague–Dawley rats when using both a chronic nerve injury model and an inflammatory pain model.[19]

Currently, no literature is available to describe the variation of withdrawal thresholds and thermal latencies among nociceptive tests in rats with different genetic backgrounds. The choice of inbred compared with outbred genetic backgrounds, in addition to the unique characteristics of the different strains/stocks of rats,

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can often have a profound impact on study outcomes. Understanding the potential response variability among nociceptive tests, and whether strain/stock genetic differences contribute to these variations, is important for both the selection of testing equipment and the choice of genetic background for animal models used in pain research. The use of a single strain/stock in a study and proper controls can help minimize this variability.

In our study, we evaluated 3 different nociceptive tests, Randall-Selitto test (RST), Hargreaves test (HT), and tail flick test (TFT) in Sprague–Dawley (SD) and Fischer 344 (F344) rats to determine if there is variation in these nociceptive testing modalities between SD and F344 rats. Our hypothesis was that withdrawal thresholds or thermal latencies for each pain nociceptive test would not differ between SD and F344 rats, but due to their outbred genetics, SD rats would have greater variation in withdrawal thresholds and thermal latencies among individual animals compared with the F344 rats.

Materials and Methods

Animals. All animal procedures were approved by the Institutional Care and Use Committee under an approved animal use protocol at the University of Missouri (Columbia, MO), an AAALAC-accredited institution, in accordance with the standards of the *Guide for the Care and Use of Laboratory Animals*, 8th ed.[16] F344/NHsd (F344) and Hsd:SD (SD) rats (*Rattus norvegicus*) were purchased from Envigo (RMS; Indianapolis, IN). The animals were housed in individually ventilated cages (Thoren Caging, Model 8-48-6-8-1-4-5TM; Hazelton, PA) with recycled paper chip bedding (Shepherd Specialty Paper; Nestle Purina, Springfield, MO) and provided ab libitum water and standard rodent diet (LabDiet 5008; Land O'Lakes, St. Louis, MO) on a 12:12-h light:dark cycle (lights on at 0630 AM). All animals were acclimated for a minimum of 1 wk before experimentation. Dirty bedding sentinels were used for health monitoring and sampled every quarter. Sentinels were negative via serology for Sendai virus, pneumonia virus of mice, sialodacryoadenitis virus, Kilhams rat virus, Toolan H-1 virus, rat parvovirus 1, rat minute virus, rat theilovirus, *Mycoplasma pulmonis*, and *Filobacterium rodentium* and negative via PCR for fur mites and pinworms.

Nociceptive testing. Three different methods of nociceptive testing were performed: the RST, HT, and TFT. Ten (5 male and 5 female) F344 and 10 (5 male and 5 female) SD, 10- to 14-wk-old rats were used for each of the 3 tests (n = 60, 20 per test). Each test was performed 2 times per animal: an initial test on day 0 and a second time repeated on day 7. These nociceptive tests were performed in a room dedicated to rodent behavioral testing within the vivarium and were performed by the same person. Procedures for the RST, HT, and TFT were based on previous publications.[8,13,28] Each rat was tested with only one of the nociceptive tests.

Randall-Selitto test. Rats undergoing this test were acclimated to handling starting 3 days before testing to reduce spontaneous movement during restraint. For the RST, rats were gently restrained in a soft cloth until spontaneous movements had ceased for one minute. The point of a handheld Randall-Selitto unit (Almemo 2429; IITC Life Science, Woodland Hills, CA) was centered on the plantar surface of a hind foot. Increasing pressure was applied manually to the foot until the rat withdrew the limb. Maximum force, in grams, was recorded as the withdrawal threshold. At least a one-minute interval occurred between replicates. Greater time passed if spontaneous movements had not ceased in one minute. Five replicates were performed on each day of testing. The highest and lowest values for each data set were excluded, and the remaining 3 values were used for data analysis, as described previously for nociceptive testing in rodents.[8] The test took approximately 10 to 15 minutes (min) to perform for each rat.

Hargreaves test. Rats were acclimated to the enclosures before testing for at least 1 hour (h) with rats of the same sex in adjacent enclosures to simulate testing conditions. Acclimation occurred 48 and 24 h before testing. This acclimation was done to reduce movement during the day of testing. On the day of testing, animals were acclimated for 15 min before starting the thermal tests to reduce spontaneous movements. If a rat urinated or defecated during the acclimation or testing period, the glass was wiped cleaned with water and dried with a paper towel. After the enclosure was cleaned, at least a 5-min waiting period occurred before testing was continued to reduce spontaneous movements. Rats were separated individually in an acrylic enclosure on an elevated glass surface. A visible light source from a plantar and TFT apparatus (Series 8 Model 336T; IITC Life Science) was focused on the plantar surface of the hind paw. The maximum temperature and time exposure were set to not exceed 55 °C and 20 seconds (s) to prevent thermal injury. Active intensity, thermal output from the light source, was set to 31% for SD rats and 28% for F344 rats. The active intensity was determined with a pilot study of 3 male and 3 female rats of each genetic background to assess which output would produce a thermal latency at approximately 10 s. Thermal latency, the elapsed time to withdraw the limb at a set temperature (active intensity), was recorded. At least 5 min elapsed between each replicate. Five replicates were performed on each day of testing. The highest and lowest values for each data set were excluded and the remaining 3 values were used for data analysis, as described previously for nociceptive testing in rodents.[8] The test took approximately 45 to 60 min to complete for one rat. Multiple rats can be tested at a time.

Tail flick test. Rats were acclimated to the enclosures before testing for at least 1 h with rats of the same sex in adjacent enclosures to simulate testing conditions. Acclimation occurred 48 and 24 h before testing. This acclimation was done to reduce movement during the day of testing. On the day of testing, animals were acclimated for 15 min before starting the thermal tests to reduce spontaneous movements. If a rat urinated or defecated during the acclimation or testing period, the glass was wiped cleaned with water and dried with a paper towel. After the enclosure was cleaned, at least a 5-min waiting period occurred before the testing continued to reduce spontaneous movements. Rats were separated individually in an acrylic enclosure on an elevated glass surface. A visible light source from a plantar and TFT apparatus (Series 8 Model 336T; IITC Life Science) was focused between the distal second and third centimeters of the tail. The maximum temperature and time exposure were set to not exceed 55 °C and 20 s to prevent thermal injury. Active intensity, thermal output from the light source, was set to 28% for SD rats and 25% for F344 rats. The active intensity was determined with a pilot study of 3 male and 3 female rats of each genetic background to assess which output would produce a thermal latency at approximately 10 s. Thermal latency, the elapsed time to withdraw the tail at a set temperature active intensity, was recorded. At least 5 min elapsed between each thermal stimulus. Five replicates were performed on each day of testing. The highest and lowest values for each data set were excluded and the remaining 3 values were used for data analysis, as described previously for nociceptive testing in rodents.[8] The test took approximately 45 to 60 min to complete for one rat, but multiple rats can be tested at a time.

Statistical analysis. Statistical analyses were performed in GraphPad Prism 9 for Windows (GraphPad Software, San Diego, CA). Analysis of the withdrawal thresholds and thermal latencies was performed with the variables of strain/stock, sex, and test via a 3-way ANOVA to assess threshold differences for each test. Variation of withdrawal thresholds and thermal latencies were assessed by calculating the coefficient of variation for each individual rat. The coefficients of variation were analyzed via a 3-way ANOVA with variables strain/stock, sex, and test to assess intertest withdrawal threshold variability and with variables strain/stock, sex, and time to assess intratest withdrawal threshold variability. Data normality was assessed by the Shapiro-Wilk test. Statistical significance was set as $P < 0.05$.

Results

Withdrawal thresholds. Withdrawal thresholds for the RST ranged from 159.5 grams (g) to 271.2 g across all groups. Female F344 rats had significantly lower thresholds compared with F344 males at day 0 for the RST with an average withdrawal at 159.5 and 271.2 g, respectively (Figure 1A; $P = 0.0134$). However, on day 7, there was no significant difference in the RST responses for male and female F344 rats (male F344: 227.0 g; female F344: 189.6 g). No differences were observed for SD male and SD female rats with the RST on either day 0 or day 7. Thermal latencies for the HT ranged from 10.43 to 15.36 seconds (s) (Figure 1B) across groups. No significant differences were observed for SD and F344 male and female rats with the HT. Thermal latencies for the TFT ranged from 8.93 to 12.72 s across groups (Figure 1C). No significant differences were observed between SD and F344 male and female rats with the TFT.

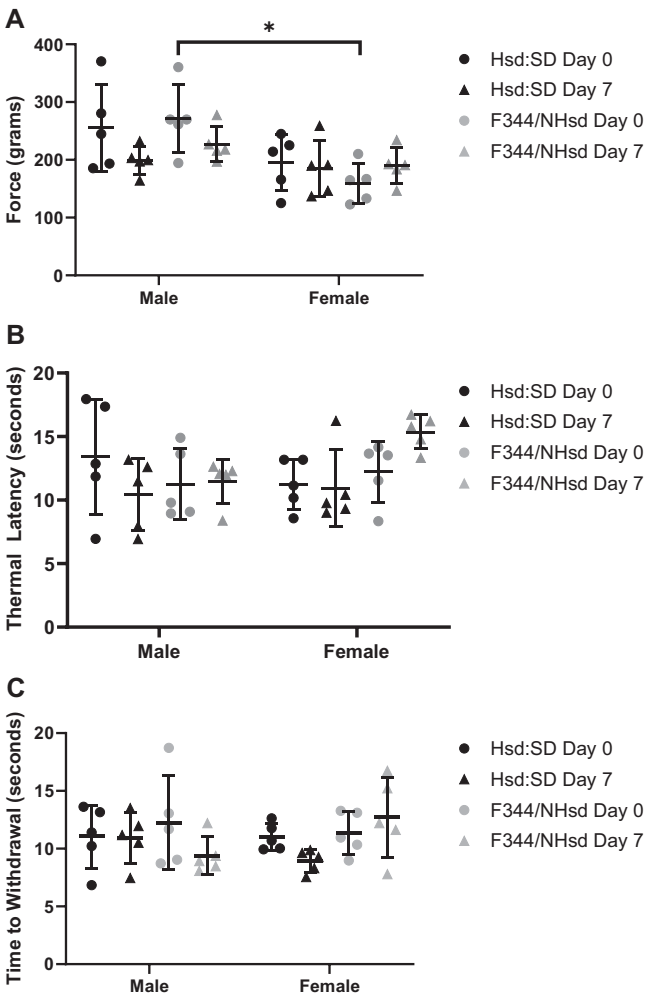

Figure 1. Comparison of withdrawal thresholds and thermal latencies for RST, HT, and TFT in male and female SD and F344 rats. (A) RST withdrawal thresholds for male and female SD and F344 rats on day 0 and day 7. (B) HT thermal latencies for male and female SD and F344 rats on day 0 and day 7. (C) TFT thermal latencies for male and female SD and F344 rats on day 0 and day 7. Data are represented as the average withdrawal threshold per animal ($n = 5$ per sex and genetic background). Error bars are the mean ± SD. *, $P < 0.05$.

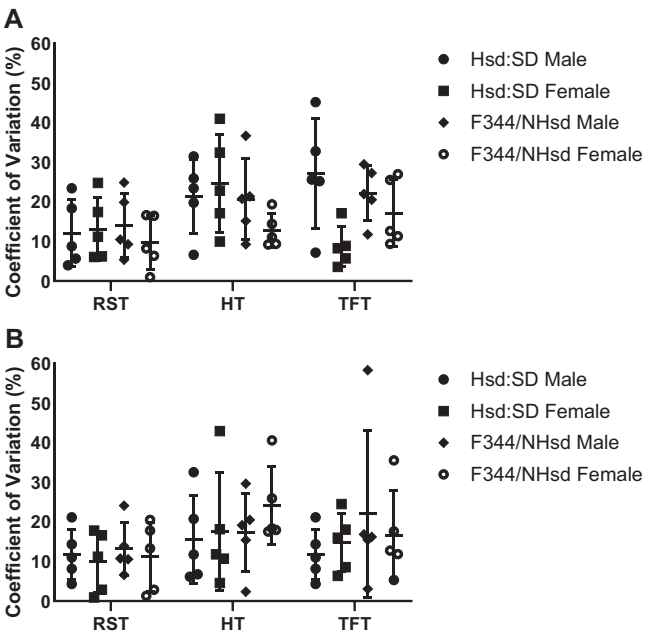

Figure 2. Intertest comparison of variation in withdrawal thresholds and thermal latencies for RST, HT, and TFT in male and female SD and F344 rats. (A) The coefficients of variation, expressed as a percentage, of male and female SD and F344 rats for the RST, HT, and TFT on day 0. (B) The coefficients of variation for male and female SD and F344 rats with the RST, HT, and TFT on day 7. Error bars are the mean ± SD; $n = 5$ per sex and genetic background.

Comparison of withdrawal threshold variation among nociceptive tests. The coefficient of variation was used to compare the variance of withdrawal thresholds or thermal latencies of each test. Across all groups, the coefficient of variation ranged from 8.72 to 27.18%. Analysis of the coefficient of variation showed there were no significant differences within each test based on sex and genetic background at day 0 (Figure 2A) and day 7 (Figure 2B) when performing the RST, HT, and TFT. In addition, there were no significant differences based on sex, genetic background, or test day when performing the RST, HT, and TFT (Figure 3A–C, respectively) for SD and F344 male and female rats.

Discussion

One of our objectives was to assess the variation in withdrawal thresholds and thermal latencies among the 3 nociceptive tests in SD and F344 rats. There is no published literature directly comparing the response variation across multiple nociceptive tests in rats. This information is valuable for the selection of the appropriate test and rat genetic background for pain studies. We have shown that SD and F344 rats do not exhibit significant variation in withdrawal thresholds or thermal latencies when using the RST, HT, and TFT. Post hoc power analysis showed that our study was underpowered. We believe that this affects the interpretation of the significance of our data; larger sample sizes would be needed

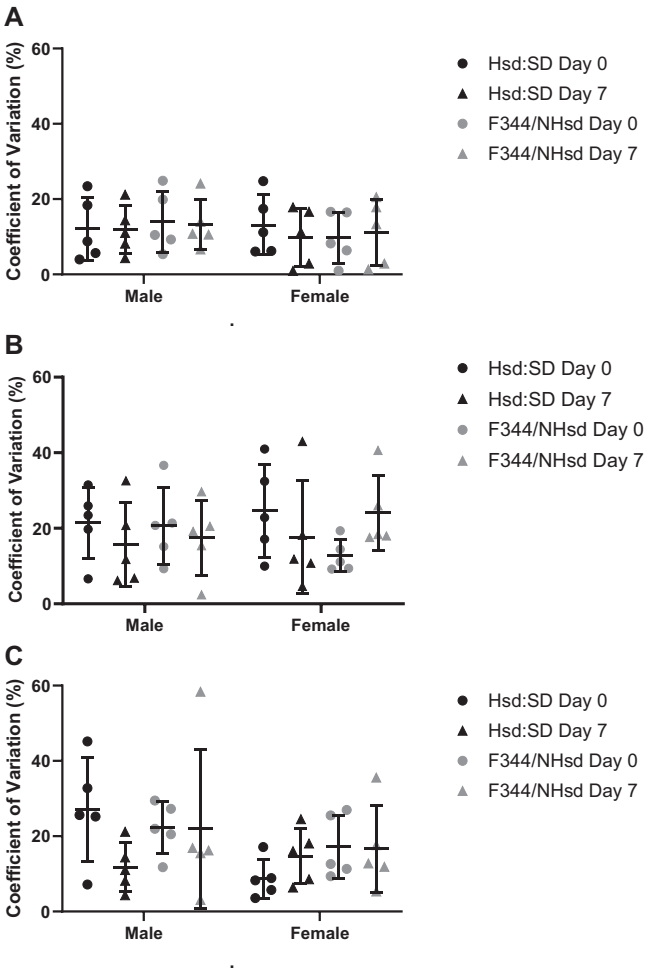

Figure 3. Intratest comparison of variation in withdrawal thresholds and thermal latencies for RST, HT, and TFT in male and female SD and F344 rats. (A) The coefficients of variation, expressed as a percentage, of male and female SD and F344 rats, using the RST on day 0 compared with day 7. (B) The coefficients of variation, expressed as a percentage, of male and female SD and F344 rats using the HT on day 0 compared with day 7. (C) The coefficient of variation, expressed as a percentage, of male and female SD and F344 rats using the TFT on day 0 compared with day 7. Error bars are the mean ± SD; $n = 5$ per sex and genetic background.

to ensure that type 1 errors were not made. However, no literature is available to describe the variation in RST, HT, and TFT that would allow researchers to perform accurate power analyses before starting their experiments. Our data can help guide future power analysis for researchers using these tests. For example, with an α of 0.05 and β 0.80, a sample size of 13 would be needed to determine the significance of the RST among 4 groups to test for a mean difference in withdrawal thresholds of 25 grams between treatment groups and the control. Under the same conditions using the HT and TFT, samples sizes of 10 and 9, respectively, would be needed to determine a significant difference of 3 seconds (s) in thermal latencies between groups. However, baseline values and those obtained after experimental manipulations, such as inflammatory pain models or the use of analgesics, could affect these values. Also, intensities can differ based on backgrounds for the HT and TFT. The active intensity for F344 rats was approximately 10% less than that of SD rats. We used these active intensities because we wanted the thermal thresholds for the HT and TFT to be around 10 s.

The data on sex differences for withdrawal threshold in rats is conflicting. The mechanical nociceptive threshold in rats after a spared nerve injury was higher in females in one study and lower in a second study.[4,10] The phase of the estrous cycle might affect the responses to painful stimuli, but evidence supporting this is unclear. The stage of estrus is a significant confounder for withdrawal thresholds in Wistar but not in SD rats.[4,32] In our study, only one significant sex difference was observed; female F344 rats had lower withdrawal thresholds than males on day 0 but not on day 7 for the RST. However, we did not assess the stage of estrus in our study. The rats may have been in different stages of estrus between days 0 and 7, which could have led to the observed differences.

The choice of nociceptive testing method and the degree of response variation as related to the genetic background are crucial for being able to design robust studies using nociceptive testing modalities. The lack of a side-by-side comparison of nociceptive testing methods in rats prompted our study. We chose the RST, HT, and TFT since they are commonly used as nociceptive tests. An important outcome of our work was the ability to evaluate the 3 tests from an operator standpoint. While performing the RST, HT, and TFT tests, we identified several logistical differences that can introduce cofounding variables when measuring nociception. These differences include animal handling and restraint, the impact of urine and feces in the testing enclosure, and the behavioral indicator, or response, denoting nociception.

First, the restraint and handling of rats differed among the nociceptive tests. The handling of rats is known to increase stress.[1] For example, holding SD rats for longer than 30 minutes (min) has been shown to increase thermal latencies using the TFT (via hot water bath) in both male and female rats.[27] Similarly, restraint devices such as cylindrical devices have been shown to impact thermal latencies in rats.[14,23] The RST requires physical restraint of each rat to perform the test whereas the HT and TFT require confinement of each rat in an acrylic enclosure. The RST differs from other nociceptive tests that deliver a mechanical noxious stimulus, such as von Frey filaments, because of how the pressure is applied. The point of the device is centered on the bottom of the foot, and pressure is applied from both the top and bottom of the foot; therefore, rats are not freely moving for the RST. Although we did not specifically evaluate stress levels in our study, the degree to which rats must be handled and restrained in each test should be considered as part of any experimental design. In our experiments, we made efforts to decrease handling stress by habituating rats to both handling and acrylic enclosures before testing. In rats, habituation to handling has been shown to decrease anxiety-like behavior.[9,26] To reduce stress during the testing procedure itself, we minimized handling, limited restraint time to less than 30 min, and did not use cylindrical restraint devices.

Second, urine and feces may complicate thermal light exposure. The HT and TFT, which rely on light-based thermal noxious stimuli, require keeping the glass stage clean to prevent light distortion caused by urine and feces. Urine and feces must be cleared to avoid light refraction or absorption, as these will alter heat transfer.[24] Cleaning the enclosures prolongs the experiment and increases the need to handle the animals. Urine and feces have no direct impact on the ability to perform the RST.

Third, behavioral indications of nociception varied among the tests. Behaviors, including an obvious withdrawal of the stimulated leg, extension of the contralateral leg, and/or audible vocalization were more consistent with the RST. In contrast, the HT and TFT primarily elicited limb withdrawal

as an indication of nociception, and less frequently, rats would look toward the stimulated foot or tail and lick the area after the thermal stimulus. This visual indication of nociception was not consistent during repeated thermal noxious stimuli to the same animal. Sensory stimulation is initiated primarily through mechanical nociceptors for the RST whereas the HT and TFT are initiated through thermal nociceptors.[12,33] Skin temperature can affect TFT withdrawal thresholds.[3] We attempted to control skin temperature by maintaining room temperature at (22 °C [72 °F]) during experimentation. Room temperature is an important consideration when using a thermal stimulus.

Experimental manipulations, such as the administration of Freund adjuvant or surgical incision, may alter nociception and pain sensation. The rats in our study were not experimentally manipulated and represent baseline variation in only 3 of many available tests. Likewise, we tested only 2 rat genetic backgrounds in the present study. Studying additional strains would provide a more comprehensive analysis of different nociceptive testing modalities in rats.

In conclusion, multiple factors must be considered when selecting tests for studies investigating pain. An investigator should choose the most suitable nociceptive test based on their specific research question and their personal experience or preference.

Conflict of Interest

The authors have no conflicts of interest to declare.

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