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Short communication

A non-reflexive method based on the variability of temperature and bioimpedance in measuring inflammatory hyperalgesia and analgesia in mice

Felipe Reitz[a], Daiana C. Salm[a,b], Daniela Dero Ludtke[a], Aureo dos Santos[a,b], Jefferson Traebert[b], Daniel F. Martins[a,b,*]

[a] Experimental Neuroscience Laboratory (LaNEx), University of Southern Santa Catarina at Palhoça, Santa Catarina, Brazil
[b] Postgraduate Program of Health Sciences. University of Southern Santa Catarina at Palhoça, Santa Catarina, Brazil

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ABSTRACT

Background: Preclinical studies measure withdrawal responses to evoking thermal and mechanical stimuli instead of the more clinically important spontaneous pain.
New method: Therefore, we studied the effect of peripheral inflammation induced by intraplantar administration of complete Freund's adjuvant (CFA) in mice on the variability of temperature and bioimpedance as an index of pain produced by inflammation. To this end, we initially determined mathematical scores based on changes in temperature and bioimpedance (STB) for animals with an inflamed paw and compared these scores with commonly used measures of inflammatory pain. We then pharmacologically validated the tool using dexamethasone.
Results: The STB analysis resembled the response found in the von Frey Hair (vFH) test. The CFA-induced increase in STB and vFH tests were reversed by intraperitoneal administration of dexamethasone. The correlation between the STB and vFH measurements showed a high correlation coefficient ($R^2 = 0.911$, $p < 0.001$).
Comparison with existing method: Our results also demonstrated that CFA paw injection induced mechanical hyperalgesia in mice and remained virtually unaltered during all time-points tested for 5 days, as measured with vFHs. The administration of CFA into the paw induced a large increase in paw volume that was apparent 1 and 5 days after the injection. The CFA injection resulted in a significant ($p < 0.05$) decrease in the response latency to the heat stimulus, as evaluated on day 4 post-CFA injection.
Conclusions: The data presented here suggest that STB may provide a novel non-invasive approach for inflammatory pain detection.

1. Introduction

There is great difficulty and ethical limitations associated with evaluating pain in humans. Therefore, animal models are employed to study the pathophysiological mechanism of pain. However, in rodents, pain cannot be directly measured, and, consequently, many methods have been developed in an attempt to quantify pain or nociceptive behavior. Such behavioral methods are based on the application of an external stimulus to verify the withdrawal response of the animal and are then defined as nociception evoked by noxious stimulus (Deuis et al., 2017).

Among the methods evoked by stimuli, we highlight von Frey Hair (vFH), Randall-Selitto and Hargreaves methods. Among these, the vFH method was the first test to be developed and continues to be used frequently (Deuis et al., 2017). However, due to concerns about clinical correlations, the need to develop and implement methods not evoked by stimulation, such as face analysis, excavations, voluntary behavior and gait analysis, has arisen (Deuis et al., 2017). All these tests mimic the clinical effects of pain but are limited by evaluating nociception in a restricted time, with difficulty in quantifying the duration and magnitude of pain (Kandasamy et al., 2016).

Despite the scientific advances made in the measurement of pain over the last few decades, the evaluation of this clinical phenomenon is supported with instruments that, although valid and reliable, possess a

* Corresponding author at: Experimental Neuroscience Laboratory, Post-Graduate Program of Health Science, Southern University of Santa Catarina, Campus Grande Florianópolis, Palhoça, Santa Catarina, Brazil.
E-mail address: daniel.martins4@unisul.br (D.F. Martins).

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considerable degree of subjectivity. Therefore, it is believed that body variables, such as temperature and bioimpedance, may become indispensable parameters for the quantification and evaluation of nociception because they present variation in frames that are altered in the presence of pain (Mikolajewska et al., 2011; Czaplik et al., 2017). Therefore, a non-invasive pain assessment protocol was developed based on the measurement of different body and environmental characteristics that, through mathematical calculations, provides us with a score for pain quantification.

To this end, we initially determined scores based on changes in temperature and drag of the voltage difference of animals with an inflamed paw and compared this with commonly used measures of inflammatory pain, including mechanical and thermal hyperalgesia and foot edema measured by the von Frey tests (a widely used outcome measure in chronic pain models) and hot plate and paw thickness tests (Martins et al., 2015), respectively. We then pharmacologically validated the tool using dexamethasone (a steroidal anti-inflammatory drug), a drug currently used in humans with analgesic and anti-inflammatory effects that reversed hyperalgesia and CFA-induced edema. Finally, the sensitivity to drug effects of the STB outcome measure was compared with changes in CFA-induced mechanical hyperalgesia determined with vFH.

2. Materials and methods

2.1. Animals

All animal care and experimental procedures were carried out in accordance with the National Institutes of Health Animal Care Guidelines (NIH publications number 80–23). All experiments were approved by the Ethics Committee of the University of Southern Santa Catarina at Palhoça, Santa Catarina, Brazil (protocol number 17.007.2.07.IV and were conducted using male Swiss mice (25–35 g, 2monthsold) housed in collective cages at 22 ± 2 °C under a 12 h light/12 h dark cycle (lights on at 6:00 a.m.) and with free access to food and water.

2.2. CFA-induced inflammation and dexamethasone treatment

Mice were injected intraplantar (i.pl.) with 80% Complete Freund's Adjuvant (CFA; Sigma-Aldrich, MO, USA) or saline (group Vehicle + vehicle), in a 20 µl injection volume, into the plantar surface of one hind paw, as previously described by Meotti et al. (2006) (Fig. 1A). Dexamethasone (Aché Laboratórios Farmacêuticos S/A, Brazil) treatments were initiated 24 h after CFA paw injection and continued for five consecutive days. The animals were treated with dexamethasone (1 mg/kg, i.p.) or sterile saline (0.9% NaCl solution). Control animals received i.p. injections of sterile physiological saline (Sigma-Aldrich, MO, USA).

2.3. Nociceptive tests

2.3.1. Evaluation of mechanical hyperalgesia by the von Frey test

To evaluate mechanical hyperalgesia, the animals were tested for response frequency to mechanical stimuli (von Frey filaments [VFFs]) applied to the plantar aspect of the right hindpaw (Martins et al., 2015). Mice were habituated in individual clear boxes (9 × 7 × 11 cm) on an elevated wire mesh platform to allow access to the ventral surface of the hind paws. The right hind paw was stimulated with a constant pressure of 0.6 g vFH, for 3 s (Stoelting, Chicago, IL, USA). The response frequency to 10 applications considered the nociceptive behavior. The results are expressed as the percentage of withdrawal frequency. Tests wereperformed 24 h after injection of CFA and 0.5 h after treatment with dexamethasone on the 1st, 3rd and 5th days post-CFA injection (Rittner et al., 2001). (Fig. 1A).

2.3.2. Evaluation of thermal hyperalgesia by the hot plate test

The thermal nociceptive threshold was assessed by the hot plate test, measuring the latency to right paw withdrawal from a noxious heat source. Mice were placed in acrylic cages (40 × 20 cm), on a metallic surface, previously heated at 50 ± 2 °C. The temperature of the hot plate used to test the role of the TRPV2 receptor was 50 ± 2 °C. In contrast to TRPV1, TRPV2 is insensitive to capsaicin and responds to higher temperature stimuli, with an activation threshold of 52 °C. Furthermore, it has been demonstrated that TRPV2 is up-regulated in DRG neurons after nerve injury and inflammation (Shimosato et al., 2005) which may indicate a potential role for TRPV2 in pain sensation. Compared to TRPV1, TRPV2 has a wider distribution pattern, including lung, spleen, bladder and immune cells, suggesting a broader range of physiological functions than TRPV1 (Frederick et al., 2007). Positive withdrawal responses included immediate licking or lifting of the right paw. A maximum duration of 30 s was chosen to prevent tissue damage (Martins et al., 2016). The test was performed on the 4th day post-CFA injection (Fig. 1A).

2.4. Hind paw edema measurement

Paw edema was measured as the difference between paw thickness (in micrometers) from the baseline evaluation using a digital micrometer. The difference between the groups indicated the degree of inflammation (Martins et al., 2015). Tests were performed 1 and 5 days post-CFA injection (Fig. 1A).

2.5. Determination of mathematical pain score based on temperature variation and bioimpedance (STB)

2.5.1. Paw surface temperature measurement and mathematical calculations

For temperature evaluation, a portable infrared camera for the acquisition of infrared images (FLIR model T420, Sweden) was used (Brioschi et al., 2003). The procedure consisted of regulating the parameters of the camera with thermal sensitivity < 0.045 to 30 °C and an infrared spectrum range of 7.5 µm–13 µm, and the scale was defined in a cold / hot palette and a temperature range of 20 °C–40 °C. The animals were recorded, and then the temperatures identified in the ventral region (central portion) of the hind and front paws of the animals were captured and recorded. The observed temperatures of the four paws were noted, and the temperature of the right hind paw was considered with the lowest temperature among the other three paws. The evaluations were carried out before injection and on the 1st, 3rd and 5th day after i.pl. CFA injection (Fig. 1A).

The determination of the temperature of the area of interest t (adi) was calculated as, 493/5000 where$\{t(adi) = t_s - t_i\}$ where t_s is the upper temperature and t_i is the lower temperature among those investigated (McAlindon et al., 2007). The value of the ADI radiation was obtained by measuring the temperature of the paws of the mice. Through the use of a protected intellectual property algorithm using multidimensional analyses, this value was added to the other values acquired within a standardization, where the computer software determined the most appropriate result for each mouse.

2.5.2. Conduction dragging measurement and mathematical calculations

When we refer here to conduction, this means a drag of the voltage difference between two points. Thus, the procedure occurred with the manual containment of the animals (without anesthesia), and then the electrodes were positioned in an intercalated fashion among the four paws, generating an exchange of six results, in which the result between the right hind paw and the left hind paw was used to foster the analysis algorithms. To determine the score, the conduction (drag) was initially measured by the electrical tension in volts (V) between the paws of the mice (as described above). Thus, a test load "q", was placed and the potential energy acquired by it was measured. This quotient was

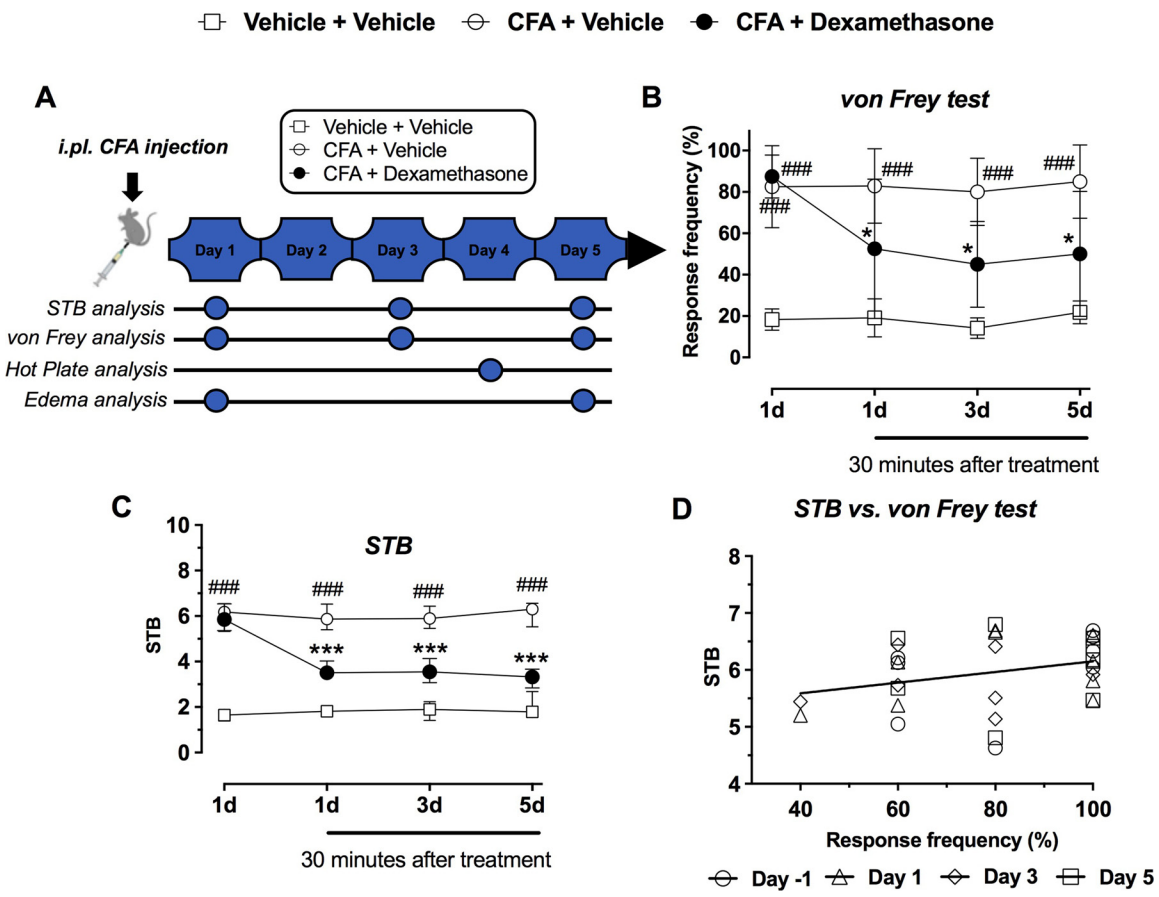

Fig. 1. Comparison and correlation between von Frey and STB measurements in inflamed mice. von Frey test (panel A, two-way ANOVA followed by the Bonferroni test - mean ± standard deviation (SD), STB analysis (panel B, nonparametric Kruskal-Wallis test - median and interquartile ranges) and correlation (panel C and D, Pearson's linear correlation and simple linear regression) are shown. The regression graph was constructed using the values of each animal response each day (Day -1, day 1, day 3 and day 5) of CFA + vehicle group. Each point represents the average of 8 animals, and the vertical bar indicates the mean ± standard deviation (SD). #$p < 0.001$ when compared to the control group (Vehicle + vehicle); *$p < 0.05$ and *** $p < 0.001$ compared to the control group (CFA + vehicle). CFA: Complete Freund's Adjuvant, d: day, day -1 = baseline.

calculated as: $V = \frac{Ep}{q}$,where V is the voltage (unit V for Volt), Ep is the electric energy (unitJ for joule) and "q" is the load (unit C for Coulomb) (Bolfe et al., 2007). To register the drag between the paws of the mice, a digital multimeter was used (Brand Emporio K and model DT830D, China), always with stabilization of the palmar and / or plantar hold of the animals. The device was calibrated to capture voltage in a scale up to two thousand millivolts. The generated result was parameterized in five groups of 0–100 nonlinear delays for data feeding in the protected intellectual property algorithm. The evaluations were performed before injection and on the 1st, 3rd and 5th day after the i.pl. injection of CFA (Fig. 1A).

2.6. Statistical analysis

Results are presented as the mean ± standard deviation (SD) of the mean or median and interquartile ranges for each group. Behavioral testing was analyzed using both one-way analysis of variance (ANOVA) following Student–Newman–Keuls test and two-way repeated measures ANOVA. The nonparametric Kruskal-Wallis test was also performed. Differences with a value of $p < 0.05$ were considered significant. Associations between variables were performed using Pearson's linear correlation and simple linear regression.

3. Results

3.1. Effects of dexamethasone on CFA-induced hyperalgesia and edema

The results show that CFA paw injection induced mechanical hyperalgesia in mice and remained virtually unaltered during all timepoints tested up to 5 days measured, as measured with vFH (Fig. 1B). Interestingly, the STB analysis resembled the response found in the test with vFH (Fig. 1C). To characterize the response of this outcome behavior specifically to anti-inflammatory compounds, we tested the effect of dexamethasone (1 mg/kg, i.p.), which elicited a reversal of the decrease in the response frequency of paw withdrawal in the vFH test and in STB of inflamed mice, for 30 min ($p < 0.05$) throughout days 1, 3 and 5 (Fig. 1B–C). Furthermore, the correlation between STB and vFH measurements, using Pearson's index, was significant ($p < 0.001$), showing a high correlation (Fig. 1D). Pearson's correlation analysis showed that the correlation coefficient was 0.95, which can be considered as very strong (Fig. 1D). In turn, the coefficient of determination ($R^2 = 0.911$, $p < 0.001$) indicated a strong statistical significance of the coefficient of correlation.

Administration of CFA into the paw induced a large increase in paw volume that was apparent at 1 (Fig. 2A) and 5 (Fig. 2B) days after the injection. In addition, the data demonstrates that mice treated with dexamethasone presented lower paw thickness ($p < 0.05$) when compared to the control animals (CFA + vehicle). This effect was also observed on the 1st and 5th days after CFA injection, as shown in Fig. 2A and B. CFA injection resulted in a significant ($p < 0.05$) decrease in

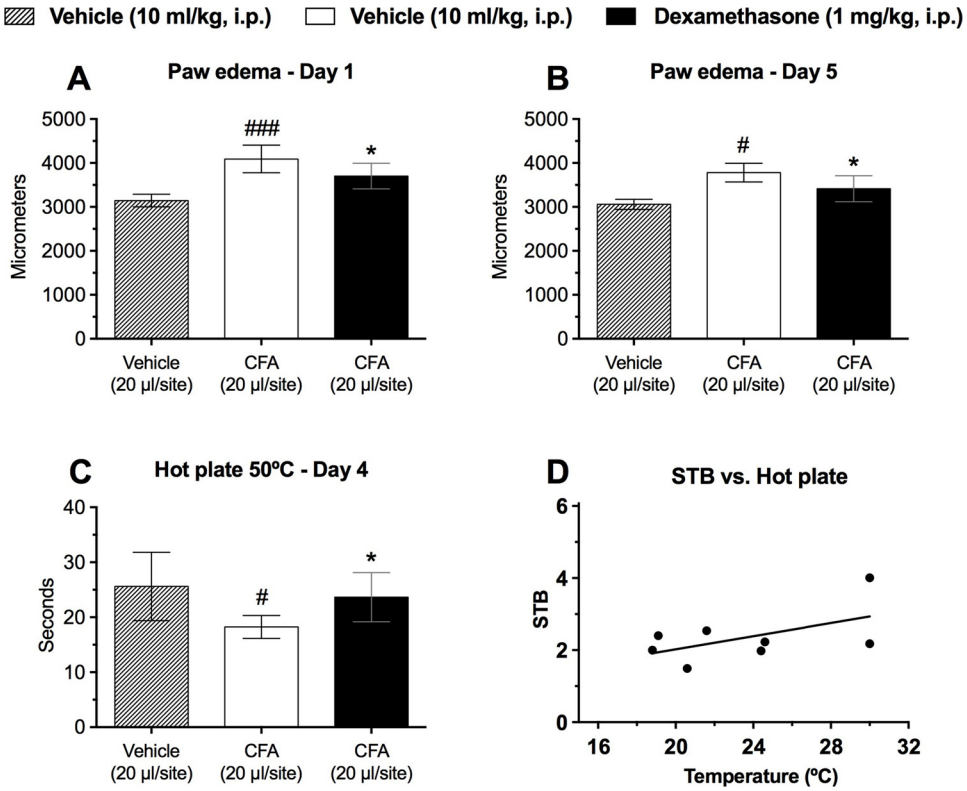

Fig. 2. Thermal hyperalgesia and edema analysis.Hot plate test (A) and measurement of paw edema (B–C). Correlation (STB vs. Hot Plate, panel D, Pearson's linear correlation and simple linear regression) is shown. The regression graph was constructed using the values of each animal on the fourth daypost-CFA injection. Each bar represents the average of 8 animals, and the vertical bar indicates the mean ± standard deviation (SD), $\#p < 0.05$ and $\#\#\#p < 0.001$ compared to the vehicle + vehicle group. $*p < 0.05$ when compared to the control group (CFA + vehicle). One-way ANOVA followed by the Newman-Keuls test. CFA: Complete Freund's Adjuvant.

the response latency to heat stimulus as evaluated on day 4 post-CFA injection when compared to the control (vehicle + vehicle group animal; Fig. 2C). Further, we demonstrated that the dexamethasone treatment increased ($p < 0.05$) the paw withdrawal latency time of the animals when compared to the control animals (CFA + vehicle; Fig. 2C). Furthermore, the correlation between STB and hot plate measurements, using Pearson's index, showed a weak correlation (Fig. 2D).

4. Discussion

Here, we present a novel non-reflexive method based on variability of temperature and bioimpedance to measure inflammatory hyperalgesia and analgesia in mice and present a validation of this score using behavioral testing and drug reversal of an inflammatory pain model applied in the hindpaw region of mice.

In our study evaluating this score, hyperalgesia was the reference endpoint for every animal since hyperalgesia was known to develop consistently for the chronic inflammatory model tested. Accordingly, the score (STB) alteration concurred with hyperalgesia onset and development. The i.p. administration of dexamethasone (1 mg/kg) caused a significant reversal of the CFA-induced changes in the STB and vFH analyses. This dose is broadly consistent with those reported to be active in other inflammatory pain models in mice. Dexamethasone is a potent steroidal anti-inflammatory agent used clinically to treat inflammatory pain conditions (Li et al., 2018), and, for this reason, it is often used as a gold-standard compound in tests of inflammatory pain. The effect of dexamethasone and its reproducibility across both of the end-points further validates the use of STB as a valid end-point for models of inflammatory pain (Sasso et al., 2012). Furthermore, the high correlation between the STB and vFH ($r = 0.911$, $P < 0.001$) analyses validates the use of the STB to assess pain-related behavior after inflammation in mice.

Methods for measuring pain more objectively have been the subject of research for many years. Riley and Richter (1975) have shown that measuring cutaneous electrical resistance can be a way of defining various types of pain. The authors verified that the areas of pain corresponded to areas of low resistance in which there was sympathetic hyperactivity. The authors also stated that the sympathetic neurovegetative nervous system presents an intimate relationship with pain, making possible the relationship among cutaneous changes related to vascular phenomena due to neurovegetative reflexes in the areas of the body with pain.

The relationship between pain and changes in skin temperature is due to neurovegetative control; a cutaneous area of the body modifies its response to pain and produces certain thermal energy because of vascular flow, resulting in greater or lesser localized infrared radiation and causing heating or cooling of the affected region. Sympathetic fibers control cutaneous microcirculation through vasoconstriction or vasodilation. A territory of sensory innervation of the skin corresponds to a microvascular territory since the sympathetic fibers follow along with the sensory fibers in the same nerve (Czaplik et al., 2017).

During an inflammatory process, such as that induced by the i.pl. of CFA, the activation of the polymodal nociceptors generates axonal reflexes in the free nerve endings of the primary afferent neurons so that the C fibers release algogenic neuropeptides, which initiate an inflammatory reaction called neurogenic inflammation. Substance P, neurokinin A and CGRP in smooth muscle vessels activate mast cells that release histamine and then potentiate vasodilation due to release of cytokines (IL-6) and nitric oxide (NO) by macrophages. Thus, all these vascular changes induced by inflammation consequently influence local temperature and pain (Cook et al., 2018). However, it is important to emphasize that pathologies that may induce an increase in local circulation without increasing nociception may influence STB, and this effect should be differentiated.

In the method of measuring pain in which we are studying, the mathematical correlation between temperature and electrical conduction of the skin means that hyperthermia is directly proportional to conductivity (Bolfe et al., 2007; Czaplik et al., 2017; Mikolajewska et al., 2011). As in the algorithm, we consider the resistance that is inversely proportional to the conductivity; thus, the hyperthermia is proportional to the inverse of the resistance, where 'R' is the resistance,

'V' is the potential or voltage differential (drag of the voltage difference between the right hind paw and the lower temperature leg between the others) and 'I' is the electric current intensity. The temperature of the right mouse paw, α, is a constant that accompanies the graph, and R is the electrical resistance of the skin. In the graph of the algorithm, we correlate these two coefficients as $C = \alpha T + \beta$, where C is the conductivity, αT is the temperature constant and β is the positioning interval on the y-axis of the graph from zero. The generated values of the temperature differential of the hind paw of the mouse with the lower temperature paw next to the drag value are important information that feed the algorithm to generate a pain score (Bolfe et al., 2007).

We observed that in the inflammatory processes induced by CFA, the temperature of the inflamed area was always greater than in the other paws. It was also observed that there was a proportional increase in potential energy between the right hind paw and the other paws. This result shows a closer correlation between hyperalgesia and electrical tension associated with the increase in temperature resulting from an inflammation.

We conclude that monitoring changes in temperature and drag of the voltage difference in mice during inflammatory pain is a simple and objective measure of physiological changes produced by inflammation. Thus, the data presented here suggest that STB may provide a novel end point for the development of new analgesics. Further work is currently ongoing to assess the use of this equipment in other more chronic models, including models of neuropathic pain.

Conflict of interest

The authors declare that they have no conflicts of interest.

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