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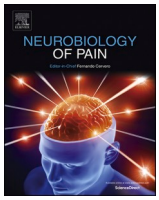

Massage-like stroking produces analgesia in mice

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ABSTRACT

Chronic pain treatment remains a major challenge and pharmacological interventions are associated with important side effects. Manual medicine treatments such as massage, acupuncture, manipulation of the fascial system (MFS), and osteopathic manipulative treatments produce pain relief in humans, but the underlying mechanism is poorly understood limiting leverage and optimization of manual medicine techniques as safe pain therapy. To decipher the physiological mechanisms of manipulative medicine treatments, we have established a preclinical model. Here, we established a murine model of massage-like stroking (MLS)-induced analgesia. We characterized that the analgesia effects were present in both sexes, and were independent of the experimenters, handling, consciousness, and opioid receptors. MLS alleviates thermal pain in naive mice and postoperative pain hypersensitivity. This novel model will allow discovery of the physiological mechanisms involved in MLS-induced analgesia and identification of new therapeutic strategies.

Introduction

Manual medicine induces pain relief in humans as evidenced by long-used treatments such as massage, acupuncture, manipulation of the fascial system (MFS), and several osteopathic manipulative treatments such as myofascial release and soft tissue technique (França et al., 2020; Tick et al., 2018; Smith et al., 2018.; Jiménez-Del-Barrio et al., 2022; Gianola et al., 2022; Cumplido-Trasmonte et al., 2021). These manual medicine treatments have been used for their analgesic effects throughout history without a clear understanding of the underlying molecular and cellular mechanisms at play (Moraska et al., 2010; Field, 2014). This lack of understanding prevents leverage of the effects potentiated by manual medicine on the endogenous analgesic system for chronic pain treatment. To decipher these mechanisms, it is necessary to establish a rodent model for assessing the analgesic effects of massage-like stroking (MLS). We adapted a published method of MLS which was able to increase thymic and splenic T cell numbers in mice that underwent the MLS procedure (Major et al., 2015). Given the critical role of immune cells and specifically T cells on pain sensitivity (Laumet et al., 2019; Laumet et al., 2020 Jan; Rosen et al., 2019), we evaluated the analgesic effects of the MLS procedure on naive mice with the hot plate test and on postoperative pain with von Frey filaments. Establishing an MLS mouse model presents potential challenges and confounding factors, therefore in this study we addressed the potential issues of sex difference, the experimenter, and stress. Finally, we tested the implication of opioid receptors.

Methods

Animal

All animal experiments were approved by Michigan State University IACUC (AUF#201900249 and #202200290) and in accordance with National Institute of Health (NIH) guidelines. Adult male and female wild-type C57Bl6 mice (JAX#000664) were bred in Michigan State University (MSU) vivarium, same-sex group housed (2–5 mice), and had *ad libitum* access to food and water.

Mice were separated into a massage group, hold-only group, or control group. The massage group underwent the MLS protocol; mice in the hold-only group were held in the handler's hand for the same duration as the massage treatment but did not receive any MLS; the control group was left untouched for the full 60 min in a new cage.

MLS protocol

Mice underwent MLS for 60 min daily over the course of one week

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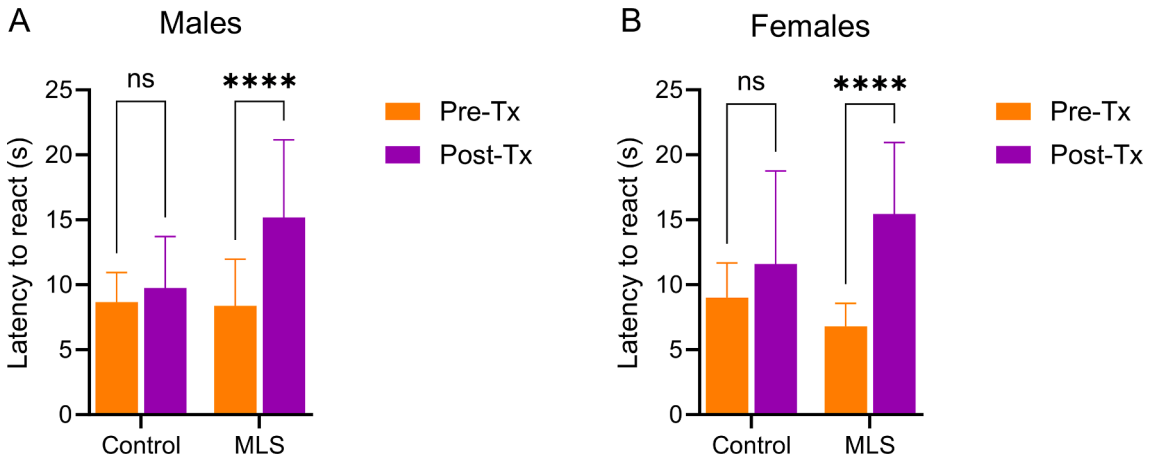

Fig. 1. MLS increased latency to react to noxious heat stimuli in both males and females. Latency to react to the 52 °C hot plate was measured 24 h before the first treatment day (Pre-Tx) and 24 h after the last treatment day (Post-Tx) in male (**A**) and female mice (**B**). Control, Males n = 11; Control, Females n = 15; MLS, Males n = 13 MLS; Females n = 17. Statistical analysis was performed via two-way ANOVA. Interaction pre/post x treatment: Males: F (1,22) = 9.32, p = 0.006. Females: F (3, 44) = 11.71, p = 0.004. Error bars represent mean ± SD. Multiple comparison represented on the figure. Ns: non-significant. MLS: Massage-like stroking.

according to a previous protocol (Major et al., 2015). Mice were brought into the testing room 30 min before treatment to allow for habituation to the testing room environment. Each mouse regardless of experimental group was separated into an individual new cage without food, water, or litter for the duration of the treatment. Mice in the massage group received 20 S per five-minute interval over the course of the 60-minute treatment at a rate of three centimeters/second in a cephalo-caudal direction along the posterior dorsal thorax to posterior hind limb using two or three fingers of the experimenter's dominant hand while holding the mouse in the palm of the non-dominant hand. Mice in the hold-only group were held for 20 s in the palm of the experimenter's hand without massage per five-minute interval over the 60-minute treatment. All experiments were carried out wearing Halyard nitrile powder-free exam gloves. The 60-minute treatment was repeated daily during the light cycle for seven days. Videos in supplemental material show the massage-like stroking procedure.

The following groups were used: Control: animals placed into a new cage; hold-only: animals placed in the palm of the experimenter, MLS: animals placed in the palm of the experimenter and receive stroking.

Treatment

Mice underwent anesthetization with 2.5 % isoflurane combined with 2.5 lpm of 100 % oxygen. Mice were kept in an induction chamber between 5-minute intervals and kept warm using a heating pad at 100F. Mouse heads were placed into a mask for maintenance oxygen and isoflurane. The MLS procedure was then performed for 20 S before returning the mice to the induction chamber. Mice in the control group were similarly transferred to the heating pad and mask without massage before returning to the induction chamber. At the conclusion of the 60-minute treatment, all mice were monitored and allowed to regain consciousness on a heating pad while breathing atmospheric air. When the mice were fully ambulatory, they were returned to their home cages.

Naloxone was used to evaluate the role of endogenous opioids in the response to treatment. Naloxone hydrochloride (#05–991-00, ThermoFisher Scientific, Ann Arbor, MI) was diluted into sterile saline. Naloxone was administered at 3 mg/kg via intraperitoneal injection one hour prior to hot plate test. A saline control group was administered 0.1 mL/g saline solution one hour prior to the hot plate test.

Postsurgical pain model

pain hypersensitivity was induced by 5-mm longitudinal incision with a number 11 scalpel blade in the skin of the left hindpaw and the underlying muscle tissue 2 mm below the heel incision under 2.5 % isoflurane anesthesia as previously described (Inyang et al., 2021 Apr; Brennan, 2004). The wound was closed using a 5-mm silk suture, followed by a 200-μl subcutaneous injection of gentamicin (5 mg/ml; Sigma-Aldrich, St. Louis, MO).

Behavioral testing and statistical analysis

Nociceptive response in naïve mice was measured after the seven-day treatment cycle via hot plate testing (Except for Fig. 1, animals were tested on the hot plate before and after the treatment). Latency to react to heat stimuli was measured as the time to either lick hind paw or to jump at 52 °C. The hot plate method is a well-established preclinical model used to measure analgesia (Barik et al., 2018). If there was no response within 60 s, mice were removed from the hot plate to prevent tissue injury. Hot plate remains the gold standard method to assess analgesia in naive mice. Mechanical sensitivity was assessed by calibrated von Frey filaments from Stoeling as previously described (Inyang et al., 2023 Sep 6). The elevated plus maze (EPM) test was used to assess anxiety-like behavior in mice following MLS procedure. EPM was carried out in accordance with a published protocol (Kraeuter et al., 2019), 24 h after the last treatment of the seven-day cycle. Behavioral responses were compared using one-way ANOVA or two-way ANOVA followed by Fisher's LSD or Tukey's multiple correction depending on experimental design in GraphPad software. Statistical comparisons are indicated on figures with brackets and starts as followed: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, and **** = $p < 0.0001$. Full statistical data are presented in supplementary tables 1 and 2.

Results

MLS significantly increased latency to react to noxious heat stimuli in both males and females compared to control (Fig. 1, **time x treatment interaction males $p < 0.006$, females $p < 0.005$**). This indicates that there is an analgesic effect induced by the MLS procedure regardless of sex. For this reason, male and female data are pooled in the rest of the study.

To determine whether confounding factors such as habituation to handling impact the latency to respond to the noxious heat, we tested the effects of stroking independently of holding and the effect of habituation to the experimenter performing the stroking. The procedure involves holding the mouse while conducting MLS, so a hold-only group

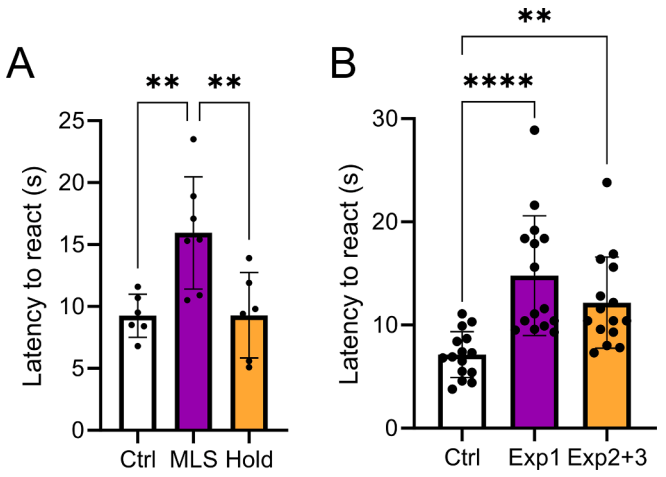

Fig. 2. Stroking is necessary to produce the analgesic effect. (A) Latency to the 52 °C hot plate was measured after treatment (hold only vs. MLS). Control = n = 6, Hold-only n = 6; MLS n = 7. Statistical analysis was performed via one-way ANOVA. F (2,16) = 7.98, p = 0.004. Error bars represent mean ± SD. (B) MLS treatment was performed by Experimenter #1 (Exp1), twenty-four hours after Exp2 and Exp3 assessed the latency to respond to 52 °C hot plate. Twenty-four hours after Exp1 repeated the hot plate testing (n = 15 mice/group). Statistical analysis was performed via one-way ANOVA. F(2,42) = 11.7, p < 0.0001. Ctrl: control. MLS: Massage-like stroking. Hold: hold only (no stroking). Error bars represent mean ± SD.

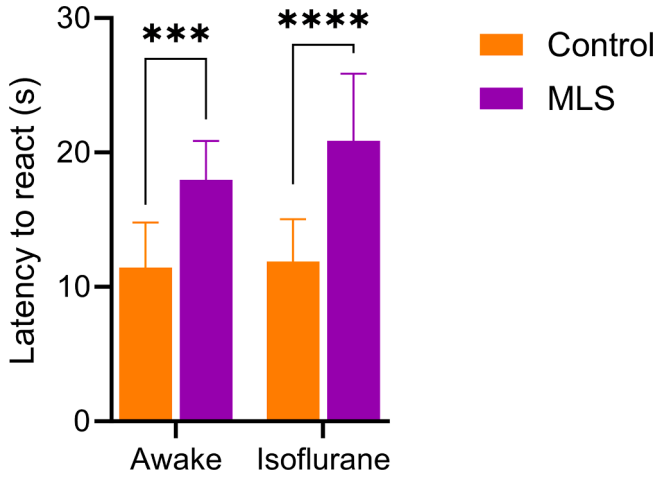

Fig. 3. MLS performed under anesthesia increased latency to react to noxious heat stimuli similar to MLS performed in awake mice. Latency to react to the 52 °C hot plate was measured 24 h after the last treatment day. Stroking treatment and control manipulation were performed in awake mice and under anesthesia. Awake n = 3F + 4 M and Isoflurane n = 3F + 3 M. Control-awake: mice placed into a new cage; control-isoflurane: mice placed into a new cage with isoflurane to induce anesthesia; MLs-awake: mice placed into the palm of the experimenter for the stroking procedure; MLS-isoflurane: stroking performed under isoflurane anesthesia. Statistical analysis was performed via two-way ANOVA. Interaction isoflurane x MLS: non-significative. MLS effect F(1,11) = 60.7, p < 0.0001. Multiple comparison represented on the figure. Error bars represent mean ± SD.

was added in which these mice were held but not stroked for the same duration as the MLS group to isolate the effects of stroking (Fig. 2**A, MLS vs. hold-only p = 0.009**). While the MLS group showed an increased latency to react, the hold-only group did not have an increased latency to react, confirming that the analgesic effect is mediated by the stroking in MLS. To exclude potential influence on latency to react from habituation to the handler over the seven-day treatment, the post-treatment

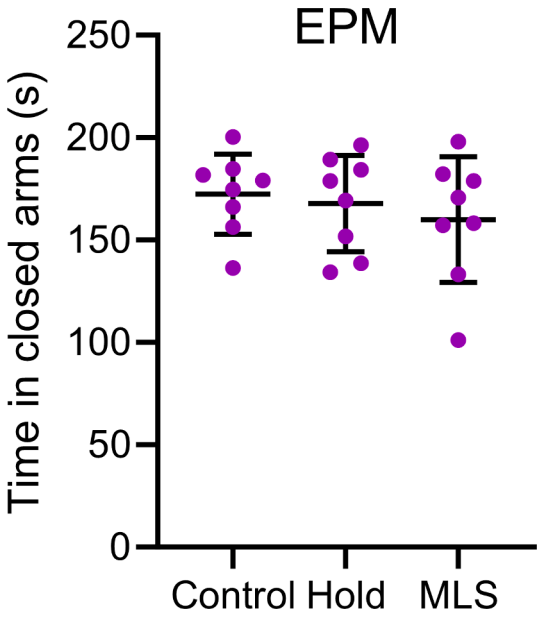

Fig. 4. MLS does not induce stress assessed by EPM test. Twenty-four hours after MLS treatment, anxiety-like behavior was measured by time spent in closed arms on the elevated plus maze (EPM). Control, Males n = 4; Females n = 4; Hold, Males n = 4; Females n = 4; MLS, Males n = 4; Females n = 4. Statistical analysis was performed via One-way ANOVA p = 0.75. Error bars represent mean ± SEM.

hot plate test was conducted by 2 other lab personnels not involved in performing MLS treatment (Fig. 2**B, Ctrl vs. Exp2 + 3p = 0.009**). Mice in this group still showed an increased latency to react to heat stimuli regardless of whether the experimenter performing the hot plate was the same as the one performing the MLS.

To determine the necessity of consciousness during MLS, MLS was performed on awake mice and on mice anesthetized by isoflurane and were compared to a control group. The group of awake mice receiving MLS showed expected enhanced latency to react. Isoflurane anesthesia did not alter the analgesic effect of MLS. The isoflurane-treated group showed similar increased latency to react to noxious heat as awake mice treated with MLS (Fig. 3**, isoflurane x MLS interaction p = 0.24**). As expected, the isoflurane anesthesia without MLS did not show an increased latency to react. These data indicate that the analgesia effects are induced physiologically and not psychologically.

To ensure that the MLS did not provoke anxiety-like behavior in mice, which could potentially introduce a confounding factor, as stress-induced analgesia might increase the response time to noxious heat stimuli, we assessed the potential anxiety-like behavior of mice subjected to MLS using the elevated plus maze paradigm (EPM) (Fig. 4**, overall effect p = 0.74**). There was no significant difference in time spent in closed arms among groups. This indicates that MLS does not induce anxiety-like behavior.

To evaluate the role of endogenous opioids in the analgesia produced by MLS, mice were administered naloxone, or saline as control, prior to hot plate testing. Naloxone is an antagonist of mu opioid receptors and thus administration prior to the hot plate test negates any effects of endogenous opioids. Naloxone does not prevent the analgesic effect of MLS (Fig. 5**, ctrl vs. NLX + MLS p = 0.0014**), suggesting a mu-opioid-independent mechanism for the analgesic effect produced by MLS.

Given the analgesic effects of MLS on acute thermal nociception, we next investigated its efficacy in alleviating surgical incision-induced pain hypersensitivity. Mice were baselined for mechanical sensitivity and received MLS for 7 days as done above. On the second day of MLS treatment, plantar incision was performed, and pain hypersensitivity was confirmed 24 h later. At this end of the MLS treatment, MLS-treated

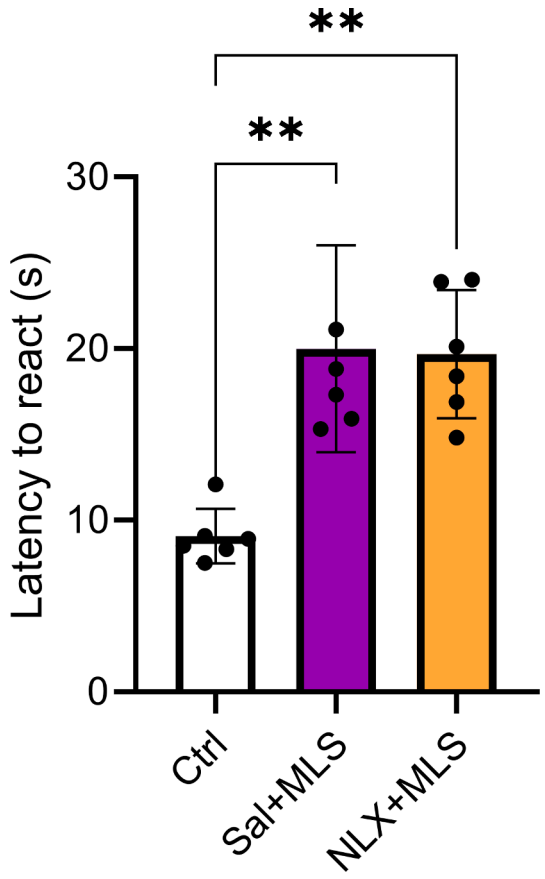

Fig. 5. Opioid receptor antagonism did not block MLS-induced increased latency to react to noxious heat stimulus in mice. Latency to react to the 52 °C hot plate was measured 24 h after MLS treatment. Mice received either intraperitoneal injection of saline or naloxone (3 mg/kg) one hour prior to the hot plate test. Control, Males n = 3; Saline, Males n = 3; Naloxone, Males n = 3. Control, Females n = 3; Saline, Females n = 3; Naloxone, Females n = 3. Statistical analysis was performed via one-way ANOVA, F(2,15) = 13.2, p = 0.0005. Multiple comparison represented on the figure. Error bars represent mean ± SD.

mice showed significantly less pain sensitivity compared to control mice and a faster recovery (Fig. 6**A**, **MLS effect p < 0.0001**). These data indicate that MLS also reduced mechanical hypersensitivity following injury. MLS has no effect on non-nociceptive mechanical stimulation (Fig. 6**B**). MLS was equally effective in alleviating incision-induced pain hypersensitivity in both male and female mice (Fig. 6**C**).

Discussion

This study established a novel preclinical model of MLS-induced analgesia in mice and opens new avenues for investigating the biological mechanisms underlying analgesia induced by massage and other manual medicine treatments. MLS can be thought of as a simplified version of a variety of manual therapy methods including MFS, massage, and osteopathic manipulative treatments such as myofascial release and soft tissue technique. MLS induced analgesia in both sexes in a manner independent of stress, habituation to the massage provider, and consciousness. More exciting, MLS also alleviated postoperative pain in both sexes but does not alter non-noxious sensitivity. These data suggest that analgesia induced by MLS is independent of cognitive processes and might be independent of the cortex. Activation of Aβ mechanoreceptors is known to inhibit C-fiber inputs into spinal projection neurons (Jie and Pei-xi, 1992). High activation of mechanoreceptors by the stroking may desensitize C-fiber-projection neuron synaptic transmission and therefore induce analgesia. MLS activates an endogenous analgesic system that does not rely on mu-opioid receptors. One may think that MLS triggers analgesia through the facilitation of serotonin, dopamine or oxytocin production (Field et al., 2005; Riem et al., 2017; Morhenn et al., 2012). MLS may also enhance the activity of the vagal nerve (Field, 2016). Alternatively, immune cells such as T cells may influence pain sensitivity (Laumet et al., 2019; Rosen et al., 2019). This is interesting because this MLS procedure has been shown to increase the number of T cells (Major et al., 2015). Pharmacological screening will test the impact of these neurochemical pathways in the next studies.

Further investigations are required to understand the mechanism underlying the analgesic effects of MLS and identify the neurochemical mediators. The validation of the model of MLS-induced analgesia will allow the scientific community to further explore these hypotheses. Our model of massage-induced analgesia is established in C57Bl6 mice, investigators will benefit from the endless genetic toolbox of this strain to explore the biological mechanisms underlying massage-induced analgesia.

Future studies will also aim at testing whether MLS impacts different types of pain such as cold pain, and spontaneous pain as well as to test models of chronic neuropathic pain. In conclusion, to our knowledge, we establish a new model of MLS-induced analgesia, and we hope that this model will foster the development of new safer therapeutic strategies urgently needed to tackle the pain/opioid crisis.

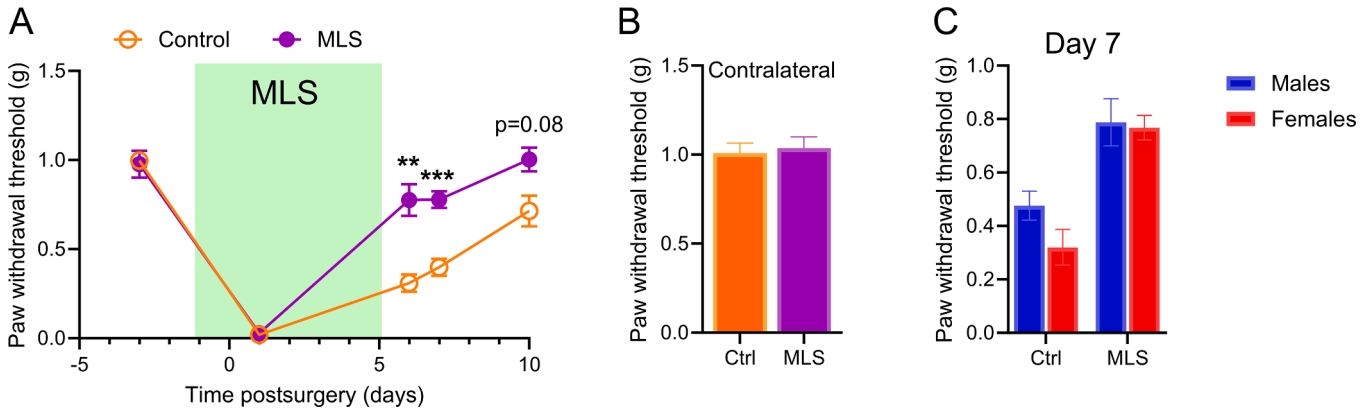

Fig. 6. MLS alleviates plantar incision-induced pain hypersensitivity. (**A**) Mechanical sensitivity was recorded by von Frey filaments. MLS started 24 h before incision and continued for 7 days. 24 h after the last MLS session mechanical sensitivity was assessed. Statistical analysis was performed via two-way ANOVA time x MLS interaction, F(4,72) = 6.89, p < 0.0001. (**B**) MLS does not affect mechanical sensitivity in the contralateral paw. (**C**) MLS had similar analgesic effects on male and female mice on day 7. Control females n = 5; Ctrl males n = 5; MLS females n = 5; MLS males n = 5. Error bars represent mean ± SEM.

Financial Disclosures

No financial conflict to disclose.

CRediT authorship contribution statement

Zachary M.S. Waarala: Conceptualization, Data curation, Formal analysis, Writing – original draft. **Logan Comins:** Data curation. **Sophie Laumet:** Data curation. **Joseph K. Folger:** Project administration, Writing – review & editing. **Geoffroy Laumet:** Conceptualization, Formal analysis, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgement

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ynpai.2023.100149.

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