Title

Sexually Dimorphic Role of Toll-like Receptor 4 (TLR4) in High Molecular Weight Hyaluronan (HMWH)-induced Anti-hyperalgesia

Journal

Journal of Pain, 22(10)

ISSN

1082-3174

Authors

Bonet, Ivan JM
Araldi, Dionéia
Green, Paul G
et al.

Publication Date

2021-10-01

DOI

10.1016/j.jpain.2021.03.152

Peer reviewed

HHS Public Access

Author manuscript

J Pain. Author manuscript; available in PMC 2022 October 01.

Published in final edited form as:

J Pain. 2021 October ; 22(10): 1273–1282. doi:10.1016/j.jpain.2021.03.152.

Sexually Dimorphic Role of Toll-like Receptor 4 (TLR4) in High Molecular Weight Hyaluronan (HMWH)-induced Anti-hyperalgesia

Ivan J. M. Bonet[1,2], **Dionéia Araldi**[1,2], **Paul G. Green**[1,2,3], **Jon D. Levine**[1,2,4,*]

[1]Departments of Medicine and Oral & Maxillofacial Surgery, San Francisco, USA

[2]UCSF Pain and Addiction Research Center, University of California at San Francisco, San Francisco, USA

[3]Departments of Preventative and Restorative Dental Sciences, University of California at San Francisco, San Francisco, USA

[4]Departments of Medicine, University of California at San Francisco, San Francisco, USA

Abstract

High molecular weight hyaluronan (HMWH), a prominent component of the extracellular matrix binds to and signals via multiple receptors, including cluster of differentiation 44 (CD44), and toll-like receptor 4 (TLR4). We tested the hypothesis that, in the setting of inflammation, HMWH acts at TLR4 to attenuate hyperalgesia. We found that the attenuation of prostaglandin E_2 (PGE_2)-induced hyperalgesia by HMWH was attenuated by a TLR4 antagonist (NBP2–26245), but only in male and ovariectomized female rats. In this study we sought to evaluated the role of the TLR4 signaling pathway in anti-hyperalgesia induced by HMWH in male rats. Decreasing expression of TLR4 in nociceptors, by intrathecal administration of an oligodeoxynucleotide (ODN) antisense to TLR4 mRNA, also attenuated HMWH-induced anti-hyperalgesia, in male and ovariectomized female rats. Estrogen replacement in ovariectomized females reconstituted the gonad-intact phenotype. The administration of an inhibitor of myeloid differentiation factor 88 (MyD88), a TLR4 second messenger, attenuated HMWH-induced anti-hyperalgesia, while an inhibitor of the MyD88-independent TLR4 signaling pathway did not. Since it has previously been shown that HMWH-induced anti-hyperalgesia is also mediated, in part by CD44 we evaluated the effect of the combination of ODN antisense to TLR4 and CD44 mRNA. This treatment completely reversed HMWH-induced anti-hyperalgesia in male rats. Our results demonstrate a sex hormone-dependent, sexually dimorphic involvement of TLR4 in HMWH-induced anti-hyperalgesia, that is MyD88 dependent.

[*]**Corresponding author:** Jon D. Levine, M.D., Ph.D., University of California, San Francisco, 513 Parnassus Avenue, San Francisco, CA 94143-0440, Phone: (415) 476-5108, Fax: (415) 476-6305, Jon.Levine@ucsf.edu.

Disclosures: All authors report no financial interests or potential conflicts of interest. This study was funded by National Institutes of Health (NIH) grant AR075334.

Keywords

Hyperalgesia; Hyaluronan; Toll-like receptor 4 (TLR4); High molecular weight hyaluronan (HMWH); Anti-hyperalgesia; Prostaglandin E_2 (PGE_2)

INTRODUCTION

Hyaluronan (HA), a negatively charged linear disaccharide polymer, is an integral component of the extracellular matrix [44, 46] that plays a role in several physiological processes. High molecular weight hyaluronan (HMWH) has been used extensively to treat osteoarthritis pain [3, 16, 19, 29, 47] and has been shown to have anti-inflammatory and immunosuppressive effects [17, 25, 30, 34, 51]. We have shown that HMWH is anti-hyperalgesic against several pro-inflammatory mediators and in chemotherapy-induced neuropathic pain [10, 21, 23]. Evidence supports the suggestion that HMWH-induced anti-hyperalgesia is due to its effect on nociceptors [9, 15, 18, 21, 23, 26]. While recent studies have increased our knowledge of how HMWH signals via plasma membrane receptors, including cluster of differentiation 44 (CD44), receptor for HA-mediated motility (RHAMM), and toll-like receptor 4 (TLR4) [9, 23, 44, 48, 49], and that HMWH reduces the excitability of the transient receptor potential vanilloid subtype 1 (TRPV1) ion channel, by stabilizing its closed state [15, 18], many details of the mechanism by which HMWH produces anti-hyperalgesia remain to be elucidated.

While CD44 is generally considered to be the cognate HA receptor [12, 13], and has been proposed to mediate effects of HA on nociceptors [21], we have recently provide evidence that TLR4 is involved in the anti-hyperalgesia induced by HMWH, in male rats [9]. This observations led us to test the hypothesis that TLR4 signaling pathways are involved in the attenuation of nociceptor sensitization by HMWH. In the current study, we provide support for sex hormone-dependence for the role of TLR4, in the anti-hyperalgesia induced by HMWH, and provide insight into its downstream signaling pathway.

METHODS

Animals

Experiments were performed on 220–400 g female and male Sprague-Dawley rats (Charles River Laboratories, Hollister, CA, USA). Experimental animals were housed three per cage, under a 12-hour light/dark cycle, in a temperature- and humidity-controlled animal care facility at the University of California, San Francisco. Food and water were available *ad libitum*. Experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California, San Francisco, and adhered to the National Institutes of Health guidelines for the care and use of laboratory animals.

Measuring nociceptive threshold

Mechanical nociceptive threshold was quantified using an Ugo Basile Analgesymeter (Stoelting, Wood Dale, IL, USA). This device applies a linearly increasing mechanical force to the dorsum of a rat's hindpaw [38, 42, 43]. Rats were placed in acrylic cylindrical restrainers, with lateral ports to allow access to the leg and hind paw, as described previously [5]. Rats

were acclimatized to the restrainers over a 3-day period to habituate them to the testing procedure. Mechanical nociceptive threshold is defined as the force in grams at which a rat withdrew its paw. Baseline threshold is defined as the mean of three readings taken before injection of test agents. Each experiment was performed on a different group of rats. Data are presented as mean ± SEM percentage change from baseline nociceptive threshold.

Drugs

The following drugs were used in the present experiments: high molecular weight hyaluronan (HMWH) [hyaluronic acid sodium salt from Streptococcus pyogenes], from Calbiochem (San Diego, CA, USA); prostaglandin E_2 (PGE_2), ST2825 (a myeloid differentiation factor 88 [MyD88] inhibitor), BX-795 (an IRF3 inhibitor), dihydrotestosterone and 17β-estradiol all from Sigma-Aldrich (Saint Louis, MO, USA); and NBP2–26245 (a TLR4 receptor antagonist) from GenScript (Piscataway, NJ, USA).

PGE_2 was dissolved in absolute ethanol to a concentration of 1 μg/μL, and immediately before experiments further diluted in 0.9% saline to the concentration used in each experiment. The ethanol concentration of the final PGE_2 solution was ~2%, a concentration previously shown to not affect mechanical nociceptive threshold [22]. HMWH, was dissolved in distilled water to a concentration of 1 μg/μL, and further diluted in 0.9% saline to the concentration used in each experiment. Aliquots containing 1 μg/μL of BX-795 and ST2825, dissolved in 100% dimethyl sulfoxide (DMSO), were diluted in 0.9% NaCl containing 10% DMSO to a concentration of 0.2 μg/μL.

All intradermal administered drugs were in a volume of 5 μL (when injected alone) or 3 μL each (when two or more drugs were co-injected), on the dorsum of the hind paw, using a 30-gauge hypodermic needle attached to a 50 μL Hamilton syringe by a segment of PE-10 polyethylene tubing (Becton Dickinson, Franklin Lakes, NJ, USA). The administration of BX-795 and ST2825 was preceded by a hypotonic shock (1 μL of distilled water, separated by a bubble of air to avoid mixing in the same syringe), distilled water produces hypo-osmotic shock, allowing drugs to penetrate the cell plasma membrane, to get these two reagents inside the submicron diameter nociceptor nerve terminal [11, 14].

Oligodeoxynucleotides (ODN) antisense to CD44 and toll-like receptor 4 (TLR4) mRNA

The role of CD44 and TLR4 in the anti-nociceptive effects of HMWH, were assessed in male and female rats treated intrathecally with ODN antisense to each receptor's mRNA. ODN antisense was directed against a unique region of the rat mRNA sequence for each receptor. ODN antisense sequences were:

- CD44 ODN antisense: 5'-GAA AAG GGT CGC GGG GG-3' (GenBank accession number NM_012924.2)
- TLR4 ODN antisense: 5'-AGG AAG TGA GAG TGC CAA CC-3' (GenBank accession number NM_019178.1)

ODN mismatch sequences correspond to the antisense sequence with mismatched bases (denoted by bold letters). ODN mismatch sequences:

- CD44 ODN mismatch: 5'-**CCC CC**G **C**GA C**C**C **TTT TC**-3'
- TLR4 ODN mismatch: 5'-A**C**G **AT**G **C**GA GAG **AGT C**A**C** C**G**-3'

ODNs were synthesized by Life Technologies (Carlsbad, CA, USA), and have been previously shown to produce a decrease in CD44 and TLR4 protein [5, 9]. Before use, ODNs were reconstituted in nuclease-free 0.9% NaCl and then administered intrathecally, for 3 consecutive days [5]. As described previously [2], rats were anesthetized with isoflurane (2.5% in O_2) and ODN (120 μg in 20 μL) injected intrathecally using a 300 μl syringe attached to a 29-gauge hypodermic needle that was inserted into the subarachnoid space between the L4 and L5 vertebrae. The intrathecal site of injection was confirmed by a sudden flick of the rat's tail, a reflex that is evoked by subarachnoid space access and bolus intrathecal injection [33]. Animals regained consciousness approximately 2 minutes after the injection and termination of anesthesia. The use of ODN antisense administered intrathecally to attenuate the expression of proteins essential for nociceptor sensitization is well supported by previous studies, by others [35, 37, 39–41] as well as our group [4–6, 8, 21–23, 36].

Gonadectomy

Gonadectomy, was performed on 22–25 day old male and female rats (i.e., before sexual maturation); gonadectomized rats were used for experiments 3 weeks later (i.e., as adults) [27]. For this surgery, animals were anesthetized with isoflurane (3% in oxygen) and received preoperative meloxicam (~5 mg/kg, s.c.), and bupivacaine (~0.1 mg/kg s.c. infiltrated at incision site) for pain control.

Ovariectomy: Briefly, ovaries were accessed by means of bilateral cutaneous and peritoneal incisions. Once ovaries were located, their vascular bundles were ligatured with 4–0 silk suture (Perma-Hand Silk® Ethicon, Johnson & Johnson, Somerville, NJ), and they were excised; the peritoneal and cutaneous incisions was then closed with 5–0 silk suture (Ethicon, Johnson & Johnson, Somerville, NJ). In some rats, we also implanted 10 mm long segments of Silastic tubing filled with crystalline 17β-estradiol, as previously described [28], to replace 17β estradiol in gonadectomized female rats.

Orchiectomy: A single cutaneous incision was made through the scrotal skin and underlying tunica to expose the testes; their vascular bundles were identified and tied off with 5–0 silk suture and severed; the testes were then removed. The cutaneous incision was closed with 5–0 silk suture. In some gonadectomized rats we also performed sex hormone reconstitution with dihydrotestosterone [28].

Statistical analysis

The dependent variable in behavioral experiments was percentage change from baseline mechanical paw-withdrawal threshold. We used 186 male and 96 female rats. In each experiment only one hind paw per rat was used. The behavioral experiments were performed with the experimenter blinded to experimental group. One-way ANOVA followed by Bonferroni's *post hoc* comparisons test, or Student's *t*-test, was used to analyze data, as appropriate, described in each figure legend. Prism 8.0 (GraphPad Software) was used

for the graphics and to perform statistical analyses; *P<0.05* was considered statistically significant. Data are presented as mean ± SEM.

RESULTS

TLR4 dependence of HMWH-induced anti-hyperalgesia

To test the hypothesis that HMWH-induced anti-hyperalgesia is TLR4 dependent in male rats, male (Fig. 1A), female (Fig. 1B) and ovariectomized female (Fig. 1C) rats received an intradermal injection of PGE_2 (100 ng, i.d.) followed by a TLR4 antagonist (NBP2–26245, 1 μg, i.d.), all injected at the site of nociceptive testing, and then 5 min later HMWH (1 μg, i.d.) was injected. Male rats receiving the TLR4 antagonist showed attenuation of HMWH-induced anti PGE_2 hyperalgesia. While in female rats, the TLR4 antagonist did not attenuate HMWH-induced anti-hyperalgesia, in ovariectomized female rats it significantly attenuated HMWH-induced anti-hyperalgesia.

TLR4 signaling pathway mediating HMWH-induced anti-hyperalgesia

Since HA can signal via TLR4 [32], we screened for a role of TLR4 second messenger pathways in HMWH-induced anti-hyperalgesia. TLR4 has two well-described second messenger signaling pathways, MyD88-dependent and MyD88-independent. To test the hypothesis that HMWH signals through the MyD88-dependent pathway, to induce anti-hyperalgesia, male rats were treated intradermally with PGE_2 (100 ng, i.d.), followed 5 min later by a MyD88 inhibitor (ST2825, 1 μg, i.d.) and then, 5 min later by HMWH (1 μg, i.d.); all injections were performed at the site of nociceptive testing, on the dorsum of the hind paw. Male rats treated with the MyD88 inhibitor showed attenuation of HMWH-induced anti-hyperalgesia (Fig. 2).

The MyD88-independent TLR4 pathway involves TIR-domain-containing adaptor inducing interferon-β (TRIF) and the recruitment of the TRIF-related Adaptor Molecule (TRAM). To determine if the MyD88-independent pathway also mediates the TLR4-dependent contribution to HMWH-induced anti-hyperalgesia, we administered PGE_2 (100 ng, i.d.), followed 5 min later by an inhibitor of IRF3, a component of the MyD88-independent signaling pathway (BX-795, 1 μg, i.d.), and then 5 min later HMWH (1 μg, i.d.). Male rats treated with the IRF3 inhibitor did not show attenuation of HMWH-induced anti-hyperalgesia (Fig. 3).

Role of sex hormones in TLR4 dependence

We compared the effect of ODN antisense to TLR4 mRNA on HMWH anti-hyperalgesia in gonad-intact and gonadectomized male and female rats, with or without same sex hormone replacement (Fig. 4). In TLR4 antisense-treated rats, we administered PGE_2 (100 ng, i.d.), followed 10 min later by HMWH (1 μg, i.d.). TLR4 ODN antisense attenuated HMWH-induced anti-hyperalgesia in gonad-intact male (Fig. 4A), but not in gonad-intact female rats (Fig. 4B). Orchiectomized male rats treated with TLR4 ODN antisense still demonstrated attenuation of HMWH-induced anti-hyperalgesia (Fig. 4A), while ovariectomized female rats now demonstrated attenuation of HMWH-induced anti-hyperalgesia (Fig. 4B). Next, the group of ovariectomized female rats were replaced with 17β-estradiol, and the group

of orchiectomized male were treated with dihydrotestosterone. In orchiectomized male rats treated with dihydrotestosterone, TLR4 ODN antisense attenuated HMWH anti-hyperalgesia (Fig. 4A). And, in hormone-replaced ovariectomized female rats, HMWH-induced anti-hyperalgesia was again not attenuated by ODN antisense to TLR4 mRNA (Fig. 4B).

Effect of combining TLR4 and CD44 antisense

Since both TLR4 and CD44 antisense partially attenuate HMWH anti-hyperalgesia, in male rats, we administered the combination of TLR4 plus CD44 ODN antisense. Male rats that received both TLR4 and CD44 ODN antisense showed almost complete reversal of the anti-hyperalgesia induced by HMWH (Fig. 5).

DISCUSSION

We have previously demonstrated that HMWH attenuates PGE_2-induced hyperalgesia in female and male rats and that while HMWH-induced anti-hyperalgesia was CD44-dependent in both sexes, TLR4 dependence was only observed in males [9]. Our current finding that decreasing expression or pharmacological antagonism of TLR4 attenuates HMWH-induced anti-hyperalgesia in male but not in female rats, provides further support that the sexually dimorphic HMWH-induced anti-hyperalgesia, is dependent, at least in part, on the TLR4 present in nociceptors [7].

While HMWH does not alone affect mechanical nociceptive threshold in the naïve control rat, it reverses nociceptor sensitization induced by diverse pronociceptive mediators (e.g. PGE_2, epinephrine, TNFα, and interleukin-6 [23]), supporting the suggestion that HMWH is acting to reverse nociceptor sensitization. The fact that in male but not in female rats HMWH-induced anti-hyperalgesia is partially dependent on TLR4 could be due, at least in part, to sex differences in downstream signaling [20, 24], and the sensitivity of TLR4 to sex hormones (e.g. reduction in TLR4 expression by estrogen [53]).

While it has previously been shown that HA can signal via TLR4 to mediate HMWH-induced anti-hyperalgesia in male rats [32], little is currently known about the second messenger signaling pathway in nociceptors that mediates this contribution of TLR4 to HMWH anti-hyperalgesia. TLR4 has two well-established second messenger signaling pathways, referred to as MyD88-dependent and MyD88-independent [1]. In the present study, we found that administration of a MyD88 inhibitor reduced HMWH-induced anti-hyperalgesia. The MyD88-independent TLR4 signaling pathway [31], which involves the TRAM-TRIF complex, leads to activation of IRF3 [52]. We found that treatment with an IRF3 inhibitor did not attenuate HMWH-induced anti-hyperalgesia, supporting the suggestion that the MyD88-dependent but not the MyD88-independent pathway plays a role in HMWH anti-hyperalgesia. The attenuation of HMWH-induced anti-hyperalgesia by blocking MyD88 could be due to interactions between CD44 and TLR4 [45].

In the present study, we demonstrated that sexual dimorphism in HMWH-induced anti-hyperalgesia is female sex hormone-dependent. Thus, while in gonad-intact females, ODN antisense against TLR4 mRNA did not attenuate HMWH-induced hyperalgesia, in ovariectomized females, it did. To test the hypothesis that this effect of ovariectomy is due

to an estrogen-dependent mechanism, we replaced estrogen in ovariectomized female rats, by administering 17β-estradiol. Ovariectomized female rats with 17β-estradiol replacement showed a similar lack of response to TLR4 ODN antisense as observed in gonad-intact females. Gonad-intact, orchiectomized or orchiectomized treated with dihydrotestosterone males, all showed attenuation of HMWH-induced anti-hyperalgesia when treated with ODN antisense against TLR4 mRNA. These results support the suggestion that sex differences in HMWH-induced anti-hyperalgesia are female but not male sex hormone-dependent.

The co-administration of ODN antisense against both CD44 and TLR4 mRNA eliminated HMWH-induced anti-hyperalgesia in male rats, supporting additive roles of CD44 and TLR4 in mediating HMWH-induced anti-hyperalgesia. Knockdown of TLR4 may decrease expression or function of CD44 in DRG, as demonstrated in mesenchymal stem cells [50], and affect the ability of HMWH to exert its anti-hyperalgesic effect.

In conclusion, in this study we demonstrate an estrogen-dependent sexual dimorphism in the role of TLR4 in HMWH anti-hyperalgesia, with a TLR4 dependence in male, but not in female rats. That TLR4-mediated HMWH-induced anti-hyperalgesia signals via MyD88 pathways opens a novel line of research into molecular targets for the treatment of pain produced by nociceptor sensitization.

Acknowledgements:

The authors would like to thank Samantha Stevens for technical assistance.

REFERENCES

1. Akira S, Takeda K. Toll-like receptor signalling. Nat Rev Immunol. 4:499–511, 2004 [PubMed: 15229469]
2. Alessandri-Haber N, Yeh JJ, Boyd AE, Parada CA, Chen X, Reichling DB, Levine JD. Hypotonicity induces TRPV4-mediated nociception in rat. Neuron. 39:497–511, 2003 [PubMed: 12895423]
3. Altman RD, Moskowitz R. Intraarticular sodium hyaluronate (Hyalgan) in the treatment of patients with osteoarthritis of the knee: a randomized clinical trial. Hyalgan Study Group. J Rheumatol. 25:2203–2212, 1998 [PubMed: 9818665]
4. Alvarez P, Green PG, Levine JD. Role for monocyte chemoattractant protein-1 in the induction of chronic muscle pain in the rat. Pain. 155:1161–1167, 2014 [PubMed: 24637038]
5. Araldi D, Bogen O, Green PG, Levine JD. Role of Nociceptor Toll-like Receptor 4 (TLR4) in Opioid-Induced Hyperalgesia and Hyperalgesic Priming. J Neurosci. 39:6414–6424, 2019 [PubMed: 31209174]
6. Araldi D, Ferrari LF, Levine JD. Hyperalgesic priming (type II) induced by repeated opioid exposure: maintenance mechanisms. Pain. 158:1204–1216, 2017 [PubMed: 28306605]
7. Barajon I, Serrao G, Arnaboldi F, Opizzi E, Ripamonti G, Balsari A, Rumio C. Toll-like receptors 3, 4, and 7 are expressed in the enteric nervous system and dorsal root ganglia. J Histochem Cytochem. 57:1013–1023, 2009 [PubMed: 19546475]
8. Bogen O, Alessandri-Haber N, Chu C, Gear RW, Levine JD. Generation of a pain memory in the primary afferent nociceptor triggered by PKCepsilon activation of CPEB. J Neurosci. 32:2018–2026, 2012 [PubMed: 22323716]
9. Bonet IJM, Araldi D, Khomula EV, Bogen O, Green PG, Levine JD. Mechanisms Mediating High-Molecular-Weight Hyaluronan-Induced Antihyperalgesia. J Neurosci. 40:6477–6488, 2020 [PubMed: 32665406]
10. Bonet IJM, Green PG, Levine JD. Sexual dimorphism in the nociceptive effects of hyaluronan. Pain. 2020

11. Borle AB, Snowdowne KW. Measurement of intracellular free calcium in monkey kidney cells with aequorin. Science. 217:252–254, 1982 [PubMed: 6806904]
12. Bourguignon LY. Matrix hyaluronan-activated CD44 signaling promotes keratinocyte activities and improves abnormal epidermal functions. Am J Pathol. 184:1912–1919, 2014 [PubMed: 24819962]
13. Bourguignon LY, Shiina M, Li JJ. Hyaluronan-CD44 interaction promotes oncogenic signaling, microRNA functions, chemoresistance, and radiation resistance in cancer stem cells leading to tumor progression. Adv Cancer Res. 123:255–275, 2014 [PubMed: 25081533]
14. Burch RM, Axelrod J. Dissociation of bradykinin-induced prostaglandin formation from phosphatidylinositol turnover in Swiss 3T3 fibroblasts: evidence for G protein regulation of phospholipase A2. Proc Natl Acad Sci U S A. 84:6374–6378, 1987 [PubMed: 2888113]
15. Caires R, Luis E, Taberner FJ, Fernandez-Ballester G, Ferrer-Montiel A, Balazs EA, Gomis A, Belmonte C, de la Pena E. Hyaluronan modulates TRPV1 channel opening, reducing peripheral nociceptor activity and pain. Nat Commun. 6:8095, 2015 [PubMed: 26311398]
16. Cohen MM, Altman RD, Hollstrom R, Hollstrom C, Sun C, Gipson B. Safety and efficacy of intra-articular sodium hyaluronate (Hyalgan) in a randomized, double-blind study for osteoarthritis of the ankle. Foot Ankle Int. 29:657–663, 2008 [PubMed: 18785414]
17. Cuff CA, Kothapalli D, Azonobi I, Chun S, Zhang Y, Belkin R, Yeh C, Secreto A, Assoian RK, Rader DJ, Pure E. The adhesion receptor CD44 promotes atherosclerosis by mediating inflammatory cell recruitment and vascular cell activation. J Clin Invest. 108:1031–1040, 2001 [PubMed: 11581304]
18. de la Pena E, Gomis A, Ferrer-Montiel A, Belmonte C. TRPV1 channel modulation by hyaluronan reduces pain. Channels (Austin). 10:81–82, 2016 [PubMed: 26517313]
19. Dougados M, Nguyen M, Listrat V, Amor B. High molecular weight sodium hyaluronate (hyalectin) in osteoarthritis of the knee: a 1 year placebo-controlled trial. Osteoarthritis Cartilage. 1:97–103, 1993 [PubMed: 8886085]
20. Doyle HH, Eidson LN, Sinkiewicz DM, Murphy AZ. Sex Differences in Microglia Activity within the Periaqueductal Gray of the Rat: A Potential Mechanism Driving the Dimorphic Effects of Morphine. J Neurosci. 37:3202–3214, 2017 [PubMed: 28219988]
21. Ferrari LF, Araldi D, Bogen O, Levine JD. Extracellular matrix hyaluronan signals via its CD44 receptor in the increased responsiveness to mechanical stimulation. Neuroscience. 324:390–398, 2016 [PubMed: 26996509]
22. Ferrari LF, Khomula EV, Araldi D, Levine JD. Marked Sexual Dimorphism in the Role of the Ryanodine Receptor in a Model of Pain Chronification in the Rat. Sci Rep. 6:31221, 2016 [PubMed: 27499186]
23. Ferrari LF, Khomula EV, Araldi D, Levine JD. CD44 Signaling Mediates High Molecular Weight Hyaluronan-Induced Antihyperalgesia. J Neurosci. 38:308–321, 2018 [PubMed: 29175954]
24. Fields CT, Chassaing B, Castillo-Ruiz A, Osan R, Gewirtz AT, de Vries GJ. Effects of gut-derived endotoxin on anxiety-like and repetitive behaviors in male and female mice. Biol Sex Differ. 9:7, 2018 [PubMed: 29351816]
25. Furuta J, Ariyoshi W, Okinaga T, Takeuchi J, Mitsugi S, Tominaga K, Nishihara T. High molecular weight hyaluronic acid regulates MMP13 expression in chondrocytes via DUSP10/MKP5. J Orthop Res. 35:331–339, 2017 [PubMed: 27101204]
26. Gomis A, Miralles A, Schmidt RF, Belmonte C. Nociceptive nerve activity in an experimental model of knee joint osteoarthritis of the guinea pig: effect of intra-articular hyaluronan application. Pain. 130:126–136, 2007 [PubMed: 17197090]
27. Green PG, Dahlqvist SR, Isenberg WM, Miao FJ, Levine JD. Role of adrenal medulla in development of sexual dimorphism in inflammation. Eur J Neurosci. 14:1436–1444, 2001 [PubMed: 11722605]
28. Green PG, Dahlqvist SR, Isenberg WM, Strausbaugh HJ, Miao FJ, Levine JD. Sex steroid regulation of the inflammatory response: sympathoadrenal dependence in the female rat. J Neurosci. 19:4082–4089, 1999 [PubMed: 10234036]
29. Huang TL, Chang CC, Lee CH, Chen SC, Lai CH, Tsai CL. Intra-articular injections of sodium hyaluronate (Hyalgan(R)) in osteoarthritis of the knee. a randomized, controlled, double-blind,

multicenter trial in the Asian population. BMC Musculoskelet Disord. 12:221, 2011 [PubMed: 21978211]

30. Kataoka Y, Ariyoshi W, Okinaga T, Kaneuji T, Mitsugi S, Takahashi T, Nishihara T. Mechanisms involved in suppression of ADAMTS4 expression in synoviocytes by high molecular weight hyaluronic acid. Biochem Biophys Res Commun. 432:580–585, 2013 [PubMed: 23438438]
31. Kawai T, Sato S, Ishii KJ, Coban C, Hemmi H, Yamamoto M, Terai K, Matsuda M, Inoue J, Uematsu S, Takeuchi O, Akira S. Interferon-alpha induction through Toll-like receptors involves a direct interaction of IRF7 with MyD88 and TRAF6. Nat Immunol. 5:1061–1068, 2004 [PubMed: 15361868]
32. Lennon FE, Singleton PA. Role of hyaluronan and hyaluronan-binding proteins in lung pathobiology. Am J Physiol Lung Cell Mol Physiol. 301:L137–147, 2011 [PubMed: 21571904]
33. Mestre C, Pelissier T, Fialip J, Wilcox G, Eschalier A. A method to perform direct transcutaneous intrathecal injection in rats. J Pharmacol Toxicol Methods. 32:197–200, 1994 [PubMed: 7881133]
34. Mizrahy S, Raz SR, Hasgaard M, Liu H, Soffer-Tsur N, Cohen K, Dvash R, Landsman-Milo D, Bremer MG, Moghimi SM, Peer D. Hyaluronan-coated nanoparticles: the influence of the molecular weight on CD44-hyaluronan interactions and on the immune response. J Control Release. 156:231–238, 2011 [PubMed: 21745506]
35. Oliveira-Fusaro MCG, Zanoni CIS, Dos Santos GG, Manzo LP, Araldi D, Bonet IJM, Tambeli CH, Dias EV, Parada CA. Antihyperalgesic effect of CB1 receptor activation involves the modulation of P2X3 receptor in the primary afferent neuron. Eur J Pharmacol. 798:113–121, 2017 [PubMed: 28131783]
36. Parada CA, Yeh JJ, Reichling DB, Levine JD. Transient attenuation of protein kinase Cepsilon can terminate a chronic hyperalgesic state in the rat. Neuroscience. 120:219–226, 2003 [PubMed: 12849754]
37. Quanhong Z, Ying X, Moxi C, Tao X, Jing W, Xin Z, Li W, Derong C, Xiaoli Z, Wei J. Intrathecal PLC(beta3) oligodeoxynucleotides antisense potentiates acute morphine efficacy and attenuates chronic morphine tolerance. Brain Res. 1472:38–44, 2012 [PubMed: 22771399]
38. Randall LO, Selitto JJ. A method for measurement of analgesic activity on inflamed tissue. Arch Int Pharmacodyn Ther. 111:409–419, 1957 [PubMed: 13471093]
39. Song MJ, Wang YQ, Wu GC. Additive anti-hyperalgesia of electroacupuncture and intrathecal antisense oligodeoxynucleotide to interleukin-1 receptor type I on carrageenan-induced inflammatory pain in rats. Brain Res Bull. 78:335–341, 2009 [PubMed: 19022353]
40. Su L, Wang C, Yu YH, Ren YY, Xie KL, Wang GL. Role of TRPM8 in dorsal root ganglion in nerve injury-induced chronic pain. BMC Neurosci. 12:120, 2011 [PubMed: 22111979]
41. Sun JL, Xiao C, Lu B, Zhang J, Yuan XZ, Chen W, Yu LN, Zhang FJ, Chen G, Yan M. CX3CL1/CX3CR1 regulates nerve injury-induced pain hypersensitivity through the ERK5 signaling pathway. J Neurosci Res. 91:545–553, 2013 [PubMed: 23361876]
42. Taiwo YO, Coderre TJ, Levine JD. The contribution of training to sensitivity in the nociceptive paw-withdrawal test. Brain Res. 487:148–151, 1989 [PubMed: 2752281]
43. Taiwo YO, Levine JD. Prostaglandin effects after elimination of indirect hyperalgesic mechanisms in the skin of the rat. Brain Res. 492:397–399, 1989 [PubMed: 2665905]
44. Tavianatou AG, Caon I, Franchi M, Piperigkou Z, Galesso D, Karamanos NK. Hyaluronan: molecular size-dependent signaling and biological functions in inflammation and cancer. FEBS J. 286:2883–2908, 2019 [PubMed: 30724463]
45. Taylor KR, Yamasaki K, Radek KA, Di Nardo A, Goodarzi H, Golenbock D, Beutler B, Gallo RL. Recognition of hyaluronan released in sterile injury involves a unique receptor complex dependent on Toll-like receptor 4, CD44, and MD-2. J Biol Chem. 282:18265–18275, 2007 [PubMed: 17400552]
46. Toole BP. Hyaluronan-CD44 Interactions in Cancer: Paradoxes and Possibilities. Clin Cancer Res. 15:7462–7468, 2009 [PubMed: 20008845]
47. Triantaffilidou K, Venetis G, Bika O. Efficacy of hyaluronic acid injections in patients with osteoarthritis of the temporomandibular joint. A comparative study. J Craniofac Surg. 24:2006–2009, 2013 [PubMed: 24220392]

48. Vigetti D, Karousou E, Viola M, Deleonibus S, De Luca G, Passi A. Hyaluronan: biosynthesis and signaling. Biochim Biophys Acta. 1840:2452–2459, 2014 [PubMed: 24513306]

49. Vigetti D, Viola M, Karousou E, De Luca G, Passi A. Metabolic control of hyaluronan synthases. Matrix Biol. 35:8–13, 2014 [PubMed: 24134926]

50. Wang Y, Abarbanell AM, Herrmann JL, Weil BR, Manukyan MC, Poynter JA, Meldrum DR. TLR4 inhibits mesenchymal stem cell (MSC) STAT3 activation and thereby exerts deleterious effects on MSC-mediated cardioprotection. PLoS One. 5:e14206, 2010 [PubMed: 21151968]

51. Wu PT, Kuo LC, Su FC, Chen SY, Hsu TI, Li CY, Tsai KJ, Jou IM. High-molecular-weight hyaluronic acid attenuated matrix metalloproteinase-1 and −3 expression via CD44 in tendinopathy. Sci Rep. 7:40840, 2017 [PubMed: 28091588]

52. Yamamoto M, Sato S, Hemmi H, Uematsu S, Hoshino K, Kaisho T, Takeuchi O, Takeda K, Akira S. TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. Nat Immunol. 4:1144–1150, 2003 [PubMed: 14556004]

53. Zhang Z, Qin P, Deng Y, Ma Z, Guo H, Guo H, Hou Y, Wang S, Zou W, Sun Y, Ma Y, Hou W. The novel estrogenic receptor GPR30 alleviates ischemic injury by inhibiting TLR4-mediated microglial inflammation. J Neuroinflammation. 15:206, 2018 [PubMed: 30001721]

Perspective

The role of TLR4 in anti-hyperalgesia induced by HMWH is a sexually dimorphic, TLR4 dependent inhibition of inflammatory hyperalgesia that provides a novel molecular target for the treatment of inflammatory pain.

Highlights

- Involvement of TLR4 in HMWH-induced anti-hyperalgesia is sexually dimorphic.
- HMWH anti-hyperalgesia is TLR4-dependent only in male rats.
- TLR4 dependence of HMWH anti-hyperalgesia is MyD88 mediated.

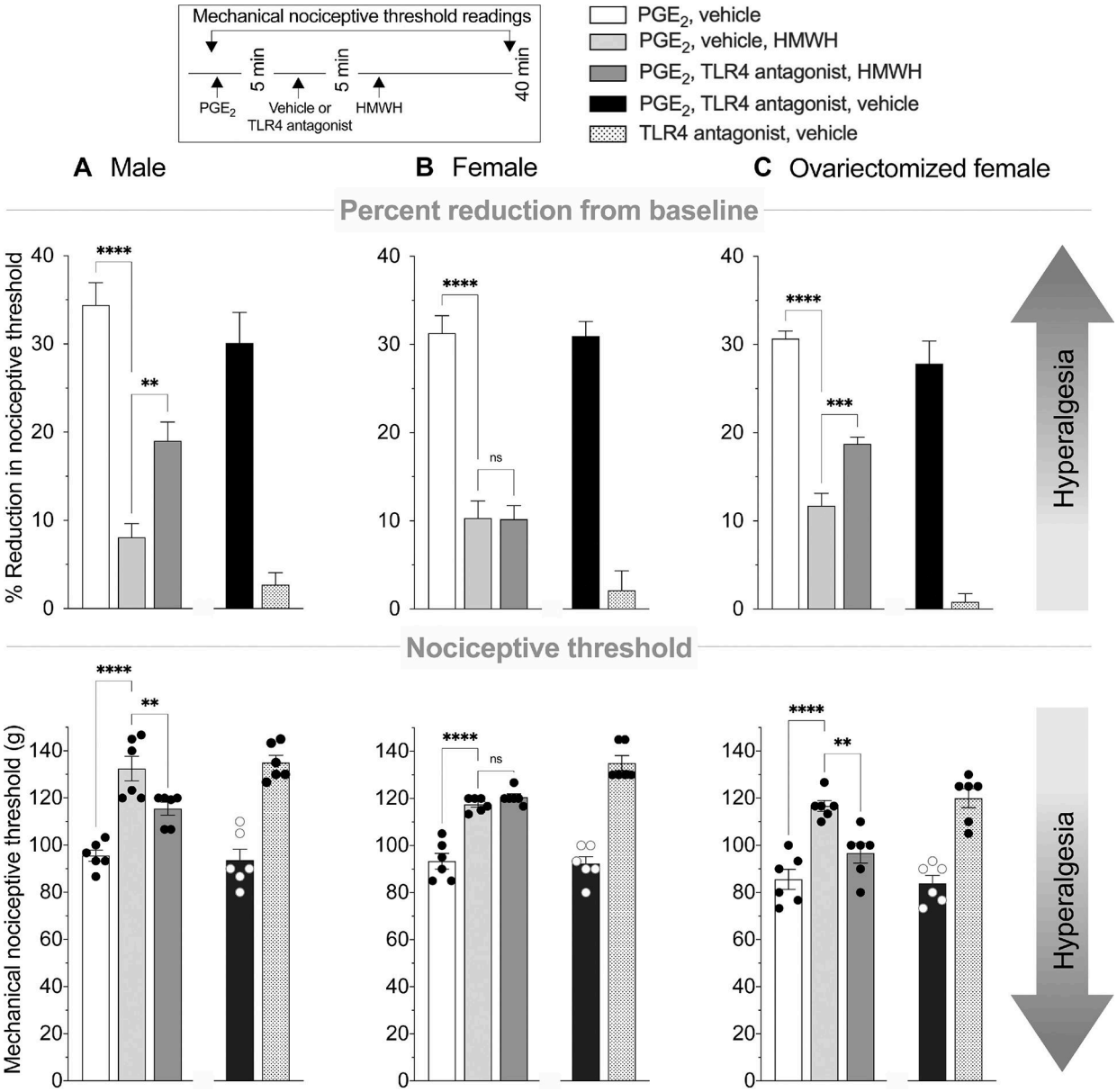

Figure 1. TLR4 antagonist attenuates HMWH-induced anti-hyperalgesia in male but not female rats.

Male, female or ovariectomized female rats were injected with PGE_2 (100 ng/ 3 μL, i.d.) followed 5 min later by vehicle (3 μL, i.d.) or TLR4 receptor antagonist (NBP2–26245, 1 μg/ 3 μL, i.d.) and then 5 min later by HMWH (1 μg/3 μL, i.d.), all at the site of nociceptive testing on the dorsum of the hind paw. One group of rats received a single injection of TLR4 receptor antagonist (NBP2–26245, 1 μg/3 μL, i.d.) to confirm that it did not produce hyperalgesia by itself, and another group received PGE_2 (100 ng/ 3 μL, i.d.) followed 5 min later by TLR4 receptor antagonist (NBP2–26245, 1 μg/ 3 μL, i.d.) to show that TLR4 receptor antagonist did not affect PGE_2-induced hyperalgesia (*dotted* and *black bars*, respectively). Mechanical nociceptive threshold was measured 40 min after injection of PGE_2.

A. Percent reduction from baseline (*top panel*) in male rats: Intradermal HMWH attenuates PGE_2-induced hyperalgesia ($F_{(2,15)}$= 43.65, ****p<0.0001, when PGE_2 was compared to PGE_2 + HMWH-treated group; one-way ANOVA followed by Bonferroni's *post hoc* comparisons test). The anti-hyperalgesic effect of HMWH for PGE_2-induced hyperalgesia was attenuated by the TLR4 antagonist in male rats (**p=0.0045, when HMWH-induced anti-hyperalgesia was compared between vehicle- and TLR4 antagonist-treated groups at 40 min after PGE_2; one-way ANOVA followed by Bonferroni's *post hoc* comparisons test). Mechanical nociceptive threshold (*bottom panel*) in male rats: Intradermal HMWH attenuates PGE_2-induced hyperalgesia ($F_{(2,15)}$= 25.42, ****p<0.0001, when PGE_2- was compared to PGE_2 + HMWH-treated group; one-way ANOVA followed by Bonferroni's *post hoc* comparisons test). Anti-hyperalgesic effect of HMWH was attenuated by the TLR4 antagonist (**p=0.01, when HMWH-induced anti-hyperalgesia was compared between vehicle- and TLR4 antagonist-treated groups at 40 min after PGE_2; one-way ANOVA followed by Bonferroni's *post hoc* comparisons test). n=6 per group.

B. Percent reduction from baseline (*top panel*) in female rats: Intradermal PGE_2-induced hyperalgesia was attenuated by HMWH, injected at the same site ($F_{(2,15)}$= 43.65, ****p<0.0001, when PGE_2 and PGE_2 + HMWH-treated groups were compared 40 min after PGE_2; one-way ANOVA followed by Bonferroni's *post hoc* comparisons test). When the TLR4 antagonist was injected before HMWH, it did not affect the anti-hyperalgesic effect of HMWH for PGE_2-induced hyperalgesia (ns, p>0.9, when HMWH-induced anti-hyperalgesia was compared between the vehicle- and TLR4 antagonist-treated groups at 40 min after PGE_2; one-way ANOVA followed by Bonferroni's *post hoc* comparisons test). Mechanical nociceptive threshold (*bottom panel*) in female rats: HMWH attenuated PGE_2-induced hyperalgesia ($F_{(2,15)}$= 46.52, ****p<0.0001, when PGE_2 and PGE_2 + HMWH-treated groups were compared 40 min after PGE_2; one-way ANOVA followed by Bonferroni's *post hoc* comparisons test). No attenuation of the anti-hyperalgesic effect of HMWH was observed in the group of female rats treated with the TLR4 antagonist (ns, p=0.67, when HMWH-induced anti-hyperalgesia was compared between vehicle- and TLR4 antagonist-treated groups at 40 min after PGE_2; one-way ANOVA followed by Bonferroni's *post hoc* comparisons test). n=6 per group.

C. Percent reduction from baseline (*top panel*) in ovariectomized females: Intradermal HMWH attenuates PGE_2-induced hyperalgesia in ovariectomized female rats ($F_{(2,15)}$= 83.20, ****p<0.0001, when PGE_2 and PGE_2 + HMWH-treated groups were compared 40 min after PGE_2; one-way ANOVA followed by Bonferroni's *post hoc* comparisons test). An attenuation of the anti-hyperalgesic effect of HMWH, for PGE_2-induced hyperalgesia, was observed in the ovariectomized female rats treated with the TLR4 antagonist (***p=0.0006, when HMWH-induced anti-hyperalgesia was compared between vehicle- and TLR4 antagonist-treated groups at 40 min after PGE_2; one-way ANOVA followed by Bonferroni's *post hoc* comparisons test). Nociceptive threshold (*bottom panel*) in ovariectomized female rats: HMWH attenuates PGE_2-induced hyperalgesia ($F_{(2,15)}$= 18.10, ****p<0.0001, when PGE_2 and PGE_2 + HMWH-treated groups were compared 40 min after PGE_2; one-way ANOVA followed by Bonferroni's *post hoc* comparisons test). Attenuation of the HMWH-induced anti-hyperalgesia was observed in the ovariectomized female rats treated with the TLR4 receptor antagonist (**p=0.0034, when HMWH-induced anti-hyperalgesia was

compared between vehicle- and TLR4 antagonist-treated groups at 40 min after PGE_2; one-way ANOVA followed by Bonferroni's *post hoc* comparisons test). n=6 per group.

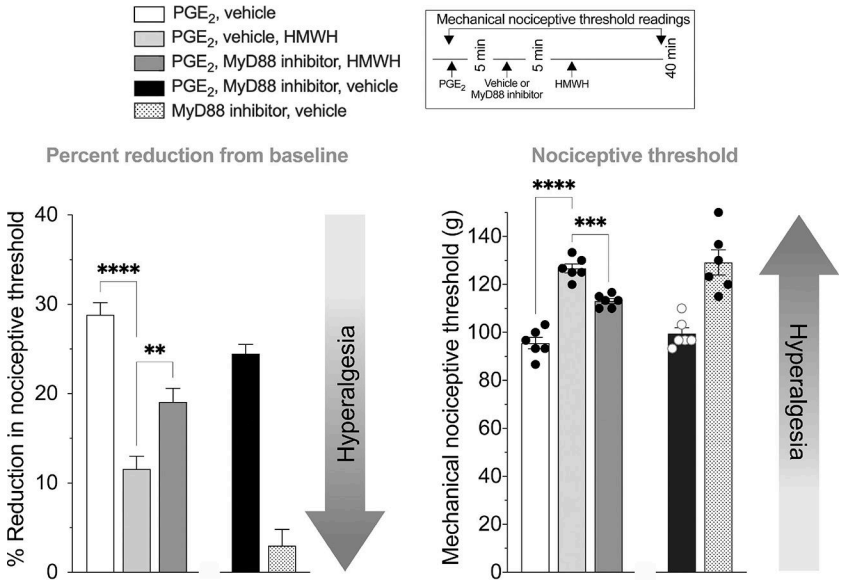

Figure 2. MyD88 inhibitor attenuates HMWH-induced anti-hyperalgesia in male rats.

Male rats received PGE_2 (100 ng/ 3 μL, i.d.) followed 5 min later by vehicle (3 μL, i.d.) or a MyD88 inhibitor (ST2825, 1 μg/ 3 μL, i.d.), and then 5 min later by HMWH (1 μg/ 3 μL, i.d.). Mechanical nociceptive threshold was measured 40 min after intradermal PGE_2. An additional group of rats received MyD88 inhibitor (1 μg/ 3 μL, i.d.) to confirm that it did not affect nociceptive threshold by itself (*dotted bar*), and another group of rats received PGE_2 (100 ng/ 3 μL, i.d.) followed 5 min later by MyD88 inhibitor (1 μg/ 3 μL, i.d.) to show that it did not affect PGE_2-induced hyperalgesia (*black bar*). Percent reduction from baseline (*left panel*): Intradermal administration of HMWH attenuate PGE_2-induced hyperalgesia ($F_{(2,15)}$= 35.30, ****p<0.0001, when PGE_2- and PGE_2 + HMWH-treated groups were compared 40 min after PGE_2; one-way ANOVA followed by Bonferroni's *post hoc* comparisons test). The anti-hyperalgesic effect of HMWH was attenuated by the MyD88 inhibitor (**p=0.0048, when HMWH-induced anti-hyperalgesia was compared between vehicle- and MyD88 inhibitor-treated groups at 40 min after PGE_2; one-way ANOVA followed by Bonferroni's *post hoc* comparisons test). Mechanical nociceptive threshold (*right panel*): Intradermal HMWH attenuated PGE_2-induced hyperalgesia ($F_{(2,15)}$= 70.50, ****p<0.0001, when PGE_2- and PGE_2 + HMWH-treated groups were compared 40 min after PGE_2; one-way ANOVA followed by Bonferroni's *post hoc* comparisons test). The anti-hyperalgesic effect of HMWH was attenuated by the MyD88 inhibitor (***p=0.0002, when HMWH-induced anti-hyperalgesia was compared between vehicle- and MyD88 inhibitor-treated groups at 40 min after PGE_2; one-way ANOVA followed by Bonferroni's *post hoc* comparisons test). n=6 per group.

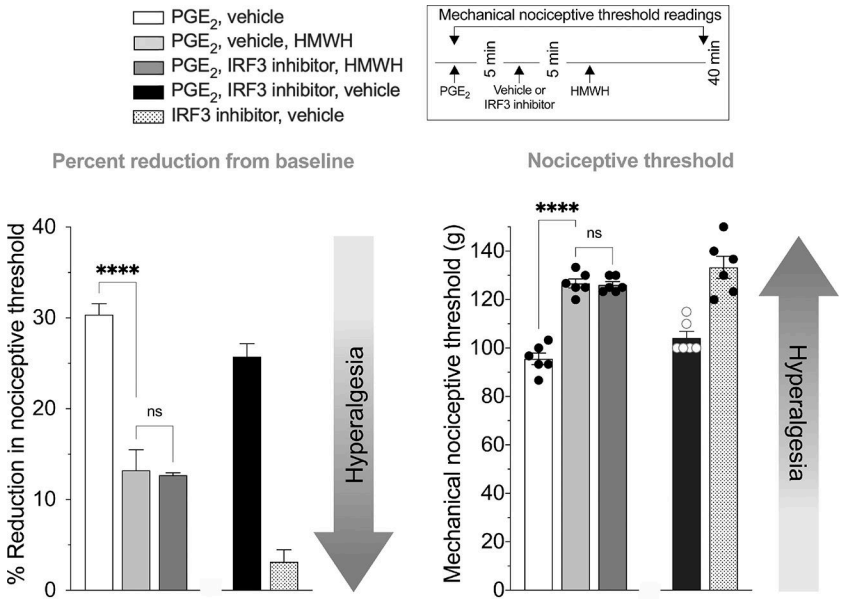

Figure 3. IRF3 inhibitor does not attenuate HMWH induced anti-hyperalgesia in male rats.

Male rats were injected with PGE_2 (100 ng/ 3 μL, i.d.) followed, 5 min later by vehicle (3 μL, i.d.) or an IRF3 inhibitor (BX-795, 1 μg/ 3 μL, i.d.), and then 5 min later by HMWH (1 μg/ 3 μL, i.d.). Mechanical nociceptive threshold was measured 40 min after intradermal PGE_2. One group of rats was treated with a single injection of the IRF3 inhibitor (1 μg/ 3 μL, i.d.) to confirm that this inhibitor alone did not affect nociceptive threshold (*dotted bar*), and another group of rats received PGE_2 (100 ng/ 3 μL, i.d.) followed 5 min later by IRF3 inhibitor (1 μg/ 3 μL, i.d.) to show that it did not affect PGE_2-induced hyperalgesia (*black bar*). Percent reduction from baseline (*left panel*): Intradermal administration of HMWH attenuates PGE_2-induced hyperalgesia ($F_{(2,15)}$= 45.55, ****p<0.0001, when PGE_2- and PGE_2, + HMWH-treated groups were compared 40 min after PGE_2; one-way ANOVA followed by Bonferroni's *post hoc* comparisons test). The IRF3 inhibitor did not affect the anti-hyperalgesic effect of HMWH (ns, p>0.9, when HMWH-induced anti-hyperalgesia was compared between vehicle- and IRF3 inhibitor-treated groups at 40 min after PGE_2; one-way ANOVA followed by Bonferroni's *post hoc* comparisons test). Mechanical nociceptive threshold (*right panel*): PGE_2-induced hyperalgesia was attenuated by intradermal HMWH ($F_{(2,15)}$= 88.25, ****p<0.0001, when the PGE_2 and PGE_2 + HMWH-treated groups were compared 40 min after PGE_2; one-way ANOVA followed by Bonferroni's post-hoc comparisons test). The IRF3 inhibitor did not affect the anti-hyperalgesic effect of HMWH (ns, p>0.9, when HWMH-induced anti-hyperalgesia was compared between vehicle- and IRF3 inhibitor-treated groups at 40 min after PGE_2; one-way ANOVA followed by Bonferroni's *post hoc* comparisons test). n=6 per group.

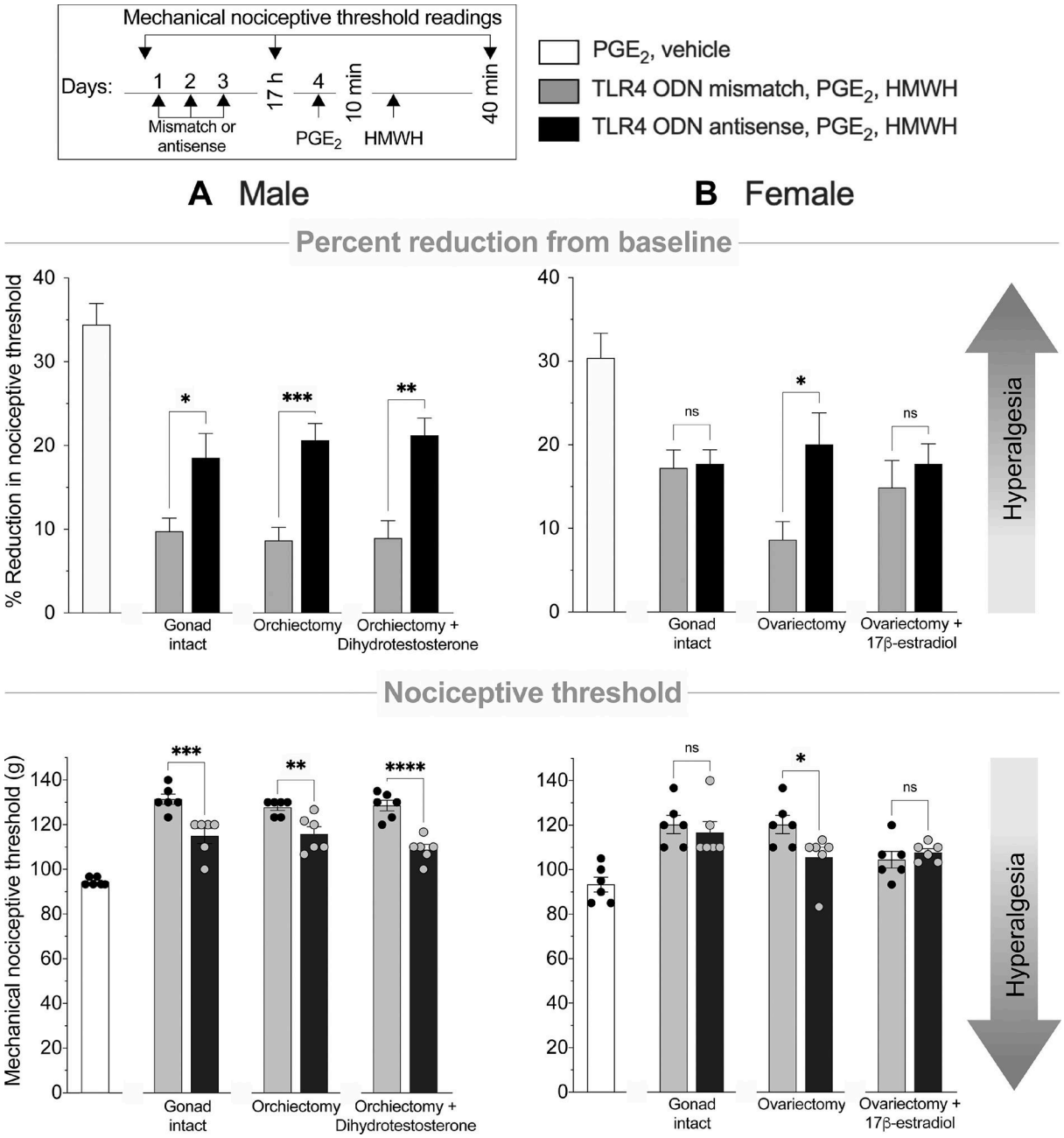

Figure 4. Involvement of TLR4 in HMWH-induced anti-hyperalgesia in female rats is sex hormone dependent.

A. Gonad intact, orchiectomized, and orchiectomized dihydrotestosterone treated male rats were injected with ODN antisense or mismatch (120 μg/ 20 μl, i.t.) for TLR4 mRNA, daily for 3 consecutive days. On the fourth day, approximately 17 h after the last ODN administration, PGE_2 (100 ng/ 5 μL, i.d.) was injected at the site of nociceptive testing, intradermally, followed 10 min later by HMWH (1 μg/ 5 μL, i.d.) injected at the same site. Mechanical nociceptive threshold was evaluated 40 min after PGE_2. Percent reduction from baseline (*top panel*): The anti-hyperalgesic effect of HMWH on PGE_2-induced hyperalgesia was attenuated by ODN antisense to TLR4 mRNA (gonad intact groups, $t_{(10)}$=

2.638, *p=0.0248; orchiectomized groups, $t_{(10)}$= 4.678, ***p=0.0009; orchiectomized + dihydrotestosterone, $t_{(10)}$= 4.200, ***p=0.0018, when the respective mismatch and antisense groups were compared 40 min after PGE_2; unpaired Student's t-test). Nociceptive threshold (*bottom panel*): Anti-hyperalgesic effect of HMWH was attenuated by ODN antisense to TLR4 mRNA (gonad intact groups, $t_{(10)}$= 3.980, **p=0.0026; orchiectomized groups, $t_{(10)}$= 3.343, **p=0.0075; orchiectomized + dihydrotestosterone, $t_{(10)}$= 4.200, ***p=0.0001, when the respective mismatch and antisense groups were compared 40 min after PGE_2; unpaired Student's t-test). n=6 per group.

B. Gonad intact, ovariectomized and 17-β estradiol replaced ovariectomized female rats were injected with ODN antisense or mismatch (120 μg/ 20 μl, i.t.) for TLR4 mRNA, daily for 3 consecutive days. On the fourth day, approximately 17 h after the last intrathecal administration of ODNs, PGE_2 (100 ng/ 5 μL, i.d.) was injected intradermally, followed 10 min later by HMWH (1 μg/ 5 μL, i.d.), at the same site. Mechanical nociceptive threshold was evaluated 40 min after PGE_2. Percent reduction from baseline (*top panel*): The anti-hyperalgesic effect of HMWH on PGE_2-induced hyperalgesia was not affected by TLR4 ODN antisense in gonad-intact and 17-β estradiol-replaced ovariectomized female rats (gonad intact groups, $t_{(10)}$= 0.1773, p=0.8628; ovariectomized + 17-β estradiol, $t_{(10)}$= 0.6999, p=0.4999, when the respective mismatch and antisense groups are compared 40 min after PGE_2; unpaired Student's t-test). However, ovariectomized female rats show an attenuation of HWMH-induced anti-hyperalgesia by intrathecal TLR4 ODN antisense (ovariectomized groups, $t_{(10)}$= 3.562, *p=0.0261; when the respective sense and antisense groups are compared 40 min after PGE_2; unpaired Student's t-test). Nociceptive threshold (*bottom panel*): TLR4 ODN antisense did not affect the anti-hyperalgesic effect of HMWH in gonad-intact and ovariectomized + 17-β estradiol females (gonad intact groups, $t_{(10)}$= 0.5633, p=0.5856; ovariectomized + 17-β estradiol, $t_{(10)}$= 0.8145, p=0.4343, when the respective mismatch and antisense groups are compared 40 min after PGE_2; unpaired Student's t-test). However, in ovariectomized female rats the intrathecal treatment with TLR4 ODN antisense attenuates HWMH-induced anti-hyperalgesia (ovariectomized group, $t_{(10)}$= 2.412, *p=0.0366; when mismatch and antisense groups are compared 40 min after PGE_2; unpaired Student's t-test). n=6 per group.

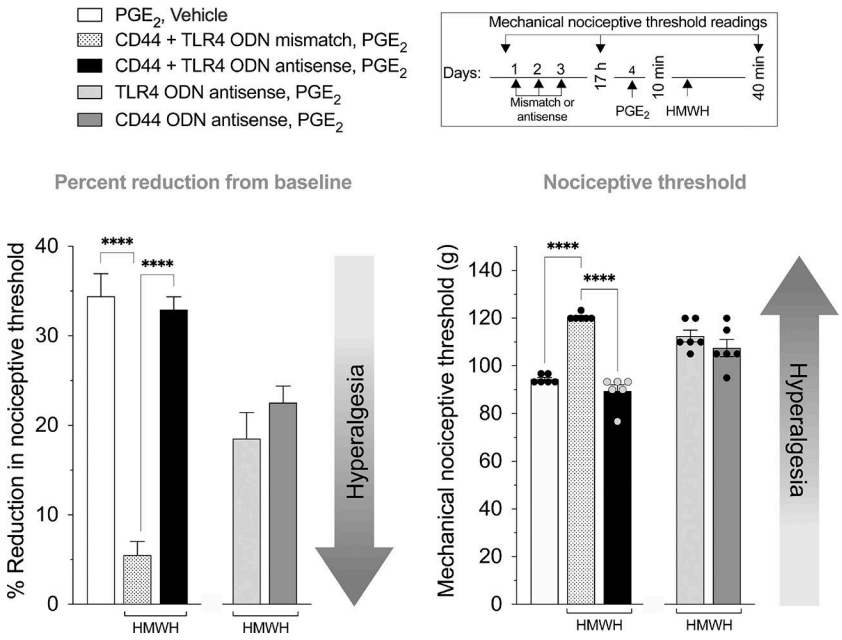

Figure 5. Reversal of PGE_2 hyperalgesia by HMWH is markedly attenuated by the combination of CD44 and TLR4 antisense in male rats.

Male rats were injected with the combination of ODN antisense or mismatch (120 μg/20 μl/each, i.t.) for TLR4 and CD44 mRNA, daily for 3 consecutive days. On the fourth day, approximately 17 h after the last intrathecal administration of ODN, PGE_2 (100 ng/5 μL, i.d.) was injected intradermally, followed 10 min later by HMWH (1 μg/ 5 μL, i.d.) at the same site. Mechanical nociceptive threshold was evaluated 40 min after intradermal PGE_2. Percent reduction from baseline (*left panel*): Intradermal HMWH attenuates PGE_2-induced hyperalgesia ($F_{(2,15)}$= 74.12, ****p<0.0001, when the PGE_2- and PGE_2 + HMWH-treated groups were compared 40 min after PGE_2; one-way ANOVA followed by Bonferroni's *post hoc* comparisons test). Intrathecal treatment with the combination of CD44 + TLR4 ODN antisense reverses the HMWH-induced anti-hyperalgesia (****p<0.0001, when HMWH-induced anti-hyperalgesia was compared between CD44 + TLR4 mismatch- and CD44 + TLR4 antisense-treated groups at 40 min after PGE_2; one-way ANOVA followed by Bonferroni's *post hoc* comparisons test). Nociceptive threshold (*rigth panel*): PGE_2-induced hyperalgesia was attenuated by HMWH ($F_{(2,15)}$= 108.3, ****p<0.0001, when the PGE_2- and PGE_2 + HMWH-treated groups were compared 40 min after PGE_2; one-way ANOVA followed by Bonferroni's *post hoc* comparisons test). The combination of CD44 + TLR4 ODN antisense reverses the HMWH-induced anti-hyperalgesia (****p<0.0001, when HMWH-induced anti-hyperalgesia was compared between CD44 + TLR4 mismatch- and CD44 + TLR4 antisense-treated groups at 40 min after PGE_2; one-way ANOVA followed by Bonferroni's post-hoc comparisons test). n=6 per group.