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# Regulation of expression of hyperalgesic priming by estrogen receptor alpha in the rat

**Luiz F. Ferrari**,

Departments of Medicine and Oral Surgery, and Division of Neuroscience, University of California at San Francisco, 513 Parnassus Avenue, San Francisco, CA 94143, USA

**Dionéia Araldi**, and

Departments of Medicine and Oral Surgery, and Division of Neuroscience, University of California at San Francisco, 513 Parnassus Avenue, San Francisco, CA 94143, USA

**Jon D. Levine***

Departments of Medicine and Oral Surgery, and Division of Neuroscience, University of California at San Francisco, 513 Parnassus Avenue, San Francisco, CA 94143, USA

## Abstract

Hyperalgesic priming, a sexually dimorphic model of transition to chronic pain, is expressed as prolongation of prostaglandin $E_2$ ($PGE_2$)-induced hyperalgesia by the activation of an additional pathway including an autocrine mechanism at the plasma membrane. The autocrine mechanism involves the transport of cAMP to the extracellular space, and its conversion to AMP and adenosine, by ecto-5′phosphodiesterase and ecto-5′nucleotidase, respectively. The end product, adenosine, activates A1 receptors, producing delayed onset prolongation of $PGE_2$ hyperalgesia. We tested the hypothesis that the previously reported, estrogen-dependent, sexual dimorphism observed in the induction of priming is present in the mechanisms involved in its expression, as a regulatory effect on ecto-5′nucleotidase by estrogen receptor alpha (EsRα), in female rats. In the primed paw AMP hyperalgesia was dependent on conversion to adenosine, being prevented by ecto-5′nucleotidase inhibitor AMPCP and A1 receptor antagonist DPCPX. To investigate an interaction between EsRα and ecto-5′nucleotidase, we treated primed female rats with ODN antisense or mismatch against EsRα mRNA. While in rats treated with antisense AMP-induced hyperalgesia was abolished, the A1 receptor agonist $N^6$-cyclopentiladenosine (CPA) still produced hyperalgesia. Thus, EsRα interacts with this autocrine pathway at the level of ecto-5′nucleotidase. These results demonstrate a sexually dimorphic mechanism for the expression of priming.

**Perspective—**This study presents evidence of an estrogen-dependent mechanism of expression of chronic pain in females, supporting the suggestion that differential targets must be considered when establishing protocols for the treatment of painful conditions in males and females.

*Corresponding author: Jon D. Levine, M.D., Ph.D., University of California, San Francisco, 513 Parnassus Avenue, San Francisco, CA 94143-0440, Phone: 415-476-4902, Fax: 476-6305, Jon.Levine@ucsf.edu.

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**Keywords**

Nociceptor; hyperalgesic priming; chronic pain; ecto-5′nucleotidase; estrogen receptor

## Introduction

One of the consequences of neuroplasticity is abnormal neuronal response to stimulation. In the experimental setting, persistent altered response to the application of inflammatory mediators to the peripheral terminal of nociceptors, producing increased and prolonged mechanical hyperalgesia, is considered an indication of such neuroplasticity [3, 31, 46]. In a preclinical model of transition to chronic pain, hyperalgesic priming [3, 46], plastic changes in nociceptors, produced by a previous inflammatory insult [5, 16, 17, 19] is expressed as prolongation of the response to hyperalgesic mediators, prototypically prostaglandin $E_2$ ($PGE_2$) [3, 18, 42]. Injection of $PGE_2$ in normal skin produces mechanical hyperalgesia that is dependent on a stimulatory G-protein coupled receptor (Gs) and protein kinase A (PKA), which lasts ~2 h [2]. However, in paws that have been previously submitted to a priming stimulus, i.e., mediators that signal through protein kinase C epsilon (PKCε) [3, 33, 43], the effect of $PGE_2$ lasts more than 4 h. This prolongation of $PGE_2$-induced hyperalgesia results from the activation of an additional, inhibitory Gi GPCR- and PKCε-dependent signaling pathway [22, 32, 42]. This pathway includes an autocrine mechanism, in which cAMP, produced by $PGE_2$-induced activation of adenyl cyclase [21], is transported to the extracellular compartment and converted, in sequence, to adenosine monophosphate (AMP) and adenosine, by the enzymes ecto-5′phosphodiesterase and ecto-5′nucleotidase, respectively [13, 25, 27]. The end product of ecto-5′nucleotidase, adenosine, in turn, activates A1 adenosine receptors to stimulate PKCε [21]. In this context, the activation of the A1 adenosine receptor by the agonist $N^6$-cyclopentyladenosine (CPA), which does not induce changes in nociceptive threshold in the normal nociceptor, produces mechanical hyperalgesia [21]. The presence of this autocrine mechanism in the nociceptor and the consequent increased response to pro-hyperalgesic mediators ($PGE_2$, adenosine, 5-hydroxytryptamine [3]) is an indication of the established nociceptor plasticity, which has been associated to several conditions in which long-term (chronic) pain is observed [36, 37, 39, 46].

Recently, it has been shown that estrogen, by acting at its cognate receptors, alpha (EsRα) and beta (EsRβ), regulates the activity of ecto-5′nucleotidase in hippocampal neurons of female rats [40]. This enzyme hydrolyzes AMP to adenosine [4, 61, 62], which, in turn, modulates hippocampal function [54] affecting, among other processes, synaptic plasticity, learning and memory [11, 49]. Since AMP and ecto-5′nucleotidase participate in the expression of hyperalgesic priming, as part of an autocrine mechanism [21], and, as previously shown EsRα plays a prominent role in the sexual dimorphism observed in hyperalgesic priming [20], in this study we investigated if EsRα also interacts with the ecto-5′nucleotidase, as in hippocampal neurons, regulating the conversion of AMP to adenosine, in primed nociceptors.

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## Methods

### Experimental Animals

Experiments were performed on adult male and female Sprague Dawley rats (220–250 g; Charles River, Hollister, CA, USA). Animals were housed three per cage, under a 12 h light/dark cycle, in a temperature- and humidity-controlled environment. Food and water were available *ad libitum*. All nociceptive testing was done between 10:00 A.M. and 4:00 P.M. All experimental protocols were approved by the University of California, San Francisco Committee on Animal Research and conformed to National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals*. All efforts were made to minimize the number of animals used and their suffering.

### Nociceptive threshold testing

Mechanical nociceptive paw withdrawal threshold was measured using an Ugo Basile Algesymeter, as previously described [45, 57]. Briefly, rats were placed in a cylindrical acrylic restrainer with holes through which the hind legs were free to extend. Four measures of nociceptive thresholds were taken at 5 min intervals, and the mean of the last three measures defined as the mechanical nociceptive threshold. The effect of hyperalgesic agents is expressed as percentage decrease in nociceptive paw-withdrawal threshold compared with the control paw-withdrawal threshold obtained before drug administration.

### Drugs and reagents

The drugs used in this study were: adenosine 5′-monophosphate disodium salt (AMP); the A1 adenosine receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX); the A1 adenosine receptor agonist $N^6$-cyclopentyladenosine (CPA) and the ryanodine receptor modulator ryanodine, all from Sigma-Aldrich (St. Louis, MO); and, the ecto-5′-nucleotidase inhibitor α,β-methyleneadenosine 5′-diphosphate sodium salt (AMPCP) (Santa Cruz Biotechnology, Santa Cruz, CA). The selection of drug doses was based on our previous studies [20, 21]. The required drug concentrations were achieved by dilutions in 0.9% NaCl. Ryanodine, used to induce priming [17, 20, 22], was first prepared as a stock solution, in absolute ethanol, and then diluted with 0.9% NaCl to the required concentration/dose. AMPCP and CPA were dissolved in distilled water. DPCPX was dissolved in DMSO and at the time of the experiments further diluted in 0.9% NaCl containing 10% DMSO. Importantly, control experiments have previously shown that the final concentration of ethanol (2%), used to prepare the solutions of ryanodine, had no effect on the mechanical threshold *per se*; DMSO, used to dissolve DPCPX, also had no effect on the mechanical threshold [20].

All drugs were administered intradermally on the dorsum of the hind paw via a beveled 30-gauge hypodermic needle that was attached to a Hamilton® microsyringe (Reno, NV) by polyethylene (PE-10) tubing. The administration of ryanodine was preceded by hypotonic shock to increase cell membrane permeability (2 μl of distilled water, separated from the drug, by an air bubble, to avoid mixing in the same syringe), facilitating its entrance into the nerve terminal [7, 9].

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### Induction of hyperalgesic priming

Hyperalgesic priming was induced as described previously [3, 19, 20]. Ryanodine was injected intradermally on the dorsum of the hind paw, at the site of nociceptive testing [18, 20]. The presence of priming was confirmed, 1 week later, by the injection of CPA or AMP (both 1 μg), at the same site. At this time, the mechanical nociceptive threshold was not different from the pre-ryanodine baseline (see *Statistics* below). The presence of hyperalgesia after injection of AMP or CPA, which in the naïve control paw (non-primed) does not induce change in the mechanical nociceptive threshold, was used as a marker for the presence of hyperalgesic priming (Fig. 1 and [21]).

### Oligodeoxynucleotides antisense and mismatch against estrogen receptor alpha (EsRα) mRNA

To investigate the role of EsRα in the expression of hyperalgesic priming, antisense oligodeoxynucleotide (ODN) against EsRα mRNA was administered to both female and male rats. As described previously [1], rats were anesthetized with isoflurane (2.5% in $O_2$), and the ODN injected using a microsyringe (20 μl) with a 30-gauge needle, inserted into the subarachnoid space, between the $L_4$ and $L_5$ vertebrae. The sequence for the EsRα, 5′-CAT-GGT-CAT-GGT-CAG-3′, antisense ODN (Invitrogen Life Technologies) was directed against a unique region of the rat EsRα (GeneBank accession number NM_012689.1), and has been previously shown to attenuate cellular levels of this receptor [14, 35]. The mismatch ODN sequence, 5′-ATC-GTG-GAT-CGT-GAC-3′, was a scrambled antisense ODN sequence that has the same base pairs and GC ratio, with the order randomized, and little or no homology to any mRNA sequence posted at GenBank.

Before use, ODNs were reconstituted in nuclease-free 0.9% NaCl, and then administered intrathecally at a dose of 6 μg/μl in a volume of 20 μl, daily for 3 consecutive days, starting 4 days after the injection of ryanodine, a time point at which priming is fully established [5, 19, 20]. Tests with AMP or CPA, injected intradermally on the dorsum of the hind paw, at the same site as ryanodine, were performed on the day following the 3rd injection of antisense or mismatch ODN (1 week after injection of ryanodine).

### Statistics

In all experiments the dependent variable was change in mechanical paw-withdrawal threshold, expressed as percentage change from baseline. 18 male and 24 female rats were used in this study. No significant difference in mechanical nociceptive thresholds was observed before the injection of the priming stimulus (ryanodine) and immediately before injection of the test agents, AMP or CPA (Males, average mechanical nociceptive threshold: before priming stimuli, 128.1 ± 2.5 g; before AMP injection, 128.1 ± 2.3 g; paired Student's *t*-test, $t_{35} = 0.05882$, $p = 0.9534$, N = 36 paws; Females, average mechanical nociceptive threshold: before priming stimuli, 129.4 ± 1.5 g; before AMP or CPA injection, 127.1 ± 1.3 g; paired Student's *t*-test, $t_{47} = 2.033$, $p = 0.0678$, N = 48 paws). Of note, in the experiments shown in Figs. 1 and 2, rats did not receive identical treatments in both paws. In Fig. 1, only the right paws received $IP_3$, while the left paws received vehicle, thus only the right paws were primed. In Fig. 2, although both paws received $IP_3$, the left paws received DPCPX + AMP, and the right paws received AMPCP + AMP. Of note, in the experiments shown in

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Figs. 3, both paws received the same treatments; AMP or CPA was injected in both paws of rats pretreated with intrathecal injections of ODN antisense or mismatch, which affects nociceptors on both sides of the experimental animal. That said, the paws of the same animal were considered as independent, as previously demonstrated, in regard to the treatments performed on the dorsum of the hind paw, in a volume of 5 μl, in the doses used in our experiments and previous work from our group [19]. As specified in the figure legends, Student's *t*-test or repeated-measures analysis of variance (ANOVA), followed by Bonferroni *post-hoc* test, was performed to compare the magnitude of the hyperalgesia induced by the injection of AMP or CPA (evaluated 10, 20, or 30 min after injection) in groups submitted to different treatments, with the control groups. All data are presented as mean ± standard error of the mean (SEM) of N independent observations. Statistical comparisons were made using GraphPad Prism 6.0 statistical software (GraphPad Software, Inc., La Jolla, CA). A $p$-value $<0.05$ was considered statistically significant.

## Results

### AMP induces hyperalgesia in primed rats

The autocrine mechanism of hyperalgesic priming expression involves the conversion of the cAMP transported to the extracellular space to AMP and adenosine, by two enzymes, ecto-5′phosphodiesterase and ecto-5′nucleotidase, respectively [21]. Adenosine activates the Gi-coupled receptor A1 adenosine, triggering PKCε-dependent signaling pathway [15, 58], which is responsible for the prolongation of $PGE_2$ hyperalgesia [3, 42, 44, 46]. Of note, the direct activation of the A1 adenosine receptor by the agonist CPA, which does not affect nociceptive threshold in the normal paw, induces hyperalgesia after priming [21, 22]. Since adenosine is a product of the conversion of AMP by the ecto-5′nucleotidase [61, 62], we evaluated if the administration of AMP in primed paws would have similar effect to that produced by the activator of the A1 adenosine receptor, i.e., induce mechanical hyperalgesia [21]. The intradermal injection of AMP (1 μg) on the dorsum of the paw in naïve male or female rats did not produce significant change in the mechanical paw withdraw threshold (Fig. 1, open symbols). However, when injected in paws that have been previously primed by intradermal injection of ryanodine (1 pg in females and 100 ng in males, injected at the same site where AMP was injected 1 week later), AMP produced significant mechanical hyperalgesia (Fig. 1, filled symbols).

### Ecto-5′nucleotidase and A1 adenosine receptor role in AMP-induced hyperalgesia

The prolongation of hyperalgesia induced by $PGE_2$ results from the serial metabolism of cAMP by the ectoenzymes expressed at the extracellular side of the nociceptor plasma membrane, and subsequent activation of the A1 adenosine receptor [21]. To confirm that the hyperalgesia induced by AMP in primed paws was the consequence of its conversion to adenosine, we treated male and female rats that had been primed with ryanodine, one week before, with the ecto-5′nucleotidase inhibitor AMPCP (1 μg), or the selective A1 receptor antagonist DPCPX (1 μg), injecting, 10 min later, AMP (1 μg) at the same site. Both AMPCP and DPCPX inhibited AMP-induced hyperalgesia, indicating that AMP is converted to adenosine, to induce hyperalgesia in primed rats by activation of A1 receptors (Fig. 2).

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### EsRα regulates the autocrine mechanism in female rats

Recent studies have shown, in female rats, that estrogen interacts with nucleotidases, such as the ecto-5′nucleotidase, modulating the conversion of AMP to adenosine in hippocampal neurons [40]. This hormone also plays a role in the sex differences observed in hyperalgesic priming, through its action at EsRα, regulating the sensitivity of female rats to develop priming in response to ryanodine [20]. We evaluated if this receptor also interacts with the ecto-5′nucleotidase, modulating the conversion of AMP to adenosine, in primed nociceptors. Male and female rats that had been primed with ryanodine, 1 week prior, were treated with ODN antisense or mismatch against EsRα for 3 consecutive days. On the 4$^{th}$ day, AMP (1 μg) was injected at the same site. In the rats treated with EsRα antisense the hyperalgesia induced by AMP was significantly attenuated, only in females (Fig. 3A), indicating a participation of this receptor in the expression of priming in females. When a different group of primed female rats that had been submitted to the same ODN protocol received an injection of the A1 receptor agonist CPA (1 μg) instead of AMP, no difference in the CPA-induced hyperalgesia was observed between the antisense and mismatch groups (Fig. 3B). This result is compatible with the hypothesis that EsRα, in female rats, interacts with the autocrine mechanism for the expression of hyperalgesic priming, at the level of the conversion of AMP to adenosine.

## Discussion

We recently demonstrated that the increased responsivity to $PGE_2$, expressed as prolonged mechanical hyperalgesia, in the primed nociceptor involves an autocrine mechanism, following activation of prostaglandin receptors on the peripheral terminal of the primary afferent nociceptor, by adding a Gi-dependent component (the subsequential activation of an A1 adenosine receptor-Gi-PKCε-dependent signaling pathway [21]) to the classical Gs-adenyl cyclase production of cAMP-PKA signaling [12, 32]. The sequence of events in the autocrine pathway, i.e., transport of cAMP to the extracellular space, its conversion to AMP and adenosine by nucleotidases, and activation of the A1 adenosine receptor, is part of the mechanism by which the changes in the nociceptor are manifested in response to $PGE_2$, and is not present in the naïve state. In this context, the direct activation of the A1 adenosine receptor by its agonist, CPA, in the primed nociceptor, in contrast to the normal state, induces mechanical hyperalgesia [21]. Also, the direct injection of AMP in the primed paw induced hyperalgesia (Fig. 1), which was prevented by the ecto-5′nucleotidase inhibitor AMPCP, or the A1 receptor antagonist DPCPX (Fig. 2). These results confirm that, in the primed paw, AMP-induced hyperalgesia depends on its conversion to adenosine, which activates A1 adenosine receptors.

The enzyme ecto-5′nucleotidase has been shown to participate in different models of neural plasticity [61, 62], by regulating the synthesis of adenosine and, consequently, modulation of neuronal function by adenosine [11, 49, 54]. Of note, several studies have demonstrated that, in the central nervous system, activity of ecto-5′nucleotidase is significantly affected by estrogen [34, 40, 41, 47]. Thus, considering our previous reports showing that both ecto-5′nucleotidase [21] and estrogen [20, 29] participate in mechanisms underlying hyperalgesic priming, we investigated if estrogen interacts with ecto-5′nucleotidase in the primed

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nociceptor. Also, as estrogen is a modulator of the sexual dimorphism in priming [20, 29], our experiments evaluated the interaction of ecto-5′nucleotidase and estrogen in both sexes. When the expression of EsRα was downregulated by ODN antisense to EsRα mRNA, the induction of hyperalgesia by AMP was attenuated, but only in female rats (Fig. 3A), demonstrating its dependence on estrogen. The lack of effect of the ODN treatment on the hyperalgesia induced by CPA (Fig. 3B) confirmed that estrogen, through EsRα, regulates the hyperalgesia induced by AMP at the level of its conversion to adenosine, by impacting ecto-5′nucleotidase. Together, these results show that, in females, without the regulation by estrogen, the activation of ecto-5′nucleotidase is impaired and, the conversion of AMP to adenosine, interrupted.

An interesting point shown by the current study is that estrogen, previously demonstrated to play a role in the inhibition of the *induction* of hyperalgesic priming in females [20], also participates in its *expression*. This is in line with the extensively described role of gonadal steroids, and their respective receptors, in the regulation of sex differences in models of neuroplasticity [23, 24, 48, 53]. In this regard, we have recently demonstrated the specific role of EsRα in the susceptibility of female rats to be primed by ryanodine [20], by increasing sensitivity to ryanodine. In that study we showed that a much smaller dose of ryanodine is able to induce priming in females, indicating the contribution of EsRα in the induction phase in our model of neuroplasticity, hyperalgesic priming. Thus, although it has been shown that both subtypes of estrogen receptor (α and β) are involved in the regulation of the ecto-5′nucleotidase in female neurons [40], our previous observations indicated the marked contribution of the EsRα in the induction of priming [20]. Still, whether other estrogen receptors play a role in this model of the transition to chronic pain remains to be determined.

Another point to be considered in the regulatory role of estrogen in neuroplasticity is its fluctuating levels during the reproductive cycle [30]. Indeed, as the presence of estrogen increases the activity of ecto-5′nucleotidase [40], changes in this interaction over the cycle might affect the levels of extracellular AMP and adenosine, as observed in hippocampal neurons [40]. Our experiments suggest that, in females, the ecto-5′nucleotidase that converts AMP to adenosine, allowing priming to be expressed, is positively regulated by circulating estrogen acting on EsRα on the nociceptor. Hence, it is possible that, depending on the phase of the reproductive cycle, the expression of priming is differentially impacted. Our current experiments do not, however, allow us to answer this question.

In this study we present evidence for a role of estrogen in the expression of hyperalgesic priming in female rats, regulating its autocrine signaling pathway through an interaction with the enzyme ecto-5′nucleotidase, as summarized in Fig. 4. Our results also suggest that, in certain conditions, considering a possible influence of the fluctuating levels of estrogen - or other factors that impact the expression of chronic pain - the absence of symptoms does not mean lack of plastic changes in nociceptors, which might affect the identification of painful syndromes in females. Also, in terms of sexual dimorphism and chronic pain mechanisms, our results contribute to the suggestion made by previous reports [6, 8, 26, 38, 51, 52] that differential targets must be considered when establishing protocols for the treatment of painful conditions in males and females.

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## Abbreviations

| | |
|---|---|
| **$PGE_2$** | prostaglandin $E_2$ |
| **Gs** | stimulatory G-protein coupled receptor |
| **PKA** | protein kinase A |
| **Gi** | inhibitory GPCR |
| **PKCε** | protein kinase C epsilon |
| **cAMP** | cyclic adenosine monophosphate |
| **AMP** | adenosine monophosphate |
| **EsRα** | estrogen receptor alpha |
| **ODN** | oligodeoxynucleotide |

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## Highlights

- Hyperalgesic priming is a sexually dimorphic model of transition to chronic pain;
- Priming involves activation of an autocrine mechanism at the nociceptor membrane;
- In females, this mechanism is regulated by estrogen acting at EsRα;
- EsRα modulates AMP conversion to adenosine by interacting with ecto-5′nucleotidase.

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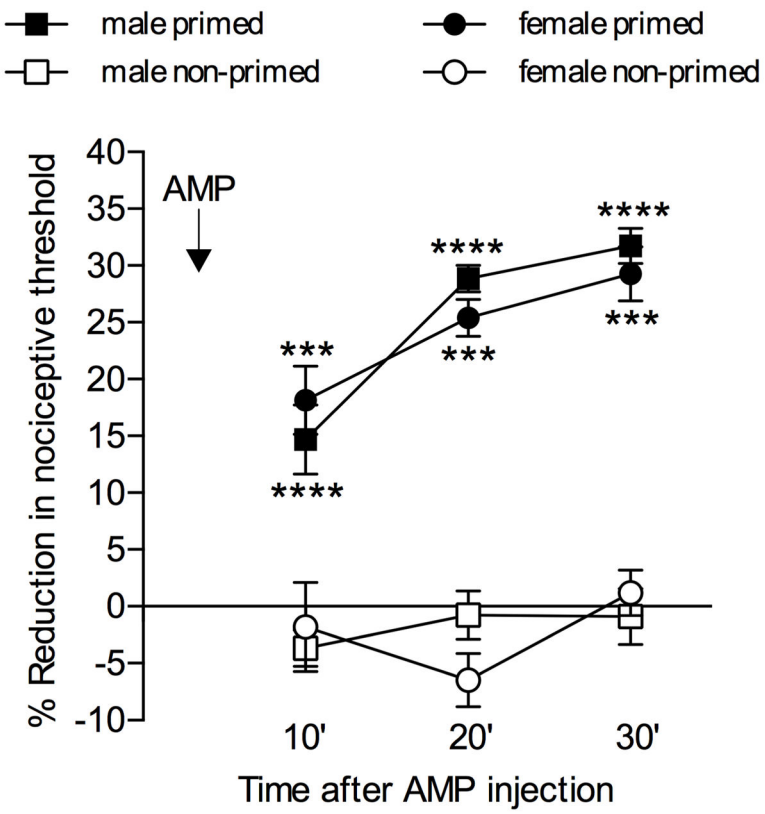


**Fig. 1. AMP induces mechanical hyperalgesia in primed male and female rats**

Male and female rats were divided in two groups, primed (filled symbols) and non-primed (open symbols). The priming inducer, ryanodine (100 ng in males and 1 pg in females), or its vehicle (saline) were injected on the dorsum of the right or left hind paw, respectively. One week later, AMP (1 μg) was injected at the same site. Mechanical nociceptive thresholds were then evaluated 10, 20 and 30 min after AMP injection. We observed that, in non-primed paws, AMP did not induce significant change in the mechanical nociceptive threshold, whereas in primed paws, hyperalgesia was observed at all time points in both male and female rats. Two-way repeated measures ANOVA followed by the Bonferroni *post-hoc* test showed significant difference between the effect of AMP in primed and non-primed paws in both male and female groups (males: $F_{3,30} = 18.18$, ****$p < 0.0001$; females: $F_{3,30} = 8.157$, ***$p = 0.0004$, when the primed male or female groups are compared to their respective non-primed groups). (N = 6 paws per group)

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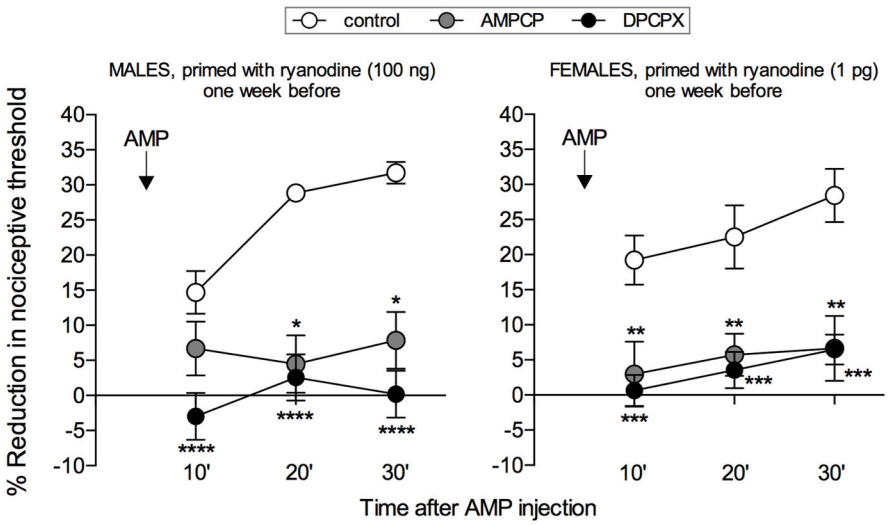


**Fig. 2. AMP-induced mechanical hyperalgesia in primed male and female rats is dependent on its conversion to adenosine and activation of A1 adenosine receptors**

Male (left panel) and female (right panel) rats received an intradermal injection of ryanodine (100 ng in males and 1 pg in females) on the dorsum of both hind paws. One week later, the ecto-5′nucleotidase inhibitor AMPCP (1 μg, gray symbols) was injected in the left paws, and the A1 adenosine receptor antagonist DPCPX (1 μg, black symbols) was injected in the right paws, at the same site as ryanodine. The control groups, represented by the white symbols, are the same primed groups shown in Fig. 1. After 10 min, AMP (1 μg) was injected in all paws and the mechanical nociceptive threshold evaluated, 10, 20 and 30 min later. While in primed paws AMP induced significant hyperalgesia (control groups), in the groups pretreated with AMPCP or DPCPX the AMP-induced hyperalgesia was markedly attenuated (males: AMPCP, $F_{1,10} = 32.22$, $^{*}p = 0.0002$; DPCPX, $F_{1,10} = 65.62$, $^{****}p < 0.0001$; females: AMPCP, $F_{1,10} = 14.96$, $^{**}p = 0.0031$; DPCPX, $F_{1,10} = 27.07$, $^{***}p = 0.0004$, when the inhibitors groups are compared to the control groups, two-way repeated measures ANOVA followed by the Bonferroni *post-hoc* test) demonstrating that the mechanical hyperalgesia induced by AMP in primed rats is dependent on its conversion to adenosine. (N = 6 paws per group)

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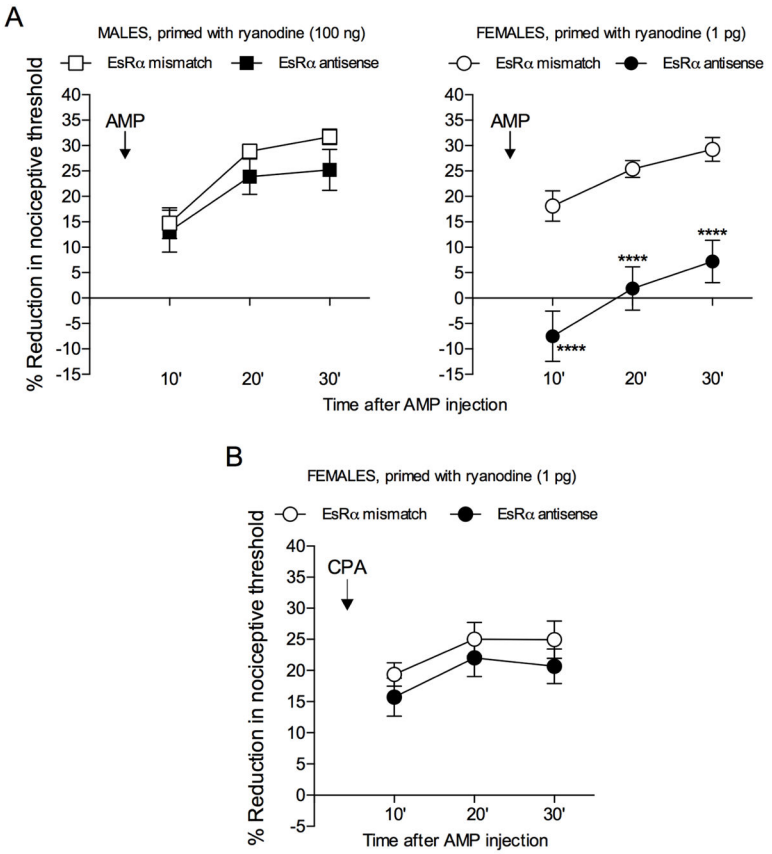


**Fig. 3. AMP-, but not CPA-, induced mechanical hyperalgesia in female primed rats is dependent on estrogen receptor alpha (EsRα)**

**A**: Male (left panel) and female (right panel) rats received an intradermal injection of ryanodine (100 ng in males and 1 pg in females) on the dorsum of both hind paws. One week later, intrathecal treatment with ODN antisense or mismatch against EsRα mRNA was performed for 3 consecutive days. On the 4$^{th}$ day, AMP (1 μg) was injected at the same site as ryanodine, and mechanical nociceptive threshold evaluated 10, 20 and 30 min later. While AMP induced significant hyperalgesia in both male and female rats treated with mismatch and in the male antisense group, in the group of females that had been treated with EsRα antisense, AMP-induced hyperalgesia was prevented [males: $F_{1,10} = 1.179$, $p = 0.3030$ (non-significant); females: $F_{1,10} = 52.35$, ****$p < 0.0001$, when the antisense and mismatch groups are compared, two-way repeated measures ANOVA followed by the Bonferroni *post-hoc* test] indicating a dependence on EsRα for the hyperalgesic effect of AMP in female, but not in male, primed rats; **B**: Female rats that had been primed with an intradermal injection of ryanodine (1 pg) on the dorsum of both hind paws, were treated, one week later, with intrathecal injections of ODN antisense or mismatch against EsRα mRNA for 3 consecutive days. On the 4$^{th}$ day, CPA (1 μg) was injected at the same site as ryanodine, and mechanical nociceptive thresholds were then evaluated 10, 20 and 30 min later. No difference in the hyperalgesia induced by CPA was observed between the antisense and mismatch groups [$F_{1,10} = 1.700$, $p = 0.2215$ (non-significant), when the antisense and mismatch groups are

compared, two-way repeated measures ANOVA followed by the Bonferroni *post-hoc* test]. (N = 6 paws per group)

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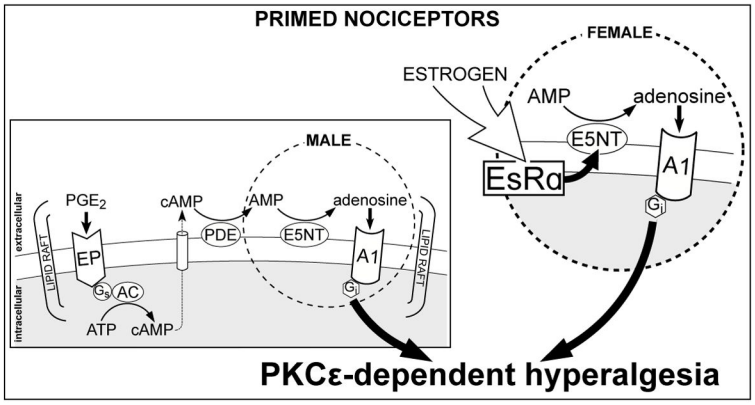


**Fig. 4. Autocrine mechanism of hyperalgesic priming expression in male and female nociceptors**

In the primed nociceptor, neuroplasticity manifests as a PKCε-dependent mechanical hyperalgesia [3, 18, 42, 46]. While in the normal nociceptor $PGE_2$ induces hyperalgesia by activating PKA, which lasts ~2h [2], in the primed nociceptor an autocrine signaling pathway at the plasma membrane [21] is also triggered, culminating in the activation of PKCε, prolonging the $PGE_2$-induced hyperalgesia to more than 4 h [3, 42]. The activation of this additional pathway involves the transport of cAMP, produced by adenyl cyclase (AC) stimulation, across the plasma membrane into the extracellular compartment. In sequence, cAMP is metabolized to AMP and then adenosine, by ecto-phosphodiesterase (PDE) and ecto-5′nucleotidase (E5NT), respectively, ultimately activating Gi-coupled A1 adenosine receptor (A1). This stimulates PKCε, responsible for the late component of $PGE_2$-induced hyperalgesia in the hyperalgesic priming. While this autocrine mechanism is present in both males and females, in females (bigger circle) it is regulated by estrogen, which acts at the estrogen receptor alpha (EsRα), regulating either the expression or the activity, or both, of the E5NT, thus modulating the conversion of AMP to adenosine, and the downstream signaling that will produce PKCε-dependent hyperalgesia. The regulation of E5NT by EsRα has been suggested to involve either a genomic mechanism, regulating the expression of this enzyme, or the interaction between EsRα and E5NT, directly or through second messengers. In the first case, transcription factors such as AP-1 and Sp1, which are active at the E5NT gene promoter [10, 56], are regulated by activation of EsRα [28, 59, 60]. Of note, this interaction has been observed in hippocampal neurons, and associated to differences in neuroplasticity between the sexes [40]. The second possibility would be the direct interaction of the receptor with the enzyme, supported by studies demonstrating co-immunoprecipitation of estrogen receptors and E5NT, suggesting that these elements can interact through physical association [40]. In addition, activation of messengers/kinases that increase either the expression or the activity of the E5NT, such as PKC, has also been reported [50, 55, 56]. Hence, even though these signaling pathways have not been demonstrated in nociceptors, it is plausible that they are involved in the regulation of processes in which E5NT plays a role, therefore explaining why the knock down of EsRα in the nociceptor impairs the expression of priming in females.