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Age-dependent Sexual Dimorphism in Susceptibility to Develop Chronic Pain in the Rat

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Abstract

Neonatal pain has been suggested to contribute to the development and/or persistence of adult pain. Observations from animal models have shown that neonatal inflammation produces long-term changes in sensory neuron function, which can affect the susceptibility of adults to develop persistent pain. We used a preclinical model of transition to chronic pain, hyperalgesic priming, in which a previous inflammatory stimulus triggers a long-lasting increase in responsiveness to pro-algesic mediators, prototypically prostaglandin E_2 (PGE_2), to investigate if post-natal age influences susceptibility of adult rats to develop chronic pain. Priming was induced by tumor necrosis factor alpha (TNFα), in male and female rats, 1, 2, 3, 4, 5 or 7 weeks after birth. When adults (8 weeks after birth), to evaluate for the presence of priming, PGE_2 was injected at the same site as TNFα. In males that had received TNFα at post-natal weeks 1, 2 or 3, priming was attenuated compared to the 4-, 5- and 7-week-old treated groups, in which robust priming developed. In contrast, in females treated with TNFα at post-natal week 1, 2, 3, or 4, but not at 5 or 7, priming was present. This age and sex difference in the susceptibility to priming was

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Author's contribution

LFF: designed research and performed experiments, analyzed the data and wrote the manuscript; **DA:** performed experiments and wrote the manuscript; **PG:** performed experiments and wrote the manuscript; **JDL:** designed research, wrote the manuscript. All authors read and approved the final version of the manuscript.

estrogen-dependent, since injection of TNFα in 3-week-old males and 5-week-old females, in the presence of the estrogen receptor antagonist ICI 182,780, did produce priming. These results suggest that estrogen levels, which vary differently in males and females over the post-natal period, until they stabilize after puberty, impact pain as an adult.

Keywords

Hyperalgesic priming; hyperalgesia; nociceptor; estrogen; chronic pain; rat

Introduction

Persistent abnormal nociceptive processing in the adult has been suggested to be, at least in part, a consequence of pain experienced during the neonatal period. Thus, inflammation in neonates has been demonstrated to produce long-term changes in sensory neuron function in rats and mice (Lidow et al., 2001; Hohmann et al., 2005; Schwaller and Fitzgerald, 2014; Weyer et al., 2016), due to the neuronal plasticity in the neonatal period (Shortland and Fitzgerald, 1994; Reynolds and Fitzgerald, 1995; Torsney and Fitzgerald, 2002; Walker et al., 2003), that might permanently affect the development and maturation of nociceptive circuitry (Beland and Fitzgerald, 2001; Brummelte et al., 2012; Schwaller and Fitzgerald, 2014). As a consequence, painful insults at early stages of development can shape the future response to noxious stimulation in adults (Bhutta et al., 2001; Lidow et al., 2001; Lidow, 2002; Ren et al., 2004; Wang et al., 2004; Peters et al., 2005). Interestingly, neonatal injury affects males and females differently, with females showing an increased vulnerability to develop long-term changes in the sensory circuits due to early life insults (LaPrairie and Murphy, 2007). A prominent role of sex hormones in such differences has been observed (Aloisi, 2003), and changes in their circulating levels over the early stages of development (Weisz and Ward, 1980; Amateau et al., 2004; Balthazart and Ball, 2006; Cornil et al., 2006), especially estrogen, shown to have a modulatory and protective effect on the nervous system (Garcia-Segura et al., 2001; Maggi et al., 2004; Amantea et al., 2005), have been suggested to strongly impact the susceptibility of adults to develop chronic pain, as a consequence of early inflammation (LaPrairie and Murphy, 2007).

Hyperalgesic priming is a preclinical model of chronic pain in which an inflammatory insult induces long-lasting neuroplasticity in the primary afferent nociceptor (Aley et al., 2000; Parada et al., 2003a; Reichling and Levine, 2009) such that its subsequent response to pro-algesic mediators, prototypically prostaglandin E_2 (PGE_2), is markedly prolonged (Aley et al., 2000; Parada et al., 2005; Ferrari et al., 2014). While the mechanical hyperalgesia induced by injection of PGE_2 in a naïve control paw lasts ~2h, in the primed paw it is markedly prolonged, lasting more than 4h, due to protein kinase C epsilon (PKCε)-triggered changes in the nociceptor (Aley and Levine, 1999; Aley et al., 2000), which strongly and permanently affects the signaling pathways activated by pro-algesic stimulation (Parada et al., 2005; Khasar et al., 2008; Dina et al., 2009; Ferrari et al., 2013c; Ferrari and Levine, 2015). Hyperalgesic priming has been shown to be sexually dimorphic (Joseph et al., 2003; Ferrari et al., 2016; Ferrari et al., 2017; Khomula et al., 2017); in female rats, the PKCε-dependent induction of priming is negatively regulated by estrogen, such that gonad intact

adult females cannot be primed by direct or receptor-mediated activation of PKCε. However, in gonadectomized females, activation of PKCε induces priming (Joseph et al., 2003).

In this study, we demonstrate that post-natal developmental stage influences the susceptibility of rats to develop priming in a sexually dimorphic manner. Also, since sex hormones vary in level during post-natal development (Weisz and Ward, 1980; Amateau et al., 2004; Balthazart and Ball, 2006; Cornil et al., 2006), and priming is sexually dimorphic and estrogen-dependent (Joseph et al., 2003; Ferrari et al., 2016; Ferrari et al., 2017; Khomula et al., 2017), we evaluated the role of estrogen in such age dependent differences in the induction of priming at young ages in males and females.

Experimental Procedures

Animals

All experiments were performed on male and female Sprague Dawley rats aged 1 to 8 weeks (< 100 g – 300 g, Charles River Laboratories, Hollister, CA). Dams were housed with their litter in standard cages on post-natal days 0–21. At the end of the third post-natal week, rats were separated by sex and housed, 3 per cage, under a 12-hour light/dark cycle in a temperature- and humidity-controlled room in the Laboratory Animal Resource Center of the University of California, San Francisco. Food and water were available *ad libitum*. Nociceptive testing was performed between 10:00 am and 5:00 pm. 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. Every effort was made to minimize the number of animals used and their suffering.

Mechanical nociceptive threshold evaluation

Mechanical nociceptive threshold was quantified using an Ugo Basile Analgesymeter® (Randall-Selitto paw-withdrawal test; Stoelting, Chicago, IL), which applies a linearly increasing mechanical force to the dorsum of the rat's hind paw (Randall and Selitto, 1957; Taiwo and Levine, 1989; Taiwo et al., 1989). Nociceptive threshold was defined as the force in grams at which the rat withdrew its paw. Baseline paw-pressure threshold was defined as the mean of the 3 readings taken before a test agent was injected. Only one paw per rat was used. Each experiment was performed on a different group of rats. Data are presented as the mean change from baseline nociceptive threshold.

Drugs

The reagents used in this study were the direct-acting hyperalgesic inflammatory mediator prostaglandin E_2 (PGE_2), and rat recombinant tumor necrosis factor α (TNFα), both from Sigma-Aldrich (St. Louis, MO), and the non-selective estrogen receptor antagonist ICI 182,780 (ICI), from R&D Systems (Minneapolis, MN). Stock solutions of PGE_2, dissolved in absolute ethanol to a concentration of 1 μg/μl, were further diluted in 0.9% NaCl (1:50, final concentration 0.2 μg/μl) immediately before injection. The ethanol concentration of the final PGE_2 solution was ~2% and the injection volume 5 μl. TNFα was dissolved in sterile phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin, and diluted in

0.9% NaCl just prior to intradermal injections. Both PGE_2 and TNFα were injected intradermally on the dorsum of the hind paw using a beveled 30-gauge hypodermic needle attached to a microsyringe (Hamilton Company, Reno, NV) by a short length of polyethylene (PE-10) tubing. ICI 182,780, dissolved in sesame oil and diluted in saline at the time of the injections, was administered subcutaneously, on the back of the neck, for 8 consecutive days, in a dose of 10 μg/day, using a 27-gauge needle. Previous work (Branham et al., 1996) has shown a significant inhibition of estrogen receptors after daily injections of this dose for 4 consecutive days. In this study we extended this treatment to 8 days (3 additional days after TNFα) in order to maintain the inhibition of estrogen receptors during the time course for the development of priming after the injection of the inducer, as previously demonstrated (Bogen et al., 2012; Ferrari et al., 2015).

Hyperalgesic priming

To induce hyperalgesic priming TNFα was injected intradermally, at the site of nociceptive testing, on the dorsum of the hind paw (Aley et al., 2000; Parada et al., 2003b; Reichling and Levine, 2009) in rats of different ages (post-natal week 1, 2, 3, 4, 5 or 7). TNFα, in addition to inducing mechanical hyperalgesia that lasts ~24h (Parada et al., 2003a), induces priming (Parada et al., 2003a; Bogen et al., 2012), which is fully developed ~3 days after TNFα injection (Bogen et al., 2012). Importantly, once priming is induced, it is permanently maintained by ongoing protein translation at the terminal of the nociceptor (Ferrari et al., 2013a). To investigate if TNFα induces priming in young rats, when adults PGE_2 was injected at the same site. While in naïve, non-primed paws, PGE_2 produces hyperalgesia that lasts approximately 2h (Aley and Levine, 1999), in primed paws it induces markedly prolonged mechanical hyperalgesia that lasts more than 4h (Aley et al., 2000; Ferrari et al., 2013c; Ferrari et al., 2014; Ferrari and Levine, 2015).

Data analysis

In all experiments, the dependent variable was change in paw-withdrawal threshold, expressed as percentage change from baseline, evaluated before the injection of PGE_2. The average paw withdrawal threshold before the injection of PGE_2, at the 8^{th} post-natal week, was 135.6 ± 1.7 g, for the males, and 126.5 ± 1.4 g for the females (N = 42 male and 41 female paws). In Figure 1, the magnitude of the hyperalgesia induced by PGE_2 in the groups that received TNFα in different post-natal weeks was compared with the control group that had received TNFα 7 weeks after birth, by one-way ANOVA followed by Bonferroni *post-hoc* test. In Figure 2, in each panel, the group treated with ICI 182,780 was compared with groups shown in Figure 1 (that received TNFα alone at the 7^{th}, 3^{rd} (males) or 5^{th} (females) post-natal week), by Student's *t*-test. The experiments were performed blind, the experimenter did not know the treatments until after the tests. GraphPad Prism 5.0 (GraphPad Software, Inc, San Diego, CA) was used for the graphics and to perform statistical analyses; $p < 0.05$ was considered statistically significant. Data are presented as mean ± standard error of the mean.

Results

Hyperalgesic priming is age- and sex-dependent

To evaluate if an inflammatory insult administered at different ages impacts nociceptor function in adults, we used the model of the transition to chronic pain, hyperalgesic priming (Aley et al., 2000; Reichling and Levine, 2009). To produce priming, we injected TNFα (100 ng in 5 μl, (Parada et al., 2003a)), intradermally, on the dorsum of the hind paw of male and female rats 1, 2, 3, 4 or 5 weeks after birth, and, at 8 weeks PGE_2 (100 ng in 5 μl) was injected at the TNFα injection site, to evaluate for priming. As a control, TNFα was injected in 7-week-old (adult) male and female rats. When the control groups were evaluated 1 week later, the hyperalgesia induced by PGE_2 was still present at the 4^{th} h in the males, but not in the females, indicating the presence of priming only in males (Figure 1, black bars). This finding is in line with our previous observation showing that, while the activation of PKCε by stimuli such as TNFα (Parada et al., 2003a) in adult males produces priming (Parada et al., 2003a; Ferrari et al., 2015), in adult females priming cannot be induced through PKCε-dependent mechanisms (Joseph et al., 2003; Ferrari et al., 2013b). We have also found, in male rats treated with TNFα 1, 2 or 3 weeks after birth, that the magnitude of the PGE_2-induced hyperalgesia was smaller when compared to the groups treated with TNFα at 4 and 5 weeks of age, and the control group, indicating that the induction of priming was attenuated when the priming stimulus was administered 1, 2, or 3 weeks postnatally (Figure 1, left panel). In contrast, in females in which TNFα was injected at post-natal weeks 1, 2, 3, or 4, but not in week 5 or adults (Figure 1, right panel), the hyperalgesia induced by PGE_2 was still present at the 4^{th} h. These results suggest that sexually dimorphic changes over the period of post-natal development affect the susceptibility of rats to develop chronic pain (Figure 1).

Estrogen prevents the induction of priming in young males

We have previously demonstrated that estrogen regulates PKCε-dependent induction of priming (Joseph et al., 2003). While direct or receptor-mediated activation of PKCε, in males, induces priming, implantation of estrogen prevents induction. Thus, considering this role of estrogen in the regulation of the induction of priming, we investigated the possibility that an effect of the circulating estrogen in early stages of the post-natal development (Weisz and Ward, 1980; Aloisi, 2003; Amateau et al., 2004; LaPrairie and Murphy, 2007) would be responsible for the decreased susceptibility of young male rats to develop priming (Figure 1). Male rats received, daily, subcutaneous injection of the non-selective estrogen receptor antagonist ICI 182,780 (10 μg/day) on the back of the neck, for 5 consecutive days before they completed 3 weeks of age, at which time TNFα (100 ng in 5 μl) was injected intradermally on the dorsum of the hind paw. Daily ICI 182,780 treatment was continued for 3 additional days, in order to keep the inhibition of estrogen receptors during the time course of the effect of TNFα to induce priming (Bogen et al., 2012). When the rats were 8 weeks old, the presence of priming was tested for by injection of PGE_2 (100 ng) at the same site as TNFα, and the mechanical hyperalgesia evaluated 4h later. Two experimental groups were used for comparison: one that received only TNFα 3 weeks after birth (Figure 2, left panel, white bar), in which the induction of priming was attenuated, and a group treated with TNFα 7 weeks after birth (Figure 2, left panel, black bar), which developed priming. We

found that, in the group of rats that received TNFα on post-natal week 3 in the presence of ICI 182,780 (gray bar), priming was robust, since the magnitude of the hyperalgesia induced by PGE_2 4h after injection was significantly higher than in the control group (TNFα alone at the 3rd post-natal week). This result suggests that the decreased susceptibility of 3-week-old male rats to be primed is dependent on circulating estrogen (Figure 2, left panel).

Role of estrogen in the induction of priming in females

While the receptor-mediated or direct activation of PKCε in intact adult females does not induce priming, in gonadectomized females, activation of such mechanisms does, indicating the inhibitory effect of circulating estrogen on the mechanisms of priming induction (Joseph et al., 2003). Since we have observed that the susceptibility of females to be primed by TNFα changes markedly from the 4th to the 5th post-natal week (Figure 1, right panel), and taking into account the preventive effect of estrogen in the induction of priming (Joseph et al., 2003), we investigated if the lack of susceptibility of 5-week-old female rats to be primed was due to an action of estrogen. A group of female rats was treated daily, for 5 consecutive days before they reached 5 weeks of age, with subcutaneous injection of ICI 182,780, 10 μg/day, on the back of the neck (Figure 2, right panel, gray bar). Then, TNFα (100 ng in 5 μl) was injected intradermally on the dorsum of the hind paw. To maintain the inhibition of estrogen receptors during the time course of the effect of TNFα to induce priming (Bogen et al., 2012), the ICI 182,780 treatment was continued for an additional 3 days. When the rats were 8 weeks old, PGE_2 was injected at the same site as TNFα and the mechanical hyperalgesia evaluated 4h later. A different group of rats, which only received TNFα, 5 weeks after birth (Figure 2, right panel, white bar), and another group treated with TNFα 7 weeks after birth (Figure 2, right panel, black bar), were used for comparison. We observed that the hyperalgesia induced by PGE_2 in the group of rats that received TNFα at post-natal week 5 in the presence of ICI 182,780 (gray bar) was present at the 4th h after injection, indicating the presence of priming. In contrast, in the other two groups the PGE_2-induced hyperalgesia was no longer present at that time point. These results suggest that an effect of estrogen is responsible for the lack of susceptibility of 5 weeks old female rats to develop priming (Figure 2, right panel).

Discussion

The elucidation of the mechanisms involved in the etiology of chronic pain is a multifactorial challenge, involving not only aspects related to its induction and maintenance, but also the identification of factors that can impact the susceptibility of individuals to develop persistent pain. Circumstances such as stress or disease can affect sensory neurons and alter their function, ultimately contributing to the development of long-lasting pain states (Khasar et al., 2009; Christianson and Davis, 2010; Chen et al., 2011; Schaible, 2012; Lutz et al., 2015; Guerrero-Alba et al., 2016). Recently, it has been demonstrated that the occurrence of inflammation in early stages of post-natal development produces significant changes in the somatosensory/pain systems (Anand and Scalzo, 2000; Anand, 2000; Whitfield and Grunau, 2000; Bhutta et al., 2001; Lidow, 2002; Walker et al., 2003; Grunau et al., 2005), resulting in altered sensitivity of individuals to noxious stimuli as adults (Ruda et al., 2000; Lidow et al., 2001; Peters et al., 2005). Such changes in the response to painful

stimulation, expressed as a higher nociceptor sensitivity when compared to individuals not submitted to the same early life insult, is a consequence of an increased vulnerability of neurons to develop plasticity in the early periods of postnatal development (Lidow, 2002; Fitzgerald, 2004; LaPrairie and Murphy, 2007), and has been associated with an increased susceptibility of adults to develop long-term pain states. With that in mind, in this study, hyperalgesic priming, a PKCε-dependent preclinical model of the transition to chronic pain in the rat, was used to investigate if the stage of post-natal development affects the susceptibility of rats to develop chronic pain. Since priming consists in a persistent nociceptor plasticity (Aley et al., 2000), which may be associated with several clinical conditions (Reichling and Levine, 2009; Diers et al., 2011), it allowed us to investigate if an inflammatory event in early post-natal life affects the response to a pro-algesic (PGE_2) agent later in life (Aley et al., 2000). Importantly, the neuroplasticity underlying hyperalgesic priming has been shown to occur in the nociceptor, i.e., at the level of the peripheral nervous system (Parada et al., 2003b; Reichling and Levine, 2009; Joseph and Levine, 2010; Ferrari et al., 2013b; Ferrari et al., 2015), and, once established, it is permanently supported by continuous protein translation at the terminals of these sensory neurons (Ferrari et al., 2013a). Although not yet determined, the protein – or proteins – translated in this process seem to be those responsible for the switch in the signaling pathways by which proinflammatory mediators induce hyperalgesia, which accounts for the increased sensitivity of the primed nociceptor (Parada et al., 2005; Dina et al., 2009; Ferrari et al., 2013c; Ferrari et al., 2014; Ferrari and Levine, 2015). To induce priming, TNFα, which activates PKCε (Parada et al., 2003a), was injected on the dorsum of the hind paw of male and female rats in different post-natal ages. When adults, the prolongation of the mechanical hyperalgesia induced by PGE_2, injected at the same site as TNFα, was used to determine the presence of priming (Parada et al., 2003a; Ferrari et al., 2015). Our first observation, attenuation of the induction of priming by TNFα administered in male rats in the 1^{st}, 2^{nd}, or 3^{rd} post-natal week, and robust priming in female rats treated with TNFα 1, 2, 3 or 4 weeks after birth, was unexpected, since in adult females, either direct or receptor-mediated activation of PKCε does not induce priming (Joseph et al., 2003). However, an increased vulnerability of female rats to long-term consequences of neonatal inflammatory injury, when compared to males, has been previously demonstrated (LaPrairie and Murphy, 2007). Those authors observed a marked increase in pain behavior in response to algogens in females previously submitted to inflammation in the neonatal period, when compared to control and male groups. Hence, our findings showing a smaller sensitivity of young males to develop priming, in contrast to young females, which fully developed priming, are supported by previous studies that suggested sexual dimorphism in the conditions that control the susceptibility of young rats to develop nociceptor plasticity, which change over the first weeks of life.

That said, there is a significant variation in the levels of circulating sex hormones in both male and female rats after birth, until they stabilize after puberty. Even in the earliest days, the blood levels of sex hormones, especially estrogen, affect several aspects of the development, including the nervous system (Weisz and Ward, 1980; Pilgrim and Hutchison, 1994; Amateau et al., 2004; Balthazart and Ball, 2006; Cornil et al., 2006). Male rats have been reported to have higher levels of estradiol in the central tissues, at birth, when

compared to females, inversely changing as the animals grow. A similar difference in sex hormone levels in the peripheral tissues has been suggested (Germain et al., 1978; Hácik, 1978; MacLusky et al., 1979; LaPrairie and Murphy, 2007), although the literature in regard to the peripheral levels of estrogen and its source in neonatal males is scarce, limited to suggestions that circulating estrogen in young males is a product of the metabolism of testosterone produced in the testes (MacLusky et al., 1979; MacLusky and Naftolin, 1981; Roselli and Resko, 1993; Amateau et al., 2004). Nevertheless, such a sexual dimorphism in the profile of estrogen levels could be associated with differences observed between males and females in pain threshold and sensitivity to develop neuroplasticity (LaPrairie and Murphy, 2007). The fact that the injection of TNFα in the presence of the estrogen receptor inhibitor ICI 182,780, in 3 week-old males and 5 week-old females did induce priming, strongly suggests the role of circulating estrogen in the regulation of nociceptor plasticity. Also, considering a neuroprotective effect of estrogen (Garcia-Segura et al., 2001; Maggi et al., 2004; Amantea et al., 2005), our data suggest that its levels in the male rat at the 3rd post-natal week are still high enough to attenuate the pathways activated by TNFα, leading to priming. On the other hand, as the levels of estrogen rise in the developing female, at the 5th post-natal week they would already be sufficient to prevent the priming effect of TNFα. Since estrogen is an important regulator/modulator of the induction of hyperalgesic priming, by acting at the level of PKCε, preventing adult females to develop priming (Joseph et al., 2003; Ferrari et al., 2013b), our results indicate that in males during early life estrogen acts as a neuroprotectant in response to an inflammatory insult. In contrast, females, which show non-detectable to low levels of estrogen in association with the nervous system at the early stages of post-natal growth (Amateau et al., 2004; LaPrairie and Murphy, 2007), are more vulnerable to the effects of noxious insults during the first 4 post-natal weeks. These results are in alignment with previous studies suggesting a significant and sexually dimorphic contribution of the neonatal endocrine environment on the impact of neonatal injury in pain later in life (Aloisi, 2003; LaPrairie and Murphy, 2007). Of note, we have recently demonstrated in cultures of dorsal root ganglion (DRG) neurons from females, but not males, that estrogen can also contribute to the induction of neuroplasticity by positively modulating the release of calcium from stores in the endoplasmic reticulum (Ferrari et al., 2016; Khomula et al., 2017), which has been associated with the mechanism involved in the induction of hyperalgesic priming (Ferrari et al., 2013b). Whether this effect of estrogen contributes to our current results remains to be determined.

Another relevant point to be addressed is how, in terms of post-natal developmental stage, the rat compares with humans. The studies correlating the age of rats and humans, defining events such as puberty, are not conclusive (Sengupta, 2011). One of the difficulties is to find a reliable parameter to determine the age of the rats, even though it is widely accepted that the average adult laboratory rat, based in the weight, is from 300 grams (Sengupta, 2011; Sengupta, 2013). However, this does not apply for puberty, for example, which is based on the period of stabilization of the blood levels of sex hormones and gonad maturation (Sengupta, 2011; Sengupta, 2013). In male rats, puberty is considered to take place somewhere between the 39th and 47th post-natal day and, for females, between 34 and 38 days after birth (Korenbrot et al., 1977; Chappel and Ramaley, 1985; Engelbregt et al., 2000; Sengupta, 2011). According to these parameters, our experiments show a transition in the

susceptibility of young rats to develop priming in a time window not exactly on, but close to, puberty, which is probably a period in which the changing circulating levels of estrogen were reaching a level high enough to impact, positively or negatively, the sensitivity of nociceptors to TNFα. To associate our observations with the occurrence of puberty, rats have a brief, accelerated childhood, with a quite variable age to enter puberty (Sengupta, 2011; Sengupta, 2013). Thus, considering the impact of the changes in the levels of estrogen in the regulation of the susceptibility to develop priming over the early postnatal period, our data indicate a critical period, close to puberty, in which a transition takes place, in both sexes: males become more susceptible to be primed and, females, insensitive.

Neuroplasticity due to early painful injuries has also been suggested as the cause of increased pain sensitivity and analgesic requirements to subsequent painful conditions observed in infants submitted to surgical procedures (Peters et al., 2005). However, it is difficult to determine whether a change in pain processing, produced in early life, responsible for a decreased pain threshold in adults, involves central or peripheral neuroplasticity (Schwaller and Fitzgerald, 2014). It is possible that, depending on the type of injury, distinct (central or peripheral) compartments, or both, of the nervous system are affected, explaining how insults in early post-natal periods can affect different modalities of response to noxious stimulation. Unfortunately, it is difficult to determine, in our experiments, whether our observations are solely due to changes in the nociceptor or also involve the central nervous system; additional work is needed to provide this information. In this regard, since priming is a phenomenon occurring at the terminals of the nociceptor (Parada et al., 2003b; Reichling and Levine, 2009; Joseph and Levine, 2010; Ferrari et al., 2013a; Ferrari et al., 2013c; Ferrari et al., 2014; Ferrari and Levine, 2015), and as the central terminals of DRG neurons are in contact with the cerebrospinal fluid in the spinal cord, an effect of estrogen present in this compartment in our observations is also feasible.

Another limitation of our protocol is the difficulty to evaluate if the inflammatory pain induced by TNFα in young rats also affected the mechanical nociceptive threshold in adults. Due to the small size of the animals, training and evaluating the mechanical threshold in early life by the methods used in this study was not possible, preventing the comparison in later age. However, when compared to the average mechanical nociceptive threshold in naïve adult rats, the early-treated rats did not show significant difference in threshold (not shown), in contrast with other studies showing that rats submitted to inflammation in early post-natal life exhibit altered nociceptive threshold as adults (Lidow et al., 2001; Lidow, 2002; Ren et al., 2004; Wang et al., 2004; Peters et al., 2005; Wollgarten-Hadamek et al., 2009). The use of different techniques to accurately evaluate the mechanical nociceptive threshold in young animals might provide the missing information from our study, and could be a direction to be assessed in further experiments. Still, our data do strongly indicate a role of early life injury in the establishment of the susceptibility of rats to develop pain later on in life.

Conclusions

In conclusion, our results suggest a strong contribution of early life events to the susceptibility of rats to develop chronic pain as adults, which involves a significant role of

estrogen, affecting differently males and females, as its levels change over post-natal development. Our work might help to explain differences between sexes in chronic pain.

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Abbreviations

PGE_2	prostaglandin E_2
PKCε	protein kinase C epsilon
TNFα	tumor necrosis factor α

References

Aley KO, Levine JD. Role of protein kinase A in the maintenance of inflammatory pain. J Neurosci. 1999; 19:2181–2186. [PubMed: 10066271]

Aley KO, Messing RO, Mochly-Rosen D, Levine JD. Chronic hypersensitivity for inflammatory nociceptor sensitization mediated by the epsilon isozyme of protein kinase C. J Neurosci. 2000; 20:4680–4685. [PubMed: 10844037]

Aloisi AM. Gonadal hormones and sex differences in pain reactivity. Clin J Pain. 2003; 19:168–174. [PubMed: 12792555]

Amantea D, Russo R, Bagetta G, Corasaniti MT. From clinical evidence to molecular mechanisms underlying neuroprotection afforded by estrogens. Pharmacol Res. 2005; 52:119–132. [PubMed: 15967377]

Amateau SK, Alt JJ, Stamps CL, McCarthy MM. Brain estradiol content in newborn rats: sex differences, regional heterogeneity, and possible de novo synthesis by the female telencephalon. Endocrinology. 2004; 145:2906–2917. [PubMed: 14988386]

Anand KJ. Pain, plasticity, and premature birth: a prescription for permanent suffering. Nat Med. 2000; 6:971–973. [PubMed: 10973310]

Anand KJ, Scalzo FM. Can adverse neonatal experiences alter brain development and subsequent behavior. Biol Neonate. 2000; 77:69–82. [PubMed: 10657682]

Balthazart J, Ball GF. Is brain estradiol a hormone or a neurotransmitter. Trends Neurosci. 2006; 29:241–249. [PubMed: 16580076]

Beland B, Fitzgerald M. Influence of peripheral inflammation on the postnatal maturation of primary sensory neuron phenotype in rats. J Pain. 2001; 2:36–45. [PubMed: 14622784]

Bhutta AT, Rovnaghi C, Simpson PM, Gossett JM, Scalzo FM, Anand KJ. Interactions of inflammatory pain and morphine in infant rats: long-term behavioral effects. Physiol Behav. 2001; 73:51–58. [PubMed: 11399294]

Bogen O, Alessandri-Haber N, Chu C, Gear RW, Levine JD. Generation of a pain memory in the primary afferent nociceptor triggered by PKCε activation of CPEB. J Neurosci. 2012; 32:2018–2026. [PubMed: 22323716]

Branham WS, Fishman R, Streck RD, Medlock KL, De George JJ, Sheehan DM. ICI 182,780 inhibits endogenous estrogen-dependent rat uterine growth and tamoxifen- induced developmental toxicity. Biol Reprod. 1996; 54:160–167. [PubMed: 8838013]

Brummelte S, Grunau RE, Chau V, Poskitt KJ, Brant R, Vinall J, Gover A, Synnes AR, Miller SP. Procedural pain and brain development in premature newborns. Ann Neurol. 2012; 71:385–396. [PubMed: 22374882]

Chappel SC, Ramaley JA. Changes in the isoelectric focusing profile of pituitary follicle-stimulating hormone in the developing male rat. Biol Reprod. 1985; 32:567–573. [PubMed: 3922443]

Chen X, Green PG, Levine JD. Stress enhances muscle nociceptor activity in the rat. Neuroscience. 2011; 185:166–173. [PubMed: 21513773]

Christianson JA, Davis BM. The Role of Visceral Afferents in Disease. In: Kruger L, Light AR, editorsTranslational Pain Research: From Mouse to Man. CRC Press/Taylor & Francis; Boca Raton, FL: 2010.

Cornil CA, Ball GF, Balthazart J. Functional significance of the rapid regulation of brain estrogen action: where do the estrogens come from. Brain Res. 2006; 1126:2–26. [PubMed: 16978590]

Diers M, Schley MT, Rance M, Yilmaz P, Lauer L, Rukwied R, Schmelz M, Flor H. Differential central pain processing following repetitive intramuscular proton/prostaglandin E_2 injections in female fibromyalgia patients and healthy controls. Eur J Pain. 2011; 15:716–723. [PubMed: 21277243]

Dina OA, Khasar SG, Gear RW, Levine JD. Activation of Gi induces mechanical hyperalgesia poststress or inflammation. Neuroscience. 2009; 160:501–507. [PubMed: 19275929]

Engelbregt MJ, Houdijk ME, Popp-Snijders C, Delemarre-van de Waal HA. The effects of intra-uterine growth retardation and postnatal undernutrition on onset of puberty in male and female rats. Pediatr Res. 2000; 48:803–807. [PubMed: 11102550]

Ferrari LF, Araldi D, Levine JD. Regulation of Expression of Hyperalgesic Priming by Estrogen Receptor α in the Rat. J Pain. 2017; 18(5):574–582. [PubMed: 28089711]

Ferrari LF, Bogen O, Chu C, Levine JD. Peripheral administration of translation inhibitors reverses increased hyperalgesia in a model of chronic pain in the rat. J Pain. 2013a; 14:731–738. [PubMed: 23664545]

Ferrari LF, Bogen O, Levine JD. Role of nociceptor αCaMKII in transition from acute to chronic pain (hyperalgesic priming) in male and female rats. J Neurosci. 2013b; 33:11002–11011. [PubMed: 23825405]

Ferrari LF, Bogen O, Levine JD. Second messengers mediating the expression of neuroplasticity in a model of chronic pain in the rat. J Pain. 2014; 15:312–320. [PubMed: 24407022]

Ferrari LF, Bogen O, Reichling DB, Levine JD. Accounting for the delay in the transition from acute to chronic pain: axonal and nuclear mechanisms. J Neurosci. 2015; 35:495–507. [PubMed: 25589745]

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. 2016; 6:31221. [PubMed: 27499186]

Ferrari LF, Levine E, Levine JD. Role of a novel nociceptor autocrine mechanism in chronic pain. Eur J Neurosci. 2013c; 37:1705–1713. [PubMed: 23379641]

Ferrari LF, Levine JD. Plasma membrane mechanisms in a preclinical rat model of chronic pain. J Pain. 2015; 16:60–66. [PubMed: 25451625]

Fitzgerald M. Painful beginnings. Pain. 2004; 110:508–509. [PubMed: 15288390]

Garcia-Segura LM, Azcoitia I, DonCarlos LL. Neuroprotection by estradiol. Prog Neurobiol. 2001; 63:29–60. [PubMed: 11040417]

Germain BJ, Campbell PS, Anderson JN. Role of the serum estrogen-binding protein in the control of tissue estradiol levels during postnatal development of the female rat. Endocrinology. 1978; 103:1401–1410. [PubMed: 84755]

Grunau RE, Holsti L, Haley DW, Oberlander T, Weinberg J, Solimano A, Whitfield MF, Fitzgerald C, Yu W. Neonatal procedural pain exposure predicts lower cortisol and behavioral reactivity in preterm infants in the NICU. Pain. 2005; 113:293–300. [PubMed: 15661436]

Guerrero-Alba R, Valdez-Morales EE, Jimenez-Vargas NN, Lopez-Lopez C, Jaramillo-Polanco J, Okamoto T, Nasser Y, Bunnett NW, Lomax AE, Vanner SJ. Stress activates pronociceptive endogenous opioid signalling in DRG neurons during chronic colitis. Gut. 2016 Sep 02. 2016. doi: 10.1136/gutjnl-2016-311456

Hácik T. Endogenous production of ovarian estradiol-17 beta and its plasms levels in young rats. Endokrinologie. 1978; 71:35–39. [PubMed: 639764]

Hohmann AG, Neely MH, Piña J, Nackley AG. Neonatal chronic hind paw inflammation alters sensitization to intradermal capsaicin in adult rats: a behavioral and immunocytochemical study. J Pain. 2005; 6:798–808. [PubMed: 16326368]

Joseph EK, Levine JD. Hyperalgesic priming is restricted to isolectin B4-positive nociceptors. Neuroscience. 2010; 169:431–435. [PubMed: 20457222]

Joseph EK, Parada CA, Levine JD. Hyperalgesic priming in the rat demonstrates marked sexual dimorphism. Pain. 2003; 105:143–150. [PubMed: 14499430]

Khasar SG, Burkham J, Dina OA, Brown AS, Bogen O, Alessandri-Haber N, Green PG, Reichling DB, Levine JD. Stress induces a switch of intracellular signaling in sensory neurons in a model of generalized pain. J Neurosci. 2008; 28:5721–5730. [PubMed: 18509033]

Khasar SG, Dina OA, Green PG, Levine JD. Sound stress-induced long-term enhancement of mechanical hyperalgesia in rats is maintained by sympathoadrenal catecholamines. J Pain. 2009; 10:1073–1077. [PubMed: 19576859]

Khomula EV, Ferrari LF, Araldi D, Levine JD. Sexual Dimorphism in a Reciprocal Interaction of Ryanodine and IP3 Receptors in the Induction of Hyperalgesic Priming. J Neurosci. 2017; 37:2032–2044. [PubMed: 28115480]

Korenbrot CC, Huhtaniemi IT, Weiner RI. Preputial separation as an external sign of pubertal development in the male rat. Biol Reprod. 1977; 17:298–303. [PubMed: 889997]

LaPrairie JL, Murphy AZ. Female rats are more vulnerable to the long-term consequences of neonatal inflammatory injury. Pain. 2007; 132(Suppl 1):S124–33. [PubMed: 17904745]

Lidow MS. Long-term effects of neonatal pain on nociceptive systems. Pain. 2002; 99:377–383. [PubMed: 12406512]

Lidow MS, Song ZM, Ren K. Long-term effects of short-lasting early local inflammatory insult. Neuroreport. 2001; 12:399–403. [PubMed: 11209957]

Lutz B, Meiler SE, Bekker A, Tao YX. Updated Mechanisms of Sickle Cell Disease-Associated Chronic pain. Transl Perioper Pain Med. 2015; 2:8–17. [PubMed: 26301256]

MacLusky NJ, Lieberburg I, McEwen BS. The development of estrogen receptor systems in the rat brain: perinatal development. Brain Res. 1979; 178:129–142. [PubMed: 497856]

MacLusky NJ, Naftolin F. Sexual differentiation of the central nervous system. Science. 1981; 211:1294–1302. [PubMed: 6163211]

Maggi A, Ciana P, Belcredito S, Vegeto E. Estrogens in the nervous system: mechanisms and nonreproductive functions. Annu Rev Physiol. 2004; 66:291–313. [PubMed: 14977405]

Parada CA, Reichling DB, Levine JD. Chronic hyperalgesic priming in the rat involves a novel interaction between cAMP and PKCepsilon second messenger pathways. Pain. 2005; 113:185–190. [PubMed: 15621379]

Parada CA, Yeh JJ, Joseph EK, Levine JD. Tumor necrosis factor receptor type-1 in sensory neurons contributes to induction of chronic enhancement of inflammatory hyperalgesia in rat. Eur J Neurosci. 2003a; 17:1847–1852. [PubMed: 12752784]

Parada CA, Yeh JJ, Reichling DB, Levine JD. Transient attenuation of protein kinase Cepsilon can terminate a chronic hyperalgesic state in the rat. Neuroscience. 2003b; 120:219–226. [PubMed: 12849754]

Peters JW, Schouw R, Anand KJ, van Dijk M, Duivenvoorden HJ, Tibboel D. Does neonatal surgery lead to increased pain sensitivity in later childhood. Pain. 2005; 114:444–454. [PubMed: 15777869]

Pilgrim C, Hutchison JB. Developmental regulation of sex differences in the brain: can the role of gonadal steroids be redefined. Neuroscience. 1994; 60:843–855. [PubMed: 7936207]

Randall LO, Selitto JJ. A method for measurement of analgesic activity on inflamed tissue. Arch Int Pharmacodyn Ther. 1957; 111:409–419. [PubMed: 13471093]

Reichling DB, Levine JD. Critical role of nociceptor plasticity in chronic pain. Trends Neurosci. 2009; 32:611–618. [PubMed: 19781793]

Ren K, Anseloni V, Zou SP, Wade EB, Novikova SI, Ennis M, Traub RJ, Gold MS, Dubner R, Lidow MS. Characterization of basal and re-inflammation-associated long-term alteration in pain responsivity following short-lasting neonatal local inflammatory insult. Pain. 2004; 110:588–596. [PubMed: 15288399]

Reynolds ML, Fitzgerald M. Long-term sensory hyperinnervation following neonatal skin wounds. J Comp Neurol. 1995; 358:487–498. [PubMed: 7593744]

Roselli CE, Resko JA. Aromatase activity in the rat brain: hormonal regulation and sex differences. J Steroid Biochem Mol Biol. 1993; 44:499–508. [PubMed: 8476764]

Ruda MA, Ling QD, Hohmann AG, Peng YB, Tachibana T. Altered nociceptive neuronal circuits after neonatal peripheral inflammation. Science. 2000; 289:628–631. [PubMed: 10915627]

Schaible HG. Mechanisms of chronic pain in osteoarthritis. Curr Rheumatol Rep. 2012; 14:549–556. [PubMed: 22798062]

Schwaller F, Fitzgerald M. The consequences of pain in early life: injury-induced plasticity in developing pain pathways. Eur J Neurosci. 2014; 39:344–352. [PubMed: 24494675]

Sengupta P. A Scientific Review of Age Determination for a Laboratory Rat: How Old is it in Comparison with Human Age? Biomedicine International. 2011; 2:81–89.

Sengupta P. The Laboratory Rat: Relating Its Age With Human's. Int J Prev Med. 2013; 4:624–630. [PubMed: 23930179]

Shortland P, Fitzgerald M. Neonatal sciatic nerve section results in a rearrangement of the central terminals of saphenous and axotomized sciatic nerve afferents in the dorsal horn of the spinal cord of the adult rat. Eur J Neurosci. 1994; 6:75–86. [PubMed: 7510571]

Taiwo YO, Coderre TJ, Levine JD. The contribution of training to sensitivity in the nociceptive paw-withdrawal test. Brain Res. 1989; 487:148–151. [PubMed: 2752281]

Taiwo YO, Levine JD. Prostaglandin effects after elimination of indirect hyperalgesic mechanisms in the skin of the rat. Brain Res. 1989; 492:397–399. [PubMed: 2665905]

Torsney C, Fitzgerald M. Age-dependent effects of peripheral inflammation on the electrophysiological properties of neonatal rat dorsal horn neurons. J Neurophysiol. 2002; 87:1311–1317. [PubMed: 11877505]

Walker SM, Meredith-Middleton J, Cooke-Yarborough C, Fitzgerald M. Neonatal inflammation and primary afferent terminal plasticity in the rat dorsal horn. Pain. 2003; 105:185–195. [PubMed: 14499435]

Wang G, Ji Y, Lidow MS, Traub RJ. Neonatal hind paw injury alters processing of visceral and somatic nociceptive stimuli in the adult rat. J Pain. 2004; 5:440–449. [PubMed: 15501426]

Weisz J, Ward IL. Plasma testosterone and progesterone titers of pregnant rats, their male and female fetuses, and neonatal offspring. Endocrinology. 1980; 106:306–316. [PubMed: 7349961]

Weyer AD, Zappia KJ, Garrison SR, O'Hara CL, Dodge AK, Stucky CL. Nociceptor Sensitization Depends on Age and Pain Chronicity(1,2,3). eNeuro. 2016; 3(1):1–26.

Whitfield MF, Grunau RE. Behavior, pain perception, and the extremely low-birth weight survivor. Clin Perinatol. 2000; 27:363–379. [PubMed: 10863655]

Wollgarten-Hadamek I, Hohmeister J, Demirakça S, Zohsel K, Flor H, Hermann C. Do burn injuries during infancy affect pain and sensory sensitivity in later childhood. Pain. 2009; 141:165–172. [PubMed: 19095356]

Highlights

- TNFα induces hyperalgesic priming in adult male, but not female, rats;
- In young males, induction of priming by TNFα is significantly attenuated;
- In young females, injection of TNFα induces robust priming;
- Estrogen modulates this difference in susceptibility to develop priming in both sexes;
- Our data help to explain development of sex differences in susceptibility to chronic pain in adults.

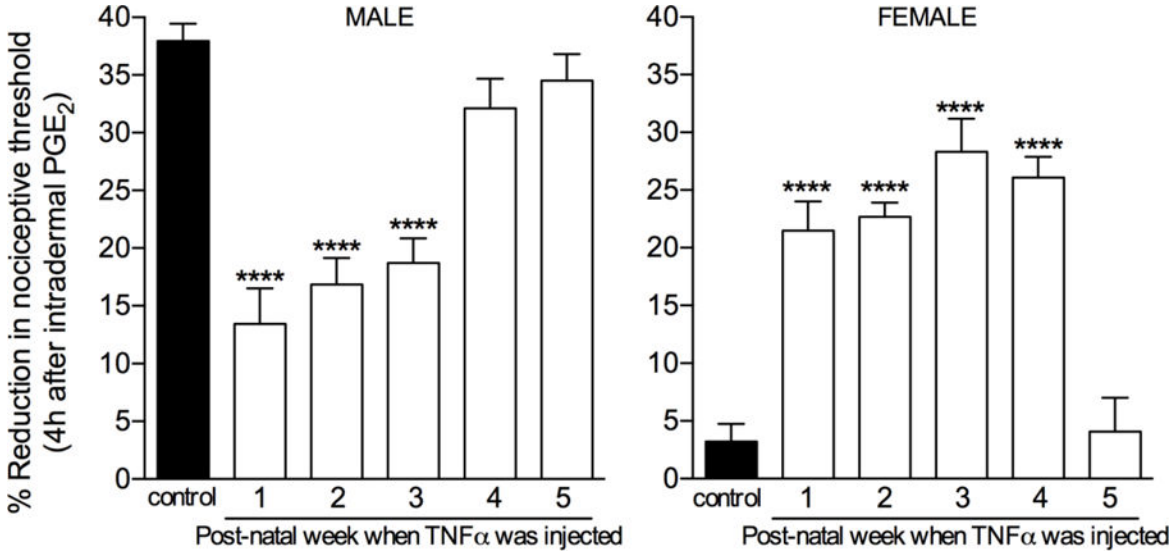

Figure 1. Age dependence for induction of hyperalgesic priming by TNFα in male and female rats

Male (left panel) and female (right panel) rats received an intradermal injection of TNFα (100 ng in 5 μl) on the dorsum of the hind paw 1, 2, 3, 4 or 5 weeks after birth (white bars). The control group (adults, black bars) received TNFα at the 7th week after birth. On the 8th week after birth, PGE_2 (100 ng) was injected at the same site as TNFα and, the mechanical hyperalgesia evaluated by the Randall-Selitto paw-withdrawal test. The detection of hyperalgesia 4h after PGE_2 injection indicated the presence of priming. We found that, in the male rats, the induction of priming by injection of TNFα 1, 2 or 3 weeks after birth was significantly attenuated, when compared to the control group and the groups treated at the 4th and 5th post-natal week, in which priming was fully developed ($F_{5,31}$ = 18.86; ****p < 0.0001, when the 1-, 2- and 3-week-old groups are compared to the control and the 4- and 5-week-old groups, one-way ANOVA followed by Bonferroni *post-hoc* test). On the other hand, while injection of TNFα in 5-week-old and adult females did not induce priming, when injected in 1-, 2-, 3- or 4-week-old females, it did produce priming ($F_{5,29}$ = 22.74; ****p < 0.0001, when the 1-, 2-, 3- and 4-week-old groups are compared to the control and 5-week-old groups). (*males*, control, TNFα/4 weeks old, and TNFα/5 weeks old groups: N = 6 paws; TNFα/1 week old, and TNFα/2 weeks old groups: N = 5 paws; TNFα/3 weeks old group: N = 9 paws; *females*, control and TNFα/1 week old group: N = 5 paws; TNFα/2 weeks old, TNFα/4 weeks old, and TNFα/5 weeks old groups: N = 6 paws; TNFα/3 weeks old group: N = 7 paws)

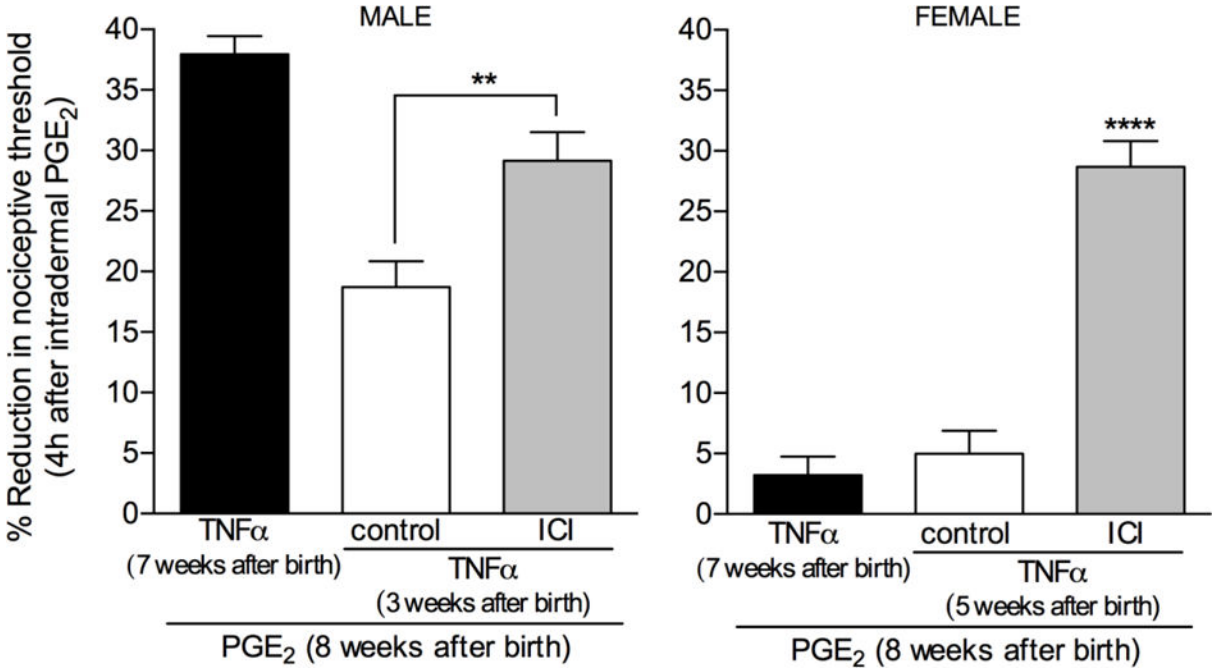

Figure 2. Estrogen attenuates the induction of priming by TNFα in 3-week-old male and 4-week-old female rats

The non-selective estrogen receptor antagonist ICI 182,780 (ICI, 10 μg/day, gray bar) was injected, subcutaneously on the back of the neck, once a day for 8 consecutive days, in male (left panel, starting 5 days before completing the third postnatal week) and female (right panel, starting 5 days before completing the 5th post-natal week) rats. The priming stimulus, TNFα (100 ng in a volume of 5 μl), was injected intradermally on the dorsum of the hind paw on the 5th day of the ICI treatment. When rats were 8 weeks old, PGE_2 (100 ng) was injected at the same site as TNFα. Mechanical nociceptive threshold was evaluated before and at the 4th h after PGE_2 injection. **Left panel:** The ICI-treated males were compared with groups shown in Figure 1 (left panel), that had received TNFα alone, at the 7th post-natal week (black bar), in which priming had fully developed, or at the 3rd post-natal week (control, white bar), in which the induction of priming was significantly attenuated, as indicated by the smaller magnitude of the PGE_2-induced hyperalgesia 4h after injection (t_{13} = 6.641; $p < 0.0001$, when the hyperalgesia in the 3- and the 7-week-old groups is compared, unpaired Student's *t*-test). We found that, when 3-week-old rats received TNFα in the presence of ICI 182,780 (gray bar), the induction of priming was robust, since the magnitude of the hyperalgesia induced by PGE_2 at the 4th h was significantly higher when compared to the 3-week- old + TNFα alone group (t_{12} = 3.106; ** p = 0.0091, when both groups are compared), indicating an inhibitory effect of estrogen in the induction of priming in young male rats. (TNFα/7 weeks old group: N = 6 paws; TNFα alone/3 weeks old group: N = 9 paws; TNFα + ICI 182,780/3 weeks old group: N = 5 paws); **Right panel:** The PGE_2-induced hyperalgesia in the rats treated with ICI + TNFα was compared with control groups, shown in Figure 1 (right panel), which did not develop priming when received TNFα alone at the 5th (white bar), or at the 7th post-natal week (black bar). In contrast to the controls, the PGE_2-induced hyperalgesia was present at the 4th h in the group that received TNFα in the presence of ICI 182,780 (t_9 = 9.297; t_{10} = 8.258; **** $p < 0.0001$ for both, when the PGE_2-

induced hyperalgesia in the 3-week-old TNFα + ICI 182,780 group is compared, respectively, with the groups that received TNFα alone 7 and 5 weeks after birth, unpaired Student's *t*-test), indicating that priming had developed. This result suggests that estrogen has an inhibitory effect on the induction of priming in 5-week-old female rats. (TNFα/7 weeks old group: N = 5 paws; TNFα alone/5 weeks old and TNFα + ICI 182,780/5 weeks old groups: N = 6 paws)