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**ROLE OF CRF1 RECEPTOR IN POST-INCISIONAL PLASMA EXTRAVASATION AND
NOCICEPTIVE RESPONSES IN MICE**

Asunción Romero¹, Juan-Antonio García-Carmona², María-Luisa Laorden², Margarita M Puig¹

¹Department of Anesthesiology, Pain Research Unit, Institut Municipal d'Investigació
Médica, Hospital del Mar, Universitat Autònoma de Barcelona, Barcelona, Spain

²Department of Pharmacology, University of Murcia, Murcia, Spain

***Corresponding author at:**

M.L. Laorden

Departamento de Farmacología

Facultad de Medicina

30100 Murcia

Spain

e-mail: laorden@um.es

ABSTRACT

The corticotropin-releasing factor (CRF) is involved in a number of physiological functions including pain perception. The purpose of this study was to evaluate the role of CRF1 receptor in the long-lasting post-surgical changes **in somatic** nociceptive thresholds and in local inflammatory responses, using genetically engineered mice lacking functional CRF1 receptor. Animals underwent a plantar incision under anaesthesia with remifentanyl (80 µg/kg s.c.) and sevoflurane. Mechanical thresholds (von Frey) and plasma extravasation (Evan's blue) were evaluated at different time points. On postoperative day 20, mechanical thresholds had returned to baseline in CD1 mice ($3.07 \pm 6.21\%$), while B6,129CRH^{tklee} mice presented significant hyperalgesia, which was similar in wild-type (WT) (**-29.81 ± 8.89%**) and CRF1 receptor knockout (KO) (**-37.10 ± 10.75%**) mice, showing strain differences. The administration of naloxone (1 mg/kg, s.c.) on postoperative day 21 **produced** hyperalgesia revealing surgery-induced latent pain sensitization. The extent of hyperalgesia was greater in KO versus WT mice, suggesting a role of CRF1 receptors in the **upward** modulation of endogenous opioid release. Furthermore, two days after surgery, plasma extravasation returned to baseline in WT mice but remained elevated in KO mice. **In non-manipulated B6,129CRH^{tklee} KO mice we observed an increase in the number of writhes (41.25±11.36) versus WT (23.80±4.71), while in the tail immersion test no differences could be detected.** Our results show that CRF/CRF1 receptors seem to be a **protective role** in latent pain sensitization induced by surgery and in the local inflammatory response to injury.

Key words: Latent pain sensitization, plasma extravasation, post-operative pain, CRF 1 receptors

Introduction

Despite the high consideration given to postoperative comfort, the use of devices such as patient controlled analgesia, and the increasing use of multimodal protocols, most patients experience some degree of postoperative pain. The purpose of the present investigation was to identify likely key mediators such as corticotropin-releasing factor (CRF) that could participate in the complex responses to the surgical injury which would facilitate the development of new therapeutic targets.

It has been well established the role of CRF in somatic pain regulation (Yakushkina et al., 2011; 2016). However, the effects of CRF in pain relief are controversial and it has been described anti- and pronociceptive effects (Ji et al., 1995; Larauche et al., 2009; Nijsen et al., 2005). CRF action is mediated through CRF receptors of type 1 and 2 (CRF1 and CRF2) distributed in central and peripheral nervous system (Perrin and Vale, 1999; Stengel and Taché, 2009). CRF1 and CRF2 receptors located within the amygdala are involved in somatic pain regulation and may mediate opposite effects (Rouwette et al., 2012). Pronociceptive effect of intra-amygdala CRF administration has been shown to be mediated by CRF1 receptors, while the antinociceptive effect of CRF is mediated by CRF2 receptors (Ji and Neugebauer, 2008; Rouwete et al., 2012). Brain CRF administration enhances colorectal distension-induced visceral pain in rats through CRF1 receptors (Martinez and Taché, 2006). In contrast with this result, it has been demonstrated that intra-dorsal periaqueductal gray matter (PAGM) administration of CRF1 receptor antagonist NBI 27914 prevents CRF-induced analgesic effect on tonic pain induced by formalin injection, suggesting the involvement of CRF1 receptors in the analgesia. However, PAGM administration of the CRF2 receptor antagonist antisauvagine 30 did not

influence in the analgesic effect (Miguel and Nunes de –de-Souza, 2011). There is evidence that peripheral injection of CRF induces visceral hypersensitivity to colorectal distention, an effect reproduced by the intraperitoneal administration of the selective CRF1 agonist, cortagine in rats and mice (Larauche et al., 2009). Pretreatment with Astressin, a nonselective CRF antagonism, blocked this CRF-induced sensitization, but Astressin 2-B, a selective CRF2 antagonist did not affect it suggesting that peripheral CRF1 signaling induced visceral sensitization were modulated by peripheral CRF2 signaling (Nozu et al., 2014).

On the other hand, CRF is a major mediator of the endocrine arm of the stress response by centrally stimulating the hypothalamic-pituitary-adrenal (HPA) axis. Briefly the hypothalamus releases CRF, which binds to CRF1 receptors localized on corticotroph cells in the anterior pituitary, then induces the secretion of adrenocorticotropin hormone (ACTH) which stimulates glucocorticoid secretion from the adrenal glands; glucocorticoid is a tonic anti-inflammatory mediator (Lightman, 2008). In the inflammatory processes the role of CRF and its receptors also remain controversial; some reports showed pro-inflammatory effects while some others indicated anti-inflammatory effects (for review see Im, 2015; Zhu et al., 2011). The local application of CRF in the brain and spinal cord has been shown to produce antinociceptive effects against inflammatory pain, and this action may be mediated by CRF receptors (Mousa et al., 2004). Thus, intracerebral injections of CRF inhibited stress-induced aggravation of trinitrobenzene-colitis, while central injection of the CRF1 and CRF2-antagonist, Astressin worsened colitis (Million et al., 1999). Taken together these results indicate that CRF can exert both pro-and anti-inflammatory functions depending on the type of receptors, the tissues, the route of administration and the disease phases. Therefore, it

would be of interest to determine the possible implication of CRF/CRF1 receptor in the modulation of pain.

In this study we have used a mouse model of post-incisional pain that closely mimics the surgical procedure in humans in order to evaluate the role of CRF/CRF1 receptors in the pro-nociceptive and inflammatory response to injury (incision), using genetically engineered mice lacking functional CRF1 receptor. In addition, phenotypic nociceptive behavior responses were evaluated in healthy B6,129CRH^{tklee} wild type (WT) and CRF1 receptor known (KO) mice, using chemical and thermal nociceptive stimuli. Swiss CD1 was used as reference strain.

Methods

Animals

Adult male B6, 129 CRH^{tklee} mice that were WT (CRF1 receptor^{+/+}) and recessive homozygous (CRF1 receptor^{-/-}) or KO mice (8-12 weeks old, 25-30 g) were derived from mating CRF1 receptor^{+/+} obtained from breeders (Jackson Laboratory, California, USA) and provided by Murcia University (Spain). Swiss CD1 strain was obtained from Charles-River (CRIFFA, France) reference strain. Because of the known variability among animal strains in their expression of either pain or anxiety/depression-like behaviors, we studied two strains of mice in the present study. The rationale for the selection of CD1 as a reference strain was: 1) it is one of the most widely used strains to assessed in a variety of various nociceptive stimuli (Noble et al., 1997) and 2) to compare behavioural phenotype in commonly used CD1 with B6,129CRH^{tklee} strain, which has not been previously characterized. The study was conducted in accordance with the European directive 2010/63/UE, the Ethical Guidelines of the International Association for the Study of Pain (IASP) and the Ethical Committee for Animal Welfare of our Institution (CEEAA-PRBB, Comité Ético de Experimentación Animal - Parc de Recerca Biomèdica de Barcelona, Barcelona, Spain) approved the protocol. The animal facility of the Institution is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care since June 2010.

WT and CRF1 receptor KO offspring from CRF1 receptor^{+/+} breeders were genotyped at weaning using a polymerase chain reaction (PCR) based method. Tail tip samples were collected at 3 weeks of age, and DNA was extracted and processed according to the manufacturer's instructions (Qiagen, Germany).

Mice were housed in groups of 4 in individual ventilated cages (Technicplast), and had free access to food and water. Autoclaved poplar softwood bedding (Souralit S.L., Barcelona, Spain) and autoclaved cellulose paper were used as nesting material, to provide maximum animal comfort. Animals were maintained in a room under a 12 h light/dark cycle (lights on at 08:00 a.m.), at controlled temperature (21 ± 1 °C), and relative humidity ($55\pm10\%$). Mice were acclimatized to the facilities at least for 5 days before beginning the experiment. Behavioral testing was performed between 9:00 a.m. and 2:00 p.m., in a quiet well illuminated room.

Nociceptive testing

In all experiments, Swiss CD1 mice were used for comparison; the investigator performing the experiments was blinded to the B6,129 CRH^{tklee} genotype (WT or KO). Animals were used only once, and were sacrificed by cervical dislocation after testing; special care was taken to use the smallest number of animals per group.

Incisional postoperative pain model (surgery)

The rat incisional pain model described in rats (Brennan et al., 1996) was adapted to mice in our laboratory and validated in previous studies (Cabañero et al., 2009a; 2009b; Campillo et al., 2010). In a sterile operating room, mice were anesthetized with sevoflurane delivered via a nose mask (induction, 3.5% v/v and surgery, 3.0% v/v) plus an infusion of subcutaneous (s.c.) remifentanyl (80 µg/kg), administered over a period of 30 min; a KD Scientific pump (KD Scientific Inc., Holliston, MA, USA) was used for the infusion. The type and doses of the anaesthetics were selected on the basis of previous experiments performed in our laboratory to obtain maximal postoperative hyperalgesia (Cabañero et al., 2009b).

After disinfection of the skin with povidone of the right hind paw, a 0.7 cm longitudinal incision was made with a number 20 blades through the skin and fascia of the plantar surface, starting 0.3 cm from the proximal edge of the heel extending toward the toes. The underlying plantaris muscle was exposed and incised longitudinally, while the muscle origin and insertion remained intact. After homeostasis with slight pressure, the skin was closed with two 6.0 silk sutures, and the wound site was covered with povidone-iodine antiseptic ointment. After surgery, the animals were allowed to recover under a heat source in cages with sterile bedding. No cardiovascular side effects or respiratory depression were observed during or after recovering from surgical anaesthesia.

Writhing test

Acetic acid-induced visceral pain models were performed as previously described at dose determined previously (Miranda et al., 2009; Noble et al., 1997; Verri et al., 2008). Briefly, mice were injected intraperitoneally (i.p.) with 0.6% acetic acid solution, in a volume of 10 ml/kg, and immediately placed in individual clear plexiglas observation cubicles (9×5×5 cm), acquired from Servei Estació (Barcelona, Spain). The induced nociceptive behavior is characterized by abdominal contractions known as a writhes, consisting of waves of contraction of the abdominal musculature, accompanied by the elongation of the body and extension of one or both hind limbs. The number of writhes over a 10 min period was recorded, starting 5 min after the acetic acid injection.

Tail flick immersion test

Before the experiments, mice were habituated for 3 days in the tail immersion device. On day 4, animals were gently held with a cloth towel and the tip of the tail (terminal 2–3 cm)

was immersed into hot water at 55 ± 0.5 °C (Le Bars et al., 2001). The time elapsing between the immersion and removal of the tail from the warm water was recorded (tail withdrawal latency). An 8 s cut-off time was established to avoid damage to the tail. A minimum of 3 tail withdrawal latency responses, obtained at 10 min intervals, were averaged for each mouse. Only animals with baseline reaction times between 2 and 3 s were used in the experiments.

Von Frey Test

Punctate stimulus was used to determine mechanical thresholds in the hind paw withdraw response to von Frey filaments (Chaplan et al., 1994). Before the experiments, mice were habituated to the equipment for 3 days. Afterwards, animals were placed in methacrylate cylinders (30 cm high, 9 cm diameter, acquired from Servei Estació, Barcelona, Spain) with a wire grid bottom through which the von Frey filaments were applied (bending force range from 0.008 to 2 g; North Coast Medical, Inc., San Jose, CA, USA). Animals were allowed to habituate for 2 h before testing to achieve immobility. Each filament was applied at the center of the (injured) paw, vertically to the plantar surface, with sufficient force to cause a slight deflection of the filament. The filament force was increased or decreased according to the response. The upper limit value (2 g) was assigned when there was no response and the threshold of response was calculated using the up-down method (Chaplan et al., 1994).

Assessment of plasma extravasation in the hind paw after surgery

Plasma extravasation was measured after the administration of 60 mg/kg of Evan's blue dye, which is a marker of protein leakage (Udaka et al., 1970). With the animals anesthetized with sevoflurane (3.5% v/v), the dye was injected intravenously (i.v.) in the retroorbital plexus, and the animals were sacrificed fifteen min later. Both hind paws

(operated/ipsilateral, and non-operated/contralateral) were divided into sections and incubated with formamide (**2mL/vial**) at 65°C for at least 3 h. Samples were then filtered, and the extracted dye measured by spectrophotometry at 620 nm (Ultrospec 2100pro UV/Visible Spectrophotometer, GE Healthcare Life Sciences, Spain). The absorbance values were calculated from a calibration curve and expressed as UA dye/g paw, of wet paw tissue. Table 1 summarizes the different tests used in both mice strains in the different experimental conditions.

Experimental protocol

Experiment 1. **Effect of surgical incision** on mechanical thresholds in CD1 and B6,129CRH^{tklee} mice (WT and KO). All mice had a plantar incision (surgery) performed under sevoflurane+remifentanyl anaesthesia. After the habituation period, mechanical thresholds were obtained during 2-3 consecutive days and the mean values calculated to obtain baseline values. **Mechanical hyperalgesia was assessed in all groups of study 3, 2 and 1 days before surgery and 4 h 2, 20 and 21 days after surgery. Non-injured hind paw was also tested for mechanical thresholds in operated mice**

Previous studies from our laboratory in CD1 mice, using the same model, have shown that the incision induces significant mechanical postoperative hyperalgesia lasting between 7-10 days, with peak effect between 4 h-2 days (Cabañero et al., 2009a, b; Campillo et al., 2010, 2011; Romero, et al., 2011). In the present study we did not perform a full time course for postoperative hyperalgesia, but tested the animals at 4 h and on days 2, 20 and 21 after surgery; this was done in order to avoid tissue damage and needless animal suffering given the inflammatory reaction observed in KO mice.

Experiment 2 Effect of naloxone on mechanical hyperalgesia induced by surgery in mice. As for assessing mechanical hyperalgesia on day 21, our group has also shown (CD1 mice), that the administration of 1 mg/kg s.c. naloxone after complete healing of the wound and when mechanical thresholds have returned to baseline values (days 14 to 150 were tested), induces again significant mechanical hyperalgesia, revealing the presence of latent pain sensitization after surgery (Campillo et al., 2011; Romero et al, 2011). Thus, we assessed mechanical hyperalgesia in all experimental groups on day 21, **20 min after saline or naloxone administration**, to establish latent pain sensitization.

Experiment 3. Effect of surgical incision on plasma extravasation in mice under sevoflurane plus remifentanil anaesthesia. In all strains (CD1 and B6,129CRH^{tklee}) of mice, plasma extravasation was evaluated **30 min before surgery (basal value), and on days 2 after surgery**, coinciding with the peak of maximal hyperalgesia (4 h-2 day).

Figure 1 shows the experimental protocol for experiments # 1-2.

Experiments 4-5 were performed in order to assess nociceptive responses in non-injured mice (CD1, WT and KO), to chemical (acetic acid) and thermal (tail flick) stimuli (Table 1).

Drugs

Acetic acid, Evan's blue dye, dimethyl sulfoxide (DMSO), formamide, Tween 80 and naloxone HCl were purchased from Sigma-Aldrich Química (Madrid, Spain). Sevoflurane (Sevorane®; Abbot Laboratories SA, Madrid, Spain), remifentanil (Ultiva®; GlaxoSmithKline, Madrid, Spain) were supplied by the Department of Anesthesiology at the Hospital del Mar (Barcelona, Spain).

Statistical analysis

The results are presented as mean values $\pm$ S.E.M. To analyse significant changes in mechanical thresholds in different groups of mice, a one-way ANOVA model for repeated measures, was used for each group. **Two-way ANOVA for the factors treatment and strains was performed to determine mechanical thresholds in the group treated with saline or naloxone following by Bonferroni analysis.** For the post hoc analyses comparing the values at each time with the baseline values, Dunnett's many-to-one test in the framework of these models was applied. Paired comparisons were performed by the non-parametric Mann Whitney test for independent samples (GraphPad Prism 4 software, San Diego, CA). A p value less than 0.05 was considered statistically significant.

Results

Behavioral testing

Mechanical hyperalgesia was evaluated in non-injured and injured hind paws from B6,129CRH^{tklee} (WT or KO) and CD1 mice. In non-injured hind paw there are no statistically significant differences in baseline mechanical thresholds nor after surgery (4h, 2 and 20 days) in CD1, WT and KO mice (data not shown). As shown in table 2 baseline mechanical thresholds in injured hind paw were also similar in the different groups of mice used in this study. Surgery similarly decreased ($p < 0.01$) mechanical thresholds in the operated paw in all groups of mice, at 4 h ($-66.34 \pm 2.38\%$, $-78.73 \pm 7.09\%$ and $-81.87 \pm 8.11\%$, for CD1, WT and KO mice, respectively), and on postoperative day 2 ($-52.51 \pm 3.29\%$, $-69.62 \pm 5.52\%$ and $-69.79 \pm 9.33\%$) (Figure 2).

Two-Way ANOVA revealed no significant main effects on mechanical thresholds for strains ($F_{(1,32)}=2.791$, $p=0.1046$) but did show a treatment effect ($F_{(1,32)}=22.42$, $p<0.0001$) and interaction between both factors ($F_{(1,32)}=11.01$ $p=0.0023$). On postoperative day 20, mechanical thresholds had returned to baseline levels in CD1 mice ($3.07 \pm 6.21\%$), while mice with the B6,129CRH^{tklee} genotype still presented significant hyperalgesia which was similar in both groups (WT $-27.15 \pm 4.30\%$, $p < 0.01$ and KO -37.46 ± 5.69 , $p < 0.001$ when compared to CD1 mice). The results show that postoperative mechanical hyperalgesia is significantly prolonged in mice with the B6,129CRH^{tklee} genotype, but the deletion of the CRF1 gen does not induce further changes, demonstrating significant differences between strains of mice on the duration of postoperative hyperalgesia.

A challenge with naloxone on day 21, induced an increased degree of hyperalgesia in CD1 ($-50.01 \pm 4.37\%$) versus CD1 saline group ($3.07 \pm 6.21\%$, $p < 0.001$) while the response in B6,129CRHtklee WT mice ($-38.15 \pm 4.10\%$) was similar to the same group injected with saline ($-29.81 \pm 8.89\%$). However, in KO mice naloxone administration produced a significant ($p < 0.01$) decrease ($-82.72 \pm 1.96\%$) in the mechanical thresholds versus the KO mice injected with saline ($-37.10 \pm 10.75\%$), WT or CD1 mice injected with naloxone; **the magnitude of difference between WT and KO mice in the response to naloxone was $57.034 \pm 12.033\%$** (Figure 2). Altogether, these results demonstrate that blockage of opioid receptors by naloxone, induces a greater degree of hyperalgesia when the CRF-1 gene is deleted.

Plasma extravasation

Postoperative plasma extravasation in the operated paw was assessed with Evans Blue in the three groups of mice. Basal values obtained before surgery were 0.43 ± 0.11 AU/g in CD1 mice, but were significantly lower in B6,129CRH^{tklee} WT (0.17 ± 0.06 AU/g, $p < 0.01$) and B6,129CRHtklee KO mice (0.19 ± 0.01 AU/g, $p < 0.05$), showing significant differences between strains (Figure 3).

Two days after surgery, plasma extravasation had almost returned to baseline in WT mice (0.31 ± 0.19 AU/g), but remained elevated in KO mice (1.13 ± 0.09 AU/g, $p < 0.01$ compared to WT); plasma extravasation in CD1 animals was significantly higher (0.87 ± 0.18 AU/g, $p < 0.01$) than baseline.

In the second part of the study, we assessed phenotypic nociceptive behaviour in healthy (non-manipulated) CD1, B6,129CRH^{tklee} WT and KO mice, exposed to different nociceptive stimulus. In Figure 5 we have represented the results obtained in the acetic acid writhing

(panel A) and tail flick (B) tests. The number of writhes in B6,129CRH^{tklee} KO mice (41.25 ± 11.36) were significantly higher ($p < 0.01$) than in B6,129CRH^{tklee} WT (23.80 ± 4.71) and CD1 mice (26.00 ± 9.36), showing that deletion of the CRF-1 gene produces a greater response to chemical / inflammatory stimulus. No differences were observed when CD1 and B6,129CRH^{tklee} WT were compared (Figure 5 A). However, when CD1 and B6,129CRH^{tklee} (WT and KO) mice were compared in the tail immersion test no differences could be detected (Figure 5B).

Discussion

Previous studies from our laboratory in CD1 mice using the incisional postoperative pain model have shown that the incision induces significant mechanical postoperative hyperalgesia lasting between 7-10 days, with peak effect between 4 h-2 days (Cabañero et al., 2009 a,b; Campillo et al., 2010; Célérier et al., 2006). **The present study confirms previous data obtained in CD1 mice and also demonstrate the development of hyperalgesia in B6,129CRH^{tklee} mice (WT and KO), similar to that described in CD1 mice, at 4 h after surgery and on postoperative day 2. According to previous data (Campillo et al., 2010), the pain hypersensitivity disappeared in CD1 mice, as demonstrated by the return of the mechanical threshold to the baseline values on postoperative day 20. However, present results demonstrate, for the first time, that postoperative mechanical hyperalgesia was significantly prolonged in mice with B6,129CRH^{tklee} genotype indicating significant differences between strains of mice. The prolongation of hyperalgesia observed in B6,129CRH^{tklee} mice could be due to a sustained and prolonged activity of the nociceptive system. Previous studies from our laboratory using CD1 mice, have demonstrated that surgery plus remifentanil anesthesia produces long lasting neuroplastic changes that could be involved in develop latent pain sensitization (Campillo et al., 2011). This long-lasting pain vulnerability was demonstrated in our study by the precipitated hyperalgesia induced after naloxone in CD1 and also in B6, 129CRH^{tklee} (WT and KO) mice. This response has been explained by an increase in endogenous opioid peptides and/or increased signaling activity at the opioid receptor (Célérier et al., 2001; Corder et al., 2013). According to a previous study (Campillo et al., 2011) hyperalgesia was observed after opioid antagonist administration 21 days after surgery in CD1 mice suggesting that latent**

sensitization is mediated by endogenous opioid system. These results provide further evidence of opioid-mediated latent sensitization and suggest that impairment of the protective endogenous opioid system may play a relevant role in the development of persistent post-surgical pain. In accordance with recent finding in animal studies it has been demonstrated persistent postsurgical pain in humans (Werner et al., 2014) and patent sensitization mediated by opioid system (Pereira et al., 2015). According to these data the long-term therapeutic goal of future research is to alleviate persistent pain by either: a) facilitating endogenous opioid analgesia, thus restricting latent pain sensitization within a state of remission; or b) extinguishing or suppress latent sensitization (Taylor and Corder, 2014). In order to contribute to the development of new therapeutic our goal has been to investigate the role of CRF/CRF1 receptor in the latent pain sensitization. Our results demonstrated similar mechanical hyperalgesia in WT or KO mice after saline injection. However, the hyperalgesia observed in KO mice after naloxone administration was higher than that observed in WT and CD1 mice suggesting that the opioid antagonist reveals a potential interaction between opioid and CRF/CRF1 receptor pathways in long lasting pain sensitization. Conversely, a role for CRF-induced antinociception has been suggested on the basis of anatomical studies that found co-localization of CRF and dynorphin terminal within central nervous system (Marchant et al., 2007; Kravets et al., 2013; Retson et al., 2013). Multiple studies have shown increased thresholds to nociceptive behaviors in nonstressed animals after exogenous administration of CRF activating CRF1 receptor, such as: increased mechanical and thermal withdrawal thresholds in Fischer and Lewis rats (Vit et al., 2006). **These finding were confirmed by showing that CRF1 receptor antagonists such as NBI-27914, MPZP or R121919 exert antinociceptive effect to mechanical stimulation in a model**

of arthritis pain and attenuated mechanical hypersensitivity in the acute dependence model (Baiamonte et al. 2014; Cohen et al., 2013; Edwards et al., 2012; Ji et al., 2013; Park et al., 2015). However, it has been clear that CRF may cause a decrease in somatic pain sensitivity (analgesic effect) in animals (Larivieri and Melzack, 2000; Vit et al., 2006) and human (Likar et al., 2007; Matejec et al., 2005). Thus, the CRF1 receptor antagonists NBI-35965 fails to attenuate swim-induced hyperalgesia in Swiss mice (Abdelhamid et al., 2013). These discrepancies could be due to the different models used. There are substantial differences between post-incisional and other models such as inflammatory and neuropathic pain (Biddlestone et al., 2007; Honore et al., 2006). In addition, it is well documented that CRF1 and CRF2 receptors located within the amygdala are involved in somatic pain regulation in rats (Rouwette et al., 2012; Yarushkina et al., 2016). In this regard, it has been proposed that initial activation of CRF1 receptor produces a clear hyperalgesia that it necessary to protect the organism against further tissue damage. Simultaneously, activation of CRF1 receptor results in transport of CRF2 receptor towards the plasma membrane where it will bind CRF2 receptor ligands such as the urocortins released in the central nervous system. This second phase of CRF receptor activation produces an analgesic effect important for the termination of pain (Rouwette et al., 2012). This concept implicates that balanced and proper sequential recruitment of CRF receptors is crucial for successful management of the pain response. Consequently, the deletion of CRF1 receptor could produce an imbalance in this process could result in an enhanced of the hyperalgesia observed after naloxone in our study.

On the other hand, surgical patients have to some extent stress during pre-and /or post-operative period. It is well known that CRF/CRF1 receptor is involved in the activation of

HPA during acute and chronic stress (Papadimitriou and Priftis, 2009; Zelena et al., 2005). CRF/CRF1 receptor stimulates ACTH and glucocorticoid release, which could be responsible of the analgesia describe in visceral and somatic pain models in response to potent and/or long-lasting stressors (Butler and Finn, 2009; Gui et al., 2004; Koolhaas et al., 2011; Larauche et al., 2012). In mice with CRF1 receptor gene deletion (KO) we have demonstrated an increased response in visceral pain without changes in somatic pain. These data are in agreement with a recent study demonstrating that mild social stress in mice, can induced analgesia in visceral but not in somatic pain models (Pitcher et al., 2017). The deletion of CRF1 receptor could cause long-lasting deficiency of stress-induced production of the CRF, ACTH and corticosterone. Thus, previous studies have demonstrated that the inhibition of CRF-induced corticosterone levels was accompanied by a disappearance of CRF-induced analgesia (Yurushkina et al., 2011). Together, these results and present data could indicate that the decreased activity of the HPA axis could be involved in surgery-induced latent pain sensitization observed in KO mice.

In this study we also demonstrated that basal values of plasma extravasation obtained before surgery were lower in B6,129CRH^{tklee} WT and KO mice versus CD1 mice demonstrating differences between strains. In addition, the plasma extravasation was increased two days after surgery in CD1 and B6,129CRH^{tklee} KO confirming that inflammation together with nociceptive sensitization are the hallmarks of tissue surrounding surgical incisions. In addition, postsurgical plasma extravasation was higher in B6,129CRH^{tklee} KO mice suggesting a relationship between CRF1 receptors and inflammation. It is known that multiple brain areas, inhibitory neurons within the dorsal horn of the spinal cord as well as immune cells that co-express CRF receptors and opioid peptides (Mousa et al., 2007). In

addition, it is known that some inflammatory mediators produce proopiomelanocortin and proenkephalin under inflammatory conditions (Chadzinska et al., 2001; Mousa et al., 2004; Rittner and Brack, 2007) and during these conditions there is an increased expression of opioid receptors in peripheral neurons (Ballet et al., 2003; Ji et al., 1995). Moreover, it has been demonstrated that CRF or stress induced the release of opioid peptides in inflamed tissues (Labuz et al., 2009; Machelska, 2003; Stein and Fuller, 1990). Taken together these results suggest that a selective stimulation of immune cells by CRF led to opioid peptide-mediated activation of opioid receptors to participate in an intense localized inflammatory response. **However, the exact mechanisms by which the CRF family and its receptors are involved in the pathophysiological processes of inflammation is not well known.** Our study clearly demonstrated that the deletion of CRF1 receptor increase the inflammatory response after surgery incision suggesting that the CRF/CRF1 receptor could be implicated in the inflammatory response to tissue injury. **In this regard, CRF reduced the nociceptive response induced by formalin injection into the hind paw in mice while NBI 2714, a selective CRF1 antagonist, completely blocked the antinociceptive effect of CRF; antisauvagine 30, a CRF2 receptor antagonist, did not alter the CRF effects. (Miguel et al., 2011).** However, previous data have demonstrated that NBI 27914 and CP-154,526, selective CRF1 receptor antagonist, were effective at alleviating acute inflammatory hyperalgesia induced by carrageenan in rats (Hummel et al., 2010). These discrepancies suggest a need for further studies with different animal models of pain to better understand the role of CRF1 receptor in the neurobiology of pain modulation.

Although, differences in several nociceptive tests have previously been described across strains (Mogil et al., 1999), it is the first time that pain sensitivity in response to chemical and

thermal stimuli has been evaluated in B6,129CRH^{tklee} mice. Our results showed no differences in pain sensitivity to chemical (**visceral pain**) and thermal stimuli (**somatic pain**) between CD1 and B6,129CRH^{tklee} mice in the behavioral tests. In the current behavioral experiments, we showed that KO mice presented more number of writhes than WT and CD1 mice. However, there is no difference between KO and WT or CD1 mice in tail immersion test. The different results obtained in chemical and thermal stimulus could be due to the different mechanisms involved in these tests. Acetic acid is a widely used for the evaluation of peripheral antinociceptive activity and pain is generated indirectly via endogenous mediators, such as bradykinin, serotonin, histamine, substance P, and PGs, especially PGI₂ as well as pro-inflammatory cytokines such as IL-1, IL-6, IL-8 and TNF- α , stimulating the peripheral nociceptor and sensitive neurons that were responsive to the inflammatory mediators (Le Bars et al., 2001). Whereas, tail flick used to evaluate central pain, is proposed to be mediated in large part by spinal mechanisms (Carsten, 2009). **Our results suggesting that CRF1 receptor could not be implicated in the central pain induced by thermal stimulus are in agree with previous results demonstrating that CRF produces not effect on thermal nociception (tail flick assay) (Ayesta and Nikolarakis, 1989; Poree et al., 1989; Song and Takemori, 1991) and they are consistent with the failure of CRF overexpression to influence thermal hyperalgesia (van Gaalen et al., 2002).**

On the other hand, the deletion of CRF1 gen induces higher responses to inflammatory irritant modality, suggesting a role of CRF/CRF1 receptor in the inflammatory processes. **In contrast to our data recently report demonstrated, in a rat visceral pain model, that subcutaneous injection of E2508, a selective CRF1 receptor antagonist, significantly decreased the number of abdominal muscle contractions induced by colonic distension,**

suggesting that this drug reduced visceral pain (Taguchi et al., 2017). In addition, CRF1 antagonist, CP-376395 to dampen the visceral hypersensitivity to colorectal distension in the wistar kyoto rats strain. (Su et al., 2014). However previous studies (Miguel et al., 2011; 2012) using a chemical stimulus (formalin test) demonstrated that NBI 27914 blocks the antinociceptive effects of CRF in Swiss mice. Altogether these data clearly indicate that the exact mechanisms by which the CRF and CRF1 receptor are involved in the pathophysiological processes of pain is not well known.

The results of present study suggest that CRF/CRF1 receptor pathways could be playing a role in the naloxone-induced hyperalgesia after surgery performed under remifentanyl plus sevoflurane anaesthesia. Moreover, the deletion of CRF1 receptor gene increased the local inflammatory response induced by injury suggesting a possible role of this receptor in the inflammatory processes. Although this finding could be relevant to improve pain relief in the postoperative period, the clinical relevance of the results should be determined in the clinical setting.

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Conflict of interest statement

The authors have declared that no competing interests exist.

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Figure legends

Figure 1. Surgery was performed under sevoflurane plus remifentanyl anaesthesia, administered for a period of 30 min. Nociceptive behaviour was assessed at 4 h and, 2, 20 and 21 days after surgery. On day 21, mice received a challenge with subcutaneous naloxone (Nx, 1mg/kg s.c.). In a different group of experiments plasma extravasation was assessed after the intravenous injection of 60 mg/kg of Evan's blue (i.v., retroorbital plexus) 30 min before surgery, and then again on postoperative day 2 (See methods)

Figure 2. Changes in mechanical thresholds after stimulation with von Frey filaments, in CD1, and B6,129CRH^{tklee} WT and CRF1 receptor-KO mice. Results expressed as percent changes (%) in mechanical thresholds over baseline, (represented by the broken horizontal line); vertical lines indicate S.E.M. Nociceptive behaviour was assessed at 4 h and on days, 2, 20 and 21, after surgery. On day 21 mice received a s.c. injection of naloxone (1mg/kg). CD1 mice, white columns (n=8); wild type, grey columns (n=10); KO, black columns (n=10). ++p<0.01, +++p<0.001 versus CD1 injected with saline. **p<0.01 versus CD1 or WT injected with naloxone; &&versus KO injected with saline. One-way ANOVA test for repeated measures was used (for each group), followed by Dunnett test. **Two-way ANOVA was performed for the factor treatment and strains followed by Bonferroni post hoc analysis.**

Figure 3. Plasma extravasation in CD1 and B6,129CRH^{tklee} (WT and KO) mice 2 days after surgery. Plasma extravasation was assessed after the injection of Evan's blue, and the dye in the manipulated paw measured by spectrophotometry (see Methods). Each column represents the mean values, obtained preoperatively (pre) and 2 days after surgery (2d); vertical lines indicate the S.E.M. CD1 mice (n=5), WT (n= 5) and KO (n=5). *p<0.05, **p<0.01

versus pre-surgery CD1; ++ $p < 0.01$ versus post-surgery Comparisons performed with non-parametric Kruskal-Wallis ANOVA and Mann Whitney tests.

Figure 4. Inflammatory response induced by surgery in B6,129CRH^{tklee} WT and CRF-1R KO mice 2 days after surgery

Figure 5. Chemical nociceptive behavior in the Acetic Acid Writhing test (A) and tail immersion (B) tests in CD-1 and B6,129CRH^{tklee} (WT and KO) mice. A) Each column represents the mean number of writhes in the Acetic Acid Writhing test, and the vertical bars indicate the S.E.M. B) Tail immersion test, the columns represent mean latency times in seconds and vertical lines indicate the S.E.M. Three groups of animals were used: CD1 (n=8), WT (n=10), and KO (n=10). Comparisons between groups performed with a non-parametric Kruskal-Wallis ANOVA test and Mann Whitney. ** $p < 0.01$ compared with CD1 and WT mice. B) S.E.M. N=12 for CD1, and n=10 each for WT and KO mice. Kruskal-Wallis ANOVA test and Mann Whitney. *** $p < 0.001$ compared with CD1 mice.

Table 1. Tests were performed in WT and KO B6,129CRH^{tklee} mice, in two experimental conditions: after a surgical incision and non-injured mice. The same tests were performed in CD1 that served as reference

Experimental condition	Manipulations
Plantar incision (surgical injury)	<ul style="list-style-type: none"> • mechanical hyperalgesia (von Frey) • plasma extravasation (Evan's Blue)
Non-injured mice	<ul style="list-style-type: none"> • Acetic Acid writhing test • Tail flick test • Mechanical hyperalgesia (von Frey)

Table 2. Baseline and postoperative mechanical thresholds in CD1 and C57BL/6 (WT and CRF1 receptor KO) mice after surgery

Hind paw (operated)	Baseline (g)	Postoperative 4 h	Mechanical 2d	Thresholds 20 d+saline 21d+Nx	
CD1	1.09±0.15	0.32±0.01**	0.44±0.01**	0.96±0.02	0.46±0.02**
C57BL/6 WT	0.97±0.16	0.19±0.06**	0.28±0.04**	0.65±0.02*	0.52±0.02*
C57BL/6 KO	0.98±0.10	0.17±0.09**	0.47±0.14**	0.60±0.05*	0.17±0.02**

Results are expressed as mean values±SEM of mechanical thresholds (g). Baseline values obtained before surgery and 4 h and on days 1,20 and 21 after manipulation. On day 21 a s.c. challenge of naloxone (Nx, 1 mg/kg) was administered. *p<0.05, **p<0.01 compared with baseline value.

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C57BL/6 KO	0.98±0.10	0.17±0.09**	0.47±0.14**	0.60±0.05*	0.17±0.02**

Results are expressed as mean values±SEM of mechanical thresholds (g). Baseline values obtained before surgery and 4 h and on days 1,20 and 21 after manipulation. On day 21 a s.c. challenge of naloxone (Nx, 1 mg/kg) was administered. *p<0.05, **p<0.01 compared with baseline value.

-The CRF is involved in different physiological functions including pain perception and inflammation but the role of CRF receptors are not well known. We have used genetically engineered mice lacking CRF1 receptor to evaluate the role of this receptor in the long-lasting post-surgical changes in nociceptive thresholds and in local inflammatory responses.

-Wild-type and KO mice underwent a plantar incision under anaesthesia with remifentanyl (80 μ g/kg sc) and sevoflurane presented hyperalgesia which was higher in KO versus wild-type mice. In addition, KO mice showed a higher response to inflammatory irritant modality. Taken into account our results suggest suggesting a role of CRF/CRF1 receptor in latent pain sensitization induced by surgery and in the inflammatory processes.

-The results of the present study may contribute to further understanding the mechanisms implicated in the latent pain sensitization and in the local inflammatory response to tissue injury.