

Original Article

The antinociceptive effect of 17β -estradiol in the nucleus paragigantocellularis lateralis of male rats may be mediated by the NMDA receptors

Roghaieh Khakpay^{1*}, Maryam Azaddar¹, Fatemeh Khakpay²

1. Department of Animal Science, Faculty of Natural Sciences, University of Tabriz, Tabriz, Iran
2. Institute for Cognitive Science Studies (ICSS), Tehran, Iran

Abstract

Introduction: The nucleus paragigantocellularis lateralis (LPGi) is involved in the descending pain modulation. The neurosteroid, 17β -estradiol found in the LPGi nucleus and modulates nociception by binding to estrogen receptors and also by allosteric interaction with NMDA receptors. In this study, the role of NMDA receptors in the 17β -estradiol-induced pain modulation was investigated by assessing the inflammatory pain responses changes after blockade of the LPGi nucleus' NMDA receptors.

Methods: In order to study the antinociceptive effect of intra-LPGi microinjection of 17β -estradiol, a guide cannula was implanted into the right LPGi nucleus. 500 nl of drugs were administered 15 minutes prior to formalin (50 μ l of 4%) injection. Then, formalin-induced paw jerking behaviour was recorded for 60 min. For assessing the role of the NMDA receptors in the pain modulation by 17β -estradiol, it was injected 15 min after the intra-LPGi administration of 0.5 nmol of AP5 (the NMDA receptor antagonist); and paw jerking frequency was recorded for 1 h.

Results: The results of the present study showed that intra-LPGi injection of 0.8 μ mol of 17β -estradiol attenuated the chronic phase ($P < 0.001$) of paw jerking behaviour. AP5 significantly reduced the antinociceptive effect of intra-LPGi 17β -estradiol both in the acute ($P < 0.001$) and in the chronic phase ($P < 0.001$) of formalin test.

Conclusion: According to the results of this study, it can be concluded that the analgesic effect of intra-LPGi injection of 17β -estradiol on the formalin-induced inflammatory pain might be mediated via NMDA receptors.

Keywords:

17β -estradiol;
Paragigantocellularis lateralis nucleus;
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*Correspondence to:

R. Khakpay

Tel: +98-04133339274-5

Fax: +98-04133356027

Email:

rkhakpai@gmail.com

Introduction

Hyperalgesia following peripheral tissue or nerve

damage is related to the increment of the sensitivity of nociceptors at the site of injury. It depends on the N-methyl-d aspartate (NMDA) receptor-mediated central changes in synaptic excitability (Parsons,

2001). The NMDA receptors play a key role in central pain transduction mechanisms (Soleimannejad et al., 2007). These receptors are selectively blocked by the drug APV (2-amino-5-phosphonovaleric acid) (Kandel et al., 2012; Ghasemi et al., 2014). The NMDA receptors are composed of NR1, NR2 (A, B, C, and D), and NR3 (A and B) subunits, which determine the functional properties of native NMDA receptors (Petrenko et al., 2003). Excitatory amino acids (EAAs) bind the NMDA receptors (Ji and Traub, 2002). There are evidences that EAAs mediate nociceptive inputs to the spinal cord (Yashpal et al., 2001). EAAs like glutamate are found in the nerve terminals of spinal nociceptive neurons and released in the spinal cord by peripheral noxious stimuli, hereby act on the NMDA receptors (Yashpal et al., 2001).

The LPGi is a reticular nucleus in the ventral portion of the rostral medulla oblongata, where it has a role in descending pain modulation through the spinal cord (Erami et al., 2012; Soleimani et al., 2013; Azhdari-Zarmehri et al., 2014; Shamsizadeh et al., 2014; Azhdari-Zarmehri et al., 2015). The LPGi nucleus is stretched in the medulla oblongata and receives its afferents from vestibular nucleus, tractus solitarius, lemniscus nucleus, and lateral hypothalamus (Azhdari-Zarmehri et al., 2013). The LPGi neurons send their efferents to important nuclei such as ventral tegmental tract, arcuate nucleus, caudal raphe nucleus, and locus coeruleus (LC) (Andrezik et al., 1981). The LPGi nucleus is involved in the cardiovascular regulation (Van Bockstaele et al., 1993), control of sleep-wake cycle, respiratory system (Arita et al., 1988), sexual behavior (Fathi-Moghaddam et al., 2006), consciousness (Van Bockstaele et al., 1993), dependence and addiction (Azizi et al., 2005), as well as pain modulation (Arita et al., 1988; Van Bockstaele et al., 1993; Fathi-Moghaddam et al., 2006; Erami et al., 2012; Azhdari-Zarmehri et al., 2013). The LPGi neurons respond to painful stimuli and relay the processed pain and sensory information into the LC nucleus. Therefore, the LPGi nucleus plays a key role in the processing of pain information associated with descending pain modulation (Aston-Jones et al., 1991).

Besides of their well-known hormonal mode of action, estrogens such as 17 β -estradiol influence brain function by direct effects on the neuronal membranes (Balthazart and Ball, 2006). Estrogenic steroids,

especially 17 β -estradiol, is synthesized in the nervous system from cholesterol through an aromatase-dependent conversion of testosterone (Grassi et al., 2010). The pain modulatory role of 17 β -estradiol is shown well (Craft et al., 2004). 17 β -estradiol interacts with glutamate and GABA neurotransmitter receptors in various brain regions. 17 β -estradiol modulates nociception by binding to its receptors and also by allosteric interaction with other membrane-bound receptors like glutamate receptors (Potes et al., 2006).

Considering the key role of LPGi nucleus in the modulation of pain (Aston-Jones et al., 1991), and the interaction between 17 β -estradiol and NMDA receptors in the modulation of pain (Potes et al., 2006; Khakpay et al., 2010b), the present study was designed to assess the possible involvement of the membrane-bound NMDA receptors in the pain modulatory effect of intra-LPGi injection of 17 β -estradiol in the male rats.

Materials and methods

Animals

Experiments were performed on adult male Wistar rats weighing 200–270 g purchased from Razi Institute (Hesarak Karj, Iran). Animals were housed at a room temperature of 22–24°C, with free access to water and food under a 12/12 h light/dark cycles. The experiments were carried out during the light phase. All research and animal care procedures were performed according to the guidelines on the use of laboratory animals and approved by Tabriz University ethical committee for animal research.

Surgery

The animals were gently handled 5 min/day for a week before the experiment for acclimatization. On the day of the surgery, the rats were anesthetized with intraperitoneal injection of ketamine (60 mg/kg) and xylazine (7.5 mg/kg). A guide cannula (23 gauge) equipped with a 30 gauge stylet was stereotaxically implanted into the right LPGi [coordinates from Bregma: AP: -11.9 mm, L: \pm 1.6 mm, DV: 10.4 mm (Paxinos and Watson, 2005)]. The guiding cannula was attached to the skull with a stainless steel screw and acrylic cement (Dentimax, the Netherlands). All animals were left to recover for 5–7 days prior to behavioral testing.

Drugs

The 4% formalin (Purchased from the Dr. Mojallaly's company) solution was injected subcutaneously into the left hind paw [50 μ l (Khakpay et al., 2014)]. Water soluble cyclodextrin-encapsulated 17 β -estradiol [0.8 μ mol; (Aloisi and Ceccarelli, 2000; Khakpay et al., 2014)], and AP5 - the NMDA receptor antagonist - [0.5 μ mol; (Khakpay et al., 2010a)] were purchased from Sigma (Sigma Chemicals, St. Louis, MO, USA). 17 β -Estradiol and AP5 were dissolved in normal saline.

Injections

Intra- LPGi injections were done as previously described by Aloisi and Ceccarelli (Aloisi and Ceccarelli, 2000). Considering both contralateral ascending of nociceptive information and left hind paw as the site of formalin injection, all injections were unilaterally done in the right side through the guide cannula using an injection needle (30 gauge) connected by polyethylene tubing to a 0.5 μ l Hamilton microsyringe (Hamilton, Switzerland). The injection needle was replaced by the stylet and its tip was 0.2 mm beyond the guide cannula. According to our previous investigations (Khakpay et al., 2014), all substances were injected in a volume of 500 nl. The needle was removed and the stylet replaced sixty seconds after infusing the substance.

Formalin test

The animals were randomly divided into 6 groups, including the control group (intact animals), the second group or sham (only cannulation into the LPGi nucleus), the third group (saline intra-LPGi injection of saline as solvent), the fourth group (intra-LPGi injection of 0.8 μ mol 17 β -estradiol), the fifth group [intra-LPGi injection of 0.5 μ mol AP5 (2-amino-5-phosphonovalerate)], and the sixth group (intra-LPGi injection of 0.5 μ mol AP5, 15 min before the intra-LPGi administration of 0.8 μ mol 17 β -estradiol). Diluted formalin was intraplantarly injected to induce nociceptive responses. Animals were adapted to the experimental room and test chamber for 20 min/day, for 2 days before the experiment. In order to study the involvement of the NMDA receptors in the antinociceptive effect of 17 β -estradiol, AP5 was injected 15 min prior to 17 β -estradiol administration, and then formalin test (Dubuisson and Dennis, 1977) was done 15 min after 17-estradiol injection.

Therefore, 50 μ l of 4% formalin solution was subcutaneously injected into the plantar surface of the left hind paw using a 30 gauge needle (Khakpay et al., 2014). Following the formalin injection, the animals were then immediately returned to their observation box (a square transparent plexiglas cage, 30 cm x 30 cm x 30 cm) and the total time spent the licking and flexing behaviors were recorded over 5 min intervals (Wheeler-Aceto and Cowan, 1991; Aloisi et al., 1998; Khakpay et al., 2010b; Khakpay et al., 2014). The responses observed were divided into two phases: first phase (0–7 min) and second phase (15–60 min) (Mahmoudi and Zarrindast, 2002; Khakpay et al., 2014). By the end of the experiment, the rats were sacrificed by diethyl ether and the brains were removed and checked for the correct cannula placement in the LPGi. Only data obtained from animals with correct placement of cannula were included in the analysis.

Statistical analysis

All results were analyzed by SPSS software and presented as mean \pm S.E.M. One-way analysis of variance (ANOVA) followed by Post Hoc Tukey's test was used to compare differences between treatments. $P < 0.05$ was considered statistically significant.

Results

Animals belonging to the sham operated (The LPGi cannulation without intra-LPGi injections) and saline groups (intra-LPGi injections of saline) did not show any significant differences compared with the control group (intact animals); therefore, they were not included in the results. The mean response of the first 7 min post-formalin injection was considered as the acute phase and the mean response over 45 min between 15 and 60 min post-formalin injection was considered as the chronic phase.

Effects of 17 β -estradiol on formalin-induced responses

Intra-LPGi injections of 0.8 μ mol 17 β -estradiol significantly reduced paw jerking frequency in the chronic phase ($P < 0.001$, Fig.1). The results indicate that 17 β -estradiol has antinociceptive effect in this dose on the paw jerking response; thus, this concentration was used for the subsequent

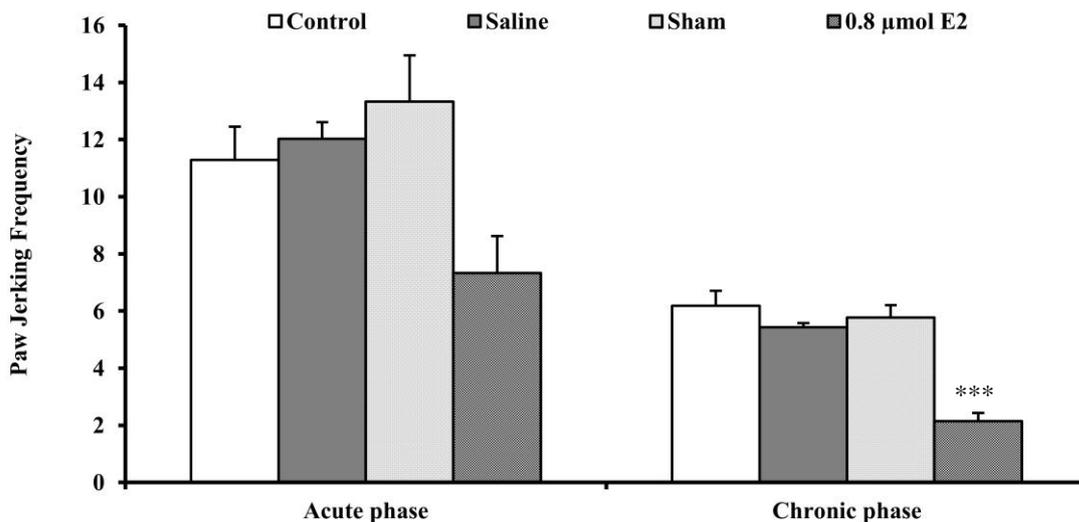


Fig.1. Effect of intra-LPGi injection of 0.8 μmol 17β-estradiol on paw jerking behavior following 50 μl of 4% formalin injected into the plantar surface of the left hind paw. The graph shows data for the acute and the chronic phase of formalin-induced responses in comparison with control, sham and saline-injected animals. The nociceptive response is presented by mean ± SEM of paw jerking frequency of six rats per group.

*** indicates significant difference from control group (P<0.001). 0.8 micromol 17β-estradiol = 0.8 μmol E2.

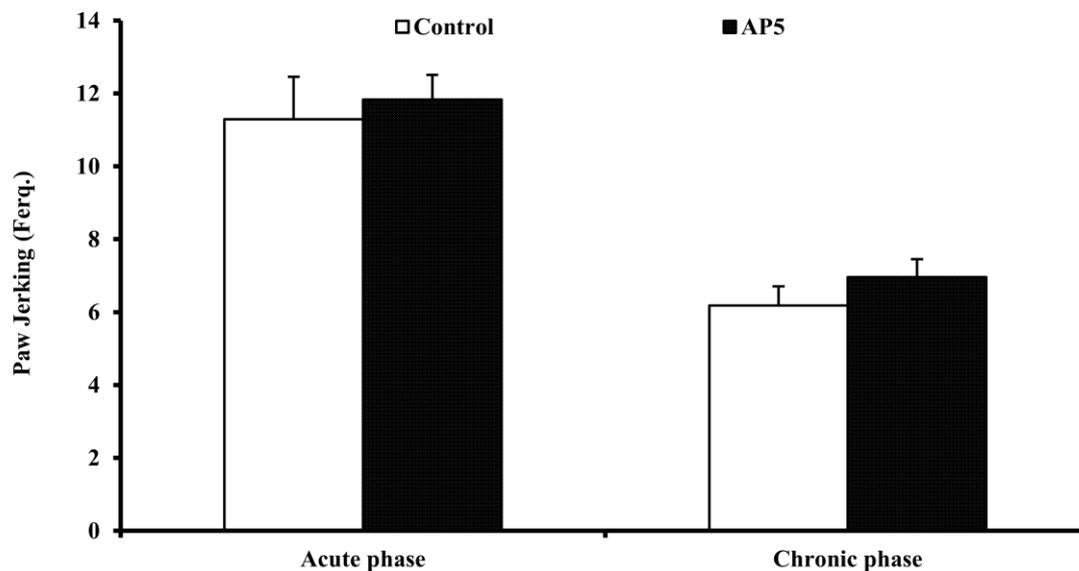


Fig.2. Nociceptive response (paw jerking) during the acute and the chronic phase of the formalin test (4%, 50 μl) in rats treated with AP5 (0.5 nmol) 15 min before formalin injection. The data are represented as mean ± SEM for six rats. 0.5 nanomol AP5 = 0.5 nmol AP5

experiments.

Effects of AP5 on formalin-induced responses

To clarify the mechanism of the antinociceptive effect of 17β-estradiol and the receptors involved, we tried to find a dose of NMDA antagonists without any significant effect on nociception. Consequently, intra-LPGi injections of 0.5 nmol of AP5 did not show any

significant differences compared with the control group (Fig. 2). In other words, AP5 had no pronociceptive effect and could not interfere with analgesic effect of 17β-estradiol.

Effects of NMDA receptor antagonists on the antinociceptive effect of 17β-estradiol

For studying the possible involvement of NMDA

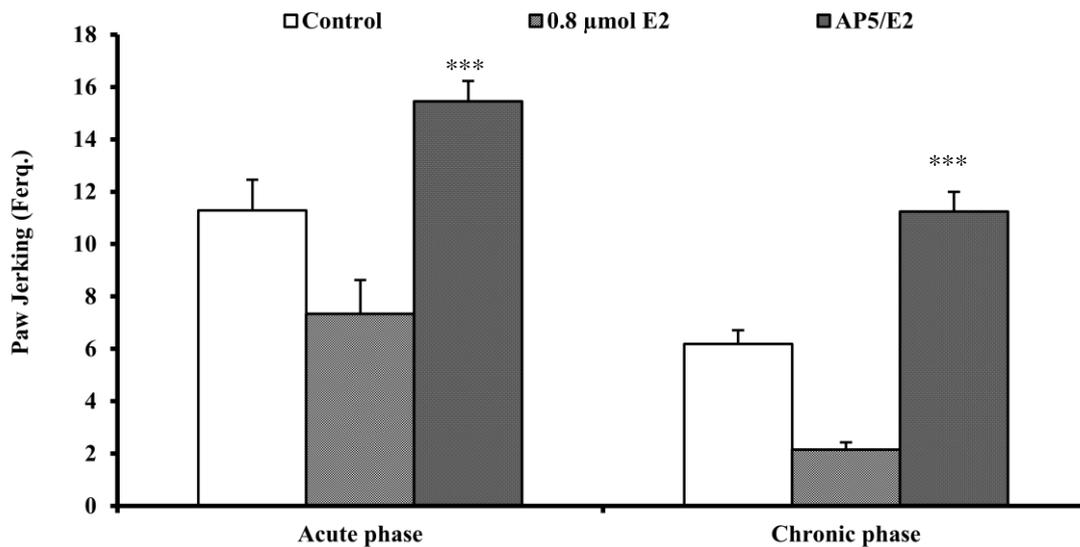


Fig.3. Effects of NMDA receptor antagonist on the paw jerking responses of 17β -estradiol. AP5 (0.5 nmol) were administered 15 min before intra-LPGi injection of 0.8 μmol 17β -estradiol. Data are presented as mean \pm SEM for six rats. *** indicates significant difference of the E2/AP5 group from the 17β -estradiol group ($P < 0.001$). 0.8 micromol 17β -estradiol = 0.8 μmol E2 and 17β -estradiol/AP5 = E2/AP5

receptors in the antinociceptive effect of 17β -estradiol, AP5 were applied 15 min before the injection of 17β -estradiol and pain-related behavior was examined following formalin injection.

Pre-treatment with 0.5 nmol AP5 significantly reverse the effect of 0.8 μmol intra-LPGi 17β -estradiol on paw jerking frequency in the both phases of formalin-induced pain ($P < 0.001$, Fig.3).

Discussion

In the present study, intra-LPGi injection of 17β -estradiol was used to assess the effect of this neuroactive steroid on centrally mediated behavioral responses to nociceptive stimulus. Our results indicated that 17β -estradiol treatment attenuated the chronic phase of paw jerking behaviour. According to the previous findings of our laboratory, 17β -estradiol microinjection into the LPGi nucleus induces strong analgesia. A part of this analgesic effect is mediated by estrogen receptors (Khakpay et al., 2014). 17β -Estradiol acts as a neuroactive steroid which plays a key role in the pain modulation (Khakpay et al., 2010a; Khakpay et al., 2010b). It controls nociception through binding to its classic receptors and by allosteric interaction with other membrane-bound receptors such as glutamate and GABA_A receptors (Potes et al., 2006; Khakpay et al., 2010b). Estradiol

increases the spinal processing of visceral nociception by increasing the NMDA receptor NR1 subunit expression and increasing site-specific receptor phosphorylation on the NR1 subunit contributing to an increase in the NMDA receptor activity (Tang et al., 2008).

In the present study, we hypothesized that the analgesic effect of intra-LPGi injection of 17β -estradiol on the formalin-induced inflammatory pain may be probably mediated via the NMDA receptors. Consistent with our findings, Khakpay et al. (2010) revealed that the analgesic effect of intra-LC injection of 17β -estradiol on the formalin-induced responses is mediated via the interaction with membrane-bound NMDA receptors (Khakpay et al., 2010b). However some studies failed to identify any changes in the NMDA receptor protein levels following 17β -estradiol administration (Snyder et al., 2011; Nebieridze et al., 2012). Therefore, it is unclear how 17β -estradiol modulates the NMDA receptor-mediated effects on the pain modulation (Nebieridze et al., 2012).

Moreover, our results indicated that NMDA receptor antagonist could not significantly affect both the acute and the chronic phases of formalin test. The NMDA receptors play a critical role in nociceptive processing (Yashpal et al., 2001). Peripheral NMDA receptors are also involved in inflammatory somatic and visceral pain (Parsons, 2001). NMDA receptor

antagonists attenuate pain behaviors in models of neuropathic and tonic pain, when applied to the CNS (Yashpal et al., 2001). Intrathecal (i.t.) administration of selective NMDA receptor antagonists produces antinociceptive effects in both phasic and tonic nociceptive tests in rats, as well as decrement of hyperalgesia associated with inflammatory or neuropathic injury in rats (Yashpal et al., 2001). Glutamate receptors - including NMDA, AMPA, and Kainate receptors- of the medullary dorsal reticular nucleus play a key role during the development and maintenance of formalin-induced secondary allodynia (Ambriz-Tututi et al., 2013). Activation of NMDA receptors via glycine sites at the supraspinal level induces an antinociceptive effect on both acute and chronic pain (Ito et al., 2014). Pre-emptive administration of ketamine -a NMDA receptor antagonist- obviously prevents the pain behavior response during the second phase of formalin test (Long et al., 2013). St-Ht31 (stearylated Ht31 peptide which inhibits AKAPs/PKA interaction) inhibits the NMDAR-mediated nociceptive transmission and effectively ameliorated CFA-induced inflammatory pain (Wang et al., 2015). The NMDA receptors of amygdala may be involved in the modulation of the minocycline-induced potentiation of morphine analgesia in the tail-flick test (Ghazvini et al., 2015). The peptide NMDA receptor antagonist SHG improves opioid antinociception, but this improvement is dependent on the animal model, behavioral endpoint, and opioid (Hama and Sagen, 2014).

To decipher the mechanism of actions of neuroactive steroids, the NMDA receptor action has attracted the most attention, in recent decades. Therefore, the aim of this study was to find out whether pretreatment of LPGi nucleus with a NMDA receptor antagonist reduces the 17β -estradiol-induced antinociceptive behaviors. For this purpose, we tried to find a dose of antagonist without any significant nociceptive effect. Microinjection of 0.5 nmol of AP5 into the LPGi nucleus did not have any significant nociceptive effect in the formalin test. Therefore, 0.5 nmol AP5 was chosen as the ideal dose.

In the present study, pretreatment of LPGi nucleus with AP5 reversed the 17β -estradiol-induced decrement in the paw jerking behavior. Our results showed that a part of the analgesic effect of intra-LPGi 17β -estradiol on the formalin-induced inflammatory pain is probably mediated by NMDA

receptors. Pretreatment of the LPGi nucleus with AP5 significantly reversed both the acute phase and the chronic phase of the paw jerking behaviour. Consistent with the results of this study, soleimannejad et al (2007) reported that microinjection of the NMDA receptor antagonist AP5 into the dentate gyrus region of the hippocampus attenuated pain behaviors both in the acute and in the tonic phases of the formalin test (Soleimannejad et al., 2007). Coderre and Van Empel (1994) showed that during the late phase of the formalin test, the spinal cord neurons release excitatory amino acids and the NMDA receptor subtypes are activated (Coderre and Van Empel, 1994). Similar to our results, they concluded that intrathecal injection of selective NMDA antagonists prevents the nociceptive behavior of the late phase of formalin test (Coderre and Van Empel, 1994). Mangiferin, a glucosylxanthone from *Mangifera indica*, shows ability to decrease tonic pain in the formalin test. Acute administration of MG reduced licking/biting exclusivity in the tonic phase of formalin test. This effect was enhanced by non-competitive NMDA antagonist ketamine (Garrido-Suárez et al., 2014). Ito et al (2014) reported that using the tail-flick test, intracerebroventricular administration of D-serine, an endogenous co-agonist at the glycine sites of NMDA receptors, elicited an antinociceptive effect on thermal nociception. In agreement with the results of the current study, they suggested that activation of NMDA receptors via glycine sites at the supraspinal level induces an antinociceptive effect on both acute and tonic pain (Ito et al., 2014). A few reports have described that the intrathecal administration of NMDA in rodents can also induce various types of analgesic responses (Lee et al., 2004). Also, intrathecal NMDA has produced a delayed antinociceptive response in the tail flick test in rats (Lee et al., 2004).

Conclusion

In conclusion, our data revealed that intra-LPGi injection of 17β -estradiol is sufficient to produce strong analgesia. The antinociceptive effect of 17β -estradiol was prevented by AP5. These data suggest that 17β -estradiol-induced analgesia in the LPGi nucleus is probably mediated by non-estrogen receptors. With regards to the membrane-bound receptors, the NMDA receptors seem to be involved

in the 17 β -estradiol-mediated antinociception in the LPGi nucleus, but further investigations by molecular and electrophysiological approaches are still required.

Acknowledgments

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Conflict of interest

The authors have declared no conflict of interest.

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