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Original Article

# The role of $GABA_A$ receptors in the analgesic effect of intra-paragigantocellularis lateralis injection of 17β-estradiol in male rat

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## Abstract

**Introduction:** 17β-Estradiol modulates nociception by binding to the estrogenic receptors and also by allosteric interaction with other membrane-bound receptors like glutamate and $GABA_A$ receptors. In addition to its autonomic functions, paragigantocellularis lateralis (LPGi) is involved in the pain modulation, too. The aim of the current study was to investigate the involvement of the membrane-bound $GABA_A$ receptors in the pain modulatory effect of intra- LPGi injection of 17β-estradiol of male rats.

**Materials and Methods:** This study was performed using male Wistar rats in the range of 200-270 g. In order to investigate the antinociceptive effect of intra-LPGi microinjection of 17β-estradiol, cannulation into the LPGi nucleus was performed. 500 nl of drugs were administered 15 minutes prior to formalin injection (50 µl of 4%). Then, formalin-induced paw jerking behaviour was recorded for 60 min. For assessing the role of the $GABA_A$ receptors in the estradiol induced pain modulation, 17β-estradiol was administered into the LPGi nucleus 15 min after the injection of 25 ng/µl bicuculline; and paw jerking frequency was recorded for 1 h.

**Results:** The results of the current study showed that intra-LPGi injection of 0.8 µmol of 17β-estradiol attenuated the chronic phase ($P<0.001$) of paw jerking behaviour. Bicuculine -the $GABA_A$ receptor antagonist- significantly reduced the antinociceptive effect of intra-LPGi 17β-estradiol in the chronic phase ($P<0.001$).

**Conclusion:** It may be concluded that the analgesic effect of intra-LPGi injection of 17β-estradiol on the formalin-induced inflammatory pain might be mediated via $GABA_A$ receptors.

**Keywords:**

17β-Estradiol;
Paragigantocellularis lateralis nucleus;
$GABA_A$ receptor;
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## Introduction

International association for the study of pain describes pain as the unpleasant sensory and emotional experiences associated with actual or potential tissue damage (Kandel *et al.*, 2012). The perception of pain is very subjective and is influenced by many factors like

estradiol and/or testosterone hormones (Aloisi and Bonifazi, 2006).

Estradiol is a neuroactive steroid found in several brain areas such as LC (Khakpay *et al.*, 2010) and LPGi nuclei (Khakpay *et al.*, 2014). It modulates nociception by binding to its classic receptors and by allosteric interaction with other membrane-bound receptors like glutamate and $GABA_A$ receptors (Potes *et al.*, 2006, Khakpay *et al.*, 2010). The neuroactive steroids like 17β-estradiol are potent positive allosteric modulators of GABA receptors because they increase the frequency and/or duration of openings of GABA-gated chloride channel (Rupprecht and Holsboer, 1999). However, very high concentrations -in the micromolar range- of these neuroactive steroids have been shown to exert a certain intrinsic agonistic activity in the absence of GABA (Rupprecht *et al.*, 2001). Intra-LC injection of 17β-estradiol has an analgesic effect on the formalin-induced inflammatory pain; and this effect is mediated through interaction with membrane-bound receptors (Khakpay *et al.*, 2010). Furthermore, intra-LPGi addition/injection of 17β-estradiol has an analgesic effect on the formalin-induced behaviours/inflammations. A Portion of this pain-relieving effect of 17β-estradiol in the LPGi nucleus is probably mediated by estrogenic receptors (Khakpay *et al.*, 2014, Khakpay *et al.*, 2015).

The paragigantocellularis nucleus (LPGi) is a reticular nucleus in the ventral portion of rostral medulla oblongata. The LPGi nucleus is extended in medulla and receives its projections from vestibular nucleus, tractus solitarus, lemniscus nucleus and lateral hypothalamus (Azhdari-Zarmehri *et al.*, 2013). LPGi neurons project 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), 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, 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).

$GABA_A$ receptors are extensively distributed in different regions of the central nervous system (Yang *et al.*, 2002) including rostral Ventrolateral Medulla (RVLM), (Fields and Basbaum, 1999, Foley *et al.*, 2003). In the rat brain, the RVM includes the nucleus raphe magnus (NRM), nucleus reticularis gigantocellularis pars α (Rgcα) and LPGi (Fields *et al.*, 1991, Mason, 1999, Yang *et al.*, 2002, Willis Jr and Coggeshall, 2004) Also, GABAergic neurons have been identified in the LPGi (Dehkordi *et al.*, 2007). Neuroactive steroids produce spinally-mediated antinociception through combination with spinal cord $GABA_A$ receptors (Nadeson and Goodchild, 2000, Goodchild *et al.*, 2009). Progestins' modulation of pain may occur via the GABA receptors (Frye and Duncan, 1994).

Since the LPGi nucleus plays a key role in the modulation of pain (Aston-Jones *et al.*, 1991), and considering the interaction between 17β-estradiol and $GABA_A$ receptors in the modulation of pain (Potes *et al.*, 2006, Khakpay *et al.*, 2010), the present study was designed to assess the extent of involvement of the membrane-bound $GABA_A$ receptors in the pain modulatory effect of intra-LPGi injection of 17β-Estradiol of male rats.

# Materials and methods

## 2.1. 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 cycle. 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.

The animals were randomly divided into 6 groups, including the control group (formalin test in the intact animals), the second group or sham (only cannulation and formalin test), the third group (saline injection as

solvent into the LPGi nucleus and formalin test), the fourth group (intra-LPGi injection of 0.8 µmol17β-estradiol and formalin test), the fifth group (intra-LPGi injection of 2.5 µmol bicuculin and formalin test), the sixth group (intra-LPGi injection of 2.5 µmol bicuculin 15 min before the administration of 0.8 µmol intra-LPGi17β-estradiol and formalin test) sets.

## 2.2. 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 in the right LPGi [coordinates from Bregma: AP: -11.9 mm, L: ±1.6 mm, DV: 10.4 mm (Paxinos and Watson, 2004)]. 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 behavioural testing.

## 2.3. Drugs

The 4% formalin (Purchased from the Dr. Mojallaly's company) solution was injected subcutaneously into the left hind paw [50 µl (Khakpay *et al.*, 2014)]. Water soluble cyclodextrin-encapsulated 17β-estradiol [0.8 µmol; (Aloisi and Ceccarelli, 1999, Khakpay *et al.*, 2014)], $GABA_A$ receptor antagonist bicuculline methiodide [2.5µmol; (Khakpay *et al.*, 2010)] were purchased from Sigma (Sigma Chemicals, St. Louis, MO, USA). 17β-Estradiol, and bicuculline were dissolved in normal saline.

## 2.4. Injections

Intra- LPGi injections were done as previously described by Aloisi and Ceccarelli, (Aloisi and Ceccarelli, 1999). 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 µl 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, Khakpay *et al.*, 2015), all substances were injected in a volume of 500 nl. The needle was removed and the stylet replaced sixty seconds after infusing the substance.

## 2.5. Formalin test

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 $GABA_A$ receptors in the antinociceptive effect of 17β-estradiol, bicuculline wasnjected 15 min prior 17β-estradiol administration, and then formalin test (Dubuisson and Dennis, 1978) was done 15 min after 17-estradiol injection. Therefore, 50 µl of 4% formalin was subcutaneously injected into the rat's left hind paw using a 30 gauge needle (Khakpay *et al.*, 2014). Following the formalin injection, the animal was returned to the test chamber (a square transparent plexiglas cage, 30 cm ×30 cm ×30 cm) and the number of paw jerking behavior was observed for 60 min (Wheeler-Aceto and Cowan, 1991, Aloisi *et al.*, 1998, Khakpay *et al.*, 2010, Khakpay *et al.*, 2014). The data collected between 0 and 7 min post-formalin injection were considered as the first phase or acute phase and the data collected between 15 and 60 min post-formalin injection were considered as the second phase or chronic phase (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.

## 2.6. Statistical analysis

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

# Results

Animals belonging to the sham operated (LPGi

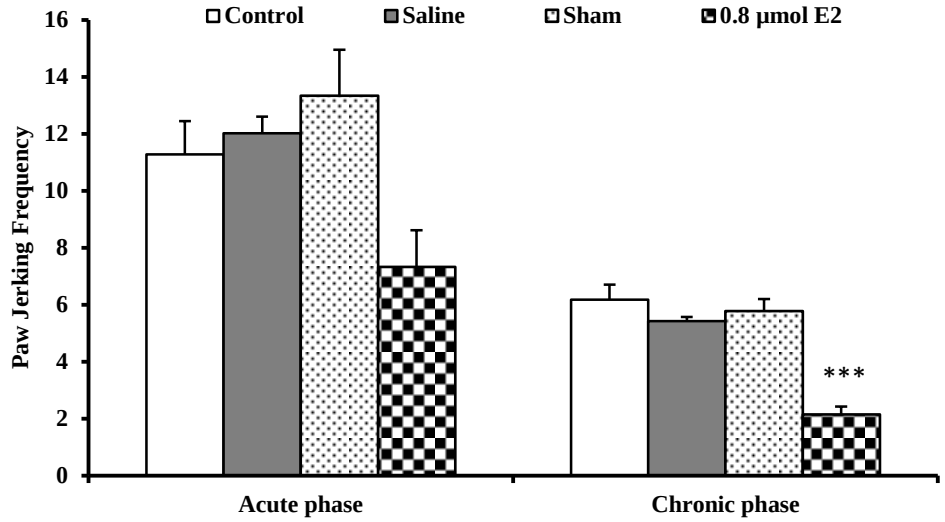


**Fig. 1.** Effect of intra-LPGi injection of 0.8 µmol 17β-estradiol on paw jerking behaviour following 50 µl of 4% formalin injected into the plantar surface of the left hind paw. The graph shows data for the acute and the chronic phase of formalin-induced responses in comparison with control, sham 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<0.05). 0.8 micromol 17β-estradiol = 0.8 µmol E2.

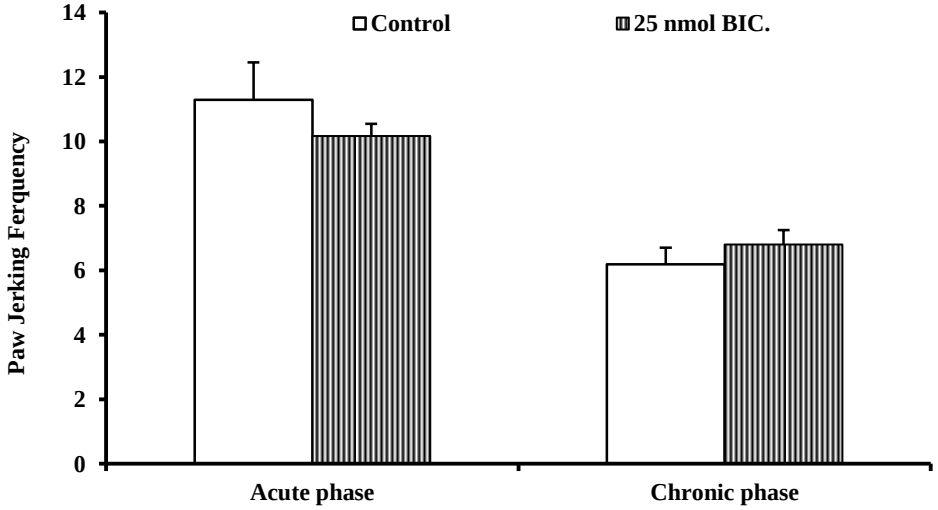


**Fig. 2.** Nociceptive response (paw jerking) during the acute and the chronic phase of the formalin test (4%, 50 µl) in rats treated with bicuculline (25 nmol) 15 min before formalin injection. The data are represented as mean ± SEM for six rats. 25 nanomol bicuculline = 25 nmol BIC.

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) and thus 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.

**3.1.** Effects of 17β-estradiol on formalin-induced responses

Intra-LPGi injections of 0.8 µmol 17β-estradiol significantly reduced paw jerking frequency in the chronic phase (P<0.001, Fig.1).

These results indicate that 17β-estradiol has antinociceptive effect in the highest applied dose in paw jerking response, thus, we used this concentration for the subsequent experiments. To clarify the

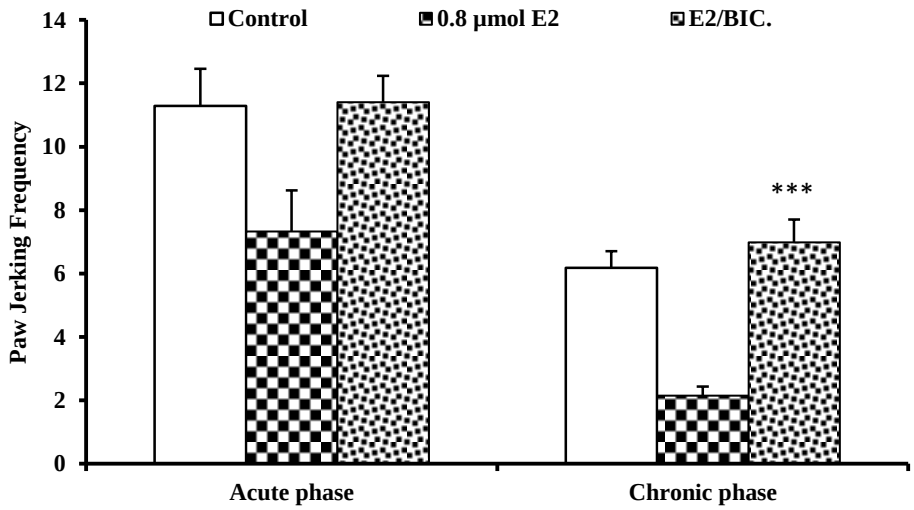


**Fig. 3.** Effects of $GABA_A$ receptor antagonists on the paw jerking responses of 17β-estradiol. Bicuculline (25 nmol) were administered 15 min before intra-LPGi injection of 0.8 µmol 17β-estradiol. Data are presented as mean ± SEM for six rats. *** indicates significant difference of the E2/BIC group from the 17β-estradiol group (P<0.001). 0.8 micromol 17β-estradiol = 0.8 µmol E2 and 17β-estradiol/bicuculline = E2/BIC.

mechanism of the antinociceptive effect of 17β-estradiol and the receptors involved, we tried to find a dose of $GABA_A$ antagonists without any significant effect on nociception. These experiments were performed for bicuculline.

**3.2.** Effects of bicuculline on formalin-induced responses Intra-LPGi injections of 25 nmol of bicuculline did not show any significant differences with the control group (Fig. 2A). That is, bicuculline had no pronociceptive effect and could not interfere with analgesic effect of 17β-estradiol.

For studying the possible involvement of membrane-bound receptors in the antinociceptive effect of 17β-estradiol, bicuculline were applied 15 min before the injection of 17β-estradiol and pain-related behavior was examined following formalin injection.

**3.3.** Effects of $GABA_A$ receptor antagonists on the antinociceptive effect of 17β-estradiol Pre-treatment with 25 nmol bicuculline significantly reverse the effect of 0.8 nmol Intra-LPGi 17β-estradiol on paw jerking frequency in the chronic phase - but not in the acute phase- 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 both the acute and 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 intracellular estrogen receptors (Khakpay *et al.*, 2014, Khakpay *et al.*, 2015). Most of the current pain literature have mentioned the role of the sex steroids in the behavioural responses to acute nociceptive stimuli, but the results have been contradictory. 17β-Estradiol acts as a neuroactive steroid which plays a key role in the pain modulation (Khakpay *et al.*, 2010, Khakpay *et al.*, 2010, Khakpay *et al.*, 2014). Estradiol increases and also decreases the threshold of responses to hot plate and latencies in the tail flick assays (Gordon and Soliman, 1996, Stoffel *et al.*, 2003, Stoffel *et al.*, 2005). 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.*, 2010). 17β-estradiol isa vigorous positive allosteric modulatorof GABA receptors since tit increases the frequency and/or duration of openings of GABA-gated chloride channel (Rupprecht and Holsboer, 1999). In the present study, we have used formalin test, a common model for both acute and persistent pain, to investigate the possible analgesic

effect of 17β-estradiol and its underlying mechanisms in the LPGi nucleus.

In the current study, we hypothesize that the analgesic effect of intra-LPGi injection of 17β-estradiol on the formalin-induced inflammatory pain might be probably mediated via $GABA_A$ receptors. Consistent with our findings, Khakpay and her colleagues revealed that the analgesic effect of intra-LC injection of 17β-estradiol on the formalin-induced responses is probably mediated via the interaction with membrane-bound $GABA_A$ receptors (Khakpay *et al.*, 2010). Involvement in the mediating of analgesic effects of neuroactive steroids like 17β-estradiol is one of the important pharmacological roles of $GABA_A$ receptors (Nadeson and Goodchild, 2000). Mitchell and her colleagues reported that the analgesic actions of neurosteroids are mediated by extrasynaptic $GABA_A$ receptors (Mitchell *et al.*, 2008). Also, Frye and Duncan suggested that the GABA receptor complex action is involved in the pain modulatory effect of progestins (Frye and Duncan, 1994).

Moreover, our results indicated that $GABA_A$ receptor antagonist could not significantly affect both the acute and the chronic phases of formalin test. $GABA_A$ receptors are the principle inhibitory ionotropic receptors in the central nervous system of mammals (Melcangic and Bowery, 1996, Mahmoudi and Zarrindast, 2000, Mahmoudi and Zarrindast, 2002, Yang *et al.*, 2002, Potes *et al.*, 2006). $GABA_A$ receptors play an important role in the mediating a variety of pharmacological events including analgesia (McGowan and Hammond, 1993, Mahmoudi and Zarrindast, 2002). Brave-Hernández et al. (Bravo-Hernández *et al.*, 2014) showed that peripheral $\alpha_5$ subunit-containing $GABA_A$ receptors play a pronociceptive role in the rat formalin test.

The $GABA_A$ receptor is part of a receptor complex that also has binding sites for certain steroids (McGowan and Hammond, 1993). to decipher the mechanism of actions of neuroactive steroids, the GABA receptor complex action has attracted the most attention, in recent decades. Therefore, this study was planned to assessing whether pretreatment with a $GABA_A$ receptor antagonist reduces the 17β-estradiol-induced antinociceptive behaviours in the LPGi nucleus. For this we tried to find a dose of antagonists without any significant nociceptive effect. Microinjection of 25 nmol of bicuculline into the LPGi nucleus did not have any significant nociceptive effect in the formalin test. Therefore, 25 nmol bicuculline was chosen as the ideal dose.

In the present experiment, pretreatment with bicuculine reversed the 17β-estradiol-induced decrement in the paw jerking behaviour. 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 $GABA_A$ receptors. Consistent with our results, McGowan et al. indicated that antinociception produced by activation of neurons in the nucleus reticularis gigantocellularis pars α (NGCpα), is mediated in part by an action of GABA on$GABA_A$ receptors in the spinal cord (McGowan and Hammond, 1993). Pretreatment of LPGi nucleus with bicuculline did not significantly influence the acute phase of formalin test; but the chronic phase of paw jerking behaviour was changed significantly. Mahmoudi and Zarrindast showed that intracerebroventricular (I.C.V.) injection of different doses of muscimol, a $GABA_A$ agonist, in a dose –dependent manner initiated a decline in both phases of formalin-induced pain behaviour. The muscimol-induced responses in both phases of formalin test were reduced by bicuculline (Mahmoudi and Zarrindast, 2002). Similar to our results, they concluded that the stimulation of $GABA_A$ receptors is responsible for antinociception in the formalin test (Mahmoudi and Zarrindast, 2002). In agreement with the results of this study, Suzukia et al. reported that inhibition of formalin-induced nociceptive behaviour is mediated by activation of $GABA_A$ receptors in the spinal cord (Suzuki *et al.*, 2009).

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 bicuculline. These data suggest that 17β-estradiol-induced analgesia in the LPGi nucleus is mostly mediated by non-estrogen receptors. With regards to the membrane-bound receptors, $GABA_A$ receptors seem to be majorly involved in 17β-estradiol-mediated antinociception in the LPGi, but it needs more investigation by molecular and electrophysiological approaches.

## Acknowledgments

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## Conflict of interest

All authors declare that there are no actual or potential conflicts of interest for this study.

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