

## Effect of Intrahippocampal $\gamma$ Aminobutyrate B Receptor on Pain Sensitivity during Estrous Cycle

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**Abstract: Problem statement:** There is a relationship between sexual hormones and pain sensitivity and also a role for hippocampal GABA<sub>B</sub> receptor in nociception. Therefore, the aim of the present investigation was to evaluate the effect of intrahippocampal injection of GABA<sub>B</sub> receptor agonist (baclofen) and GABA<sub>B</sub> receptor antagonist (CGP35348) on pain sensitivity during the estrous cycle. **Approach:** Forty eight adult female rats were used. The animals were divided into four groups: (1) Control; (2) Sham; (3) Baclofen 4.25 or 8.54  $\mu\text{g rat}^{-1}$ ; (4) CGP35348 alone with doses 3 or 5  $\mu\text{g rat}^{-1}$  and (5) CGP35348 3 or 5  $\mu\text{g rat}^{-1}$  after 0.75  $\mu\text{L}$  of baclofen 8.54  $\mu\text{g rat}^{-1}$ . Data were analyzed by two ways ANOVA measuring. The level of significance was  $p < 0.05$ . **Results:** Our data showed that baclofen significantly decreased pain sensitivity in all stages of the estrous cycle, but this analgesic effect was higher during estrus. CGP35348 significantly increased pain sensitivity in all stages of the estrous cycle at 5  $\mu\text{g rat}^{-1}$ , but this hyperalgesic was least effective during the estrus stages of the estrous cycle. Administration of CGP35348 doses 3 or 5  $\mu\text{g rat}^{-1}$  after a high dose of baclofen significantly increased pain sensitivity; This hyperalgesic effect was greater than CGP35348 alone. **Conclusion/Recommendations:** According to our results, the GABA<sub>B</sub> receptor in the hippocampus can modulate pain sensitivity during the estrous cycle.

**Key words:** Estrous cycle, plasma concentrations, intrahippocampal injection, pain sensitivity, receptor agonist, female rats, baclofen significantly, hyperalgesic effect, modulate pain, nociceptive stimulation

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### INTRODUCTION

It is widely recognized that experience of pain is different in women and men. For example, women are generally considered to have a lower pain threshold than men in response to most forms of nociceptive stimulation (Wang *et al.*, 2009). Evidence suggests that gonadal hormones can modulate sensitivity to nociceptive stimuli and opioid antinociception (Terner *et al.*, 2005). The plasma concentrations of these hormones vary during the estrous cycle in rodents. On the other hand, the levels of enkephalin and dynorphins in the hippocampal granule cell mossy fiber pathway can regulate by ovarian steroids (Torres-Reverone *et al.*, 2009). The hippocampal formation has long been implicated in learning and memory functions, as well as in affective motivational response to noxious stimulus (McKenna and Melzack, 2001). An interaction of estradiol, important in the modulation of pain mechanisms, is that with the GABA system in the CNS, although the

effects of estrogens on the GABA system are controversial (Aloisi and Bonifazi, 2006). The GABA action is due to the activation of GABA receptors including ionotropic type-A (GABA<sub>A</sub>) and metabotropic type-B (GABA<sub>B</sub>) (Kumamoto, 1997). Kirouac *et al.* (2004) showed that both the GABA<sub>A</sub> and GABA<sub>B</sub> receptor coexist in A delta and C afferent fibers that conduct pain stimuli (Kirouac *et al.*, 2004). GABA<sub>B</sub> receptors can be located pre-synaptically, acting through inhibition of high voltage activated Ca<sup>2+</sup> channels and causing suppression of neurotransmitter release (Wu and Saggau, 1997). Alternatively, they can also be postsynaptic, mediating slow and long lasting inhibitory postsynaptic potentials by activation of inwardly rectifying K<sup>+</sup> channels, therefore inhibiting neuronal excitability (Bowery and Enna, 2000). Formalin-induced inflammatory pain increases the expression of heterodimeric GABA<sub>B</sub> receptor subunit mRNAs and protein in the dorsal horn of the rat spinal cord (Sands *et al.*, 2003). In the brainstem, an increase in the

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expression of GABA<sub>B</sub> receptors was found in several nuclei after nociceptive stimulation (Pinto *et al.*, 2003). In addition, in an acute pain test, baclofen microinjection into the nucleus raphe Magnus and nucleus reticularis gigantocellularis pars alpha of the medulla, had either antinociceptive or hyperalgesic effects depending on the dose administered (Thomas *et al.*, 1996). There is ample evidence from animal studies concerning a primary antinociceptive action of baclofen. It has been demonstrated that baclofen is antinociceptive in rats by using the hotplate and tail-flick tests. Intraperitoneal injection of baclofen was also found to be antinociceptive to noxious thermal stimulation in cats and in formalin-injection tests in monkeys (Bowery, 2006). Baclofen has long been known to have antinociceptive activity in acute and chronic pain models (Hwang and Yaksh, 1997). On the other hand, pretreatment with estrogen can save GABA<sub>B</sub> receptor function, probably by neuroprotection of neurons containing postsynaptic GABA<sub>B</sub> receptors (Velisek and Veliskova, 2002). Noriega *et al.* (2010) reported that estrogen decreases the functional coupling of the mu-opioid and GABA<sub>B</sub> receptors to the inwardly rectifying K<sup>+</sup> channel, possibly through an action on the G-protein (Noriega *et al.*, 2010). On the other hand progesterone and their metabolite have different effect on pain sensitivity (Frye and Duncan, 1994). Al-Dahan and Thalmann (1996) reported that progesterone variation during the estrous cycle could be responsible for one component of the regulation of GABA<sub>B</sub> receptors that occurs in neocortex during the estrous cycle of the rat (Al-Dahan and Thalmann 1996). Therefore, the aim of the present study was to investigate the effect of baclofen (GABA<sub>B</sub> agonist) and CGP35348 (GABA<sub>B</sub> antagonist) on pain sensitivity during the estrous cycle in rats; it means that we examine the effect of natural change of ovarian hormone on pain sensitivity.

## MATERIALS AND METHODS

**Animals:** Forty eight female Sprague Dawley rats weighing 200-220g were used. Food and water were made available ad libitum. Rats were housed under a 12h light/dark (light on at 6 am) and controlled temperature (20±4°C) condition. Animals were divided into five groups: (1) control group (intact animal, n = 6); (2) sham (received 0.75 µL of Artificial Cerebrospinal Fluids (ACSF), n = 6); (3) experimental 1 (received 0.75 µL of baclofen 4.25 or 8.54 µg rat<sup>-1</sup>, n = 12); (4) experimental 2 (received 0.75 µL of CGP35348 3 or 5 µg rat<sup>-1</sup>, n = 12) and (5) experimental 3 (received 0.75 µL of CGP35348 3 or 5 µg rat<sup>-1</sup> 15 min after 0.75 baclofen 8.54 µg rat<sup>-1</sup>, n =

12). In all animals, the formalin test was performed in all stages of the estrous cycle. Each group consists of 5 rats and every rat was received formalin test 4 times; the duration between the tests was 4-5 days.

**Determination of the estrous cycle stage in female rats:** Before the experiment, different stages of the estrous cycle were detected by microscopic examination of vaginal smear based on the relative frequency of leukocyte, cornfield and nucleated epithelial cells (Eldridge *et al.*, 1999).

**Formalin test:** Five min after injection of ACSF or drugs, 50 µL of 2.5% formalin solution was injected subcutaneously into the plantar surface of right hind paw using a 30 gauge needle. A Pain score was determined for each 5 min block (each block consisted of a mean of 20 records in every 15 sec) during 60 min, by measuring the number of each of the following four behavioral categories: 0, the injected paw is not favored; (1) the injected paw has little or no weight on it; (2) the injected paw is elevated and is not in contact with any surface; (3) the injected paw is licked, bitten or shaken (Abbott *et al.*, 1995). The records of the first 10 min were considered as phase 1 of the formalin test, while the records after 10 min were considered as phase 2 of the formalin test.

**Surgery:** The rats were anesthetized with an IP injection of ketamine 35mg kg<sup>-1</sup> and xylazine 5mg kg<sup>-1</sup> and, after mounting in a stereotaxic instrument (Stoelting, USA) a cannula (23 gauge) was implanted unilaterally at the right hippocampus (AP: 3.5 mm behind the bregma, lateral: 3.1 mm and vertical: 4.5 mm from cerebral cortex). Two screws were placed in the skull and each cannula was anchored into place with dental cement poured around the outer cannula and screws. A stainless steel extending just beyond the tip of the cannula was inserted and left in place until injection. The animals were allowed to recover for at least 7 days after surgery (Soignier *et al.*, 2000). Rats were given a lethal dose of ether following testing. The microinjection site was marked by injecting of cresyl violet (0.2 µL) into the hippocampus. The brain was removed, placed in formalin (10%) and a coronal section was prepared to determine correction of surgery. This protocol has been approved by our animal use committee.

**Data analysis:** For analysis of data SPSS program were used. Data were analyzed by one way Analysis Of Variance (ANOVA). Tukey test was performed as post-hoc. Significant value was p<0.05. Data were considered in results as mean ± SEM.

## RESULTS

### Effect of intrahippocampal injection of baclofen on pain sensitivity during estrous cycle:

Intrahippocampal injection of two doses of baclofen in phase 1 of the formalin test has no effect on pain score in postures [F (3) = 0.4, p = 0.754], but significantly decreased pain sensitivity during the estrus [F (3) = 14.5, P = 0.000], met estrus [F (3) = 10.6, p = 0.000] and diestrus [F (3) = 5.5, p = 0.006] stages of the estrous cycle (Fig. 1). Intrahippocampal injection of baclofen (8.54  $\mu\text{g rat}^{-1}$ ) in phase 2 of the formalin test significantly ( $p < 0.05$ ) decreased pain sensitivity during proestrus [F (3) = 5.78, P = 0.005], estrus [F (3) = 8.8, P = 0.001] and metestrus [F (3) = 15, P = 0.000] and diestrus [F (3) = 14.6, P = 0.000] stages of the estrous cycle (Fig. 1).

Analgesic effect of baclofen 4.27 and 8.54  $\mu\text{g rat}^{-1}$  in phase 1 of formalin test was significantly higher during estrus [F (3) = 13.9, P = 0.001] in the phases 2 of the formalin test the similar effect were shown [F (3) = 9.33, p = 0.001] (Fig. 1).

### Effect of intrahippocampal injection of CGP35348 on pain sensitivity during estrous cycle:

Intrahippocampal injection of CGP35348 (5  $\mu\text{g rat}^{-1}$ ) in phase 1 of the formalin test significantly ( $p < 0.05$ ) increased pain sensitivity during the proestrus [F (3) = 12, p = 0.000] and estrus [F (3) = 3.4, P = 0.038] stages of the estrous cycle, but it has no significant effect during metestrus [F (3) = 0.6, p = 0.602] and diestrus [F (3) = 1.6, p = 0.22] (Fig. 2). Intrahippocampal injection of CGP35348 (5  $\mu\text{g rat}^{-1}$ ) in phase 2 of the formalin test significantly ( $p < 0.05$ ) increased pain sensitivity during the proestrus [F (3) = 3.2, p = 0.047] and estrus [F (3) = 4.3, p = 0.017] stages of the estrous cycle, but it has no significant effect during metestrus [F (3) = 2.2, p = 0.12] and diestrus [F (3) = 1.1, p = 0.373] (Fig. 2). Also, there was a significant ( $p < 0.05$ ) difference between the two doses of CGP35348 (Fig. 1).

Hyperalgesic effect of CGP35348 (3 and 5  $\mu\text{g rat}^{-1}$ ) was significantly ( $p < 0.05$ ) higher during the proestrus stage of the estrous cycle in phase 1 of formalin test [F (3) = 11.7, p = 0.000]. Hyperalgesic effect of CGP35348 (3 and 5  $\mu\text{g rat}^{-1}$ ) was significantly ( $p < 0.05$ ) higher during the metestrus stage of the estrous cycle in phase 2 of formalin test [F (3) = 14.2, p = 0.000].

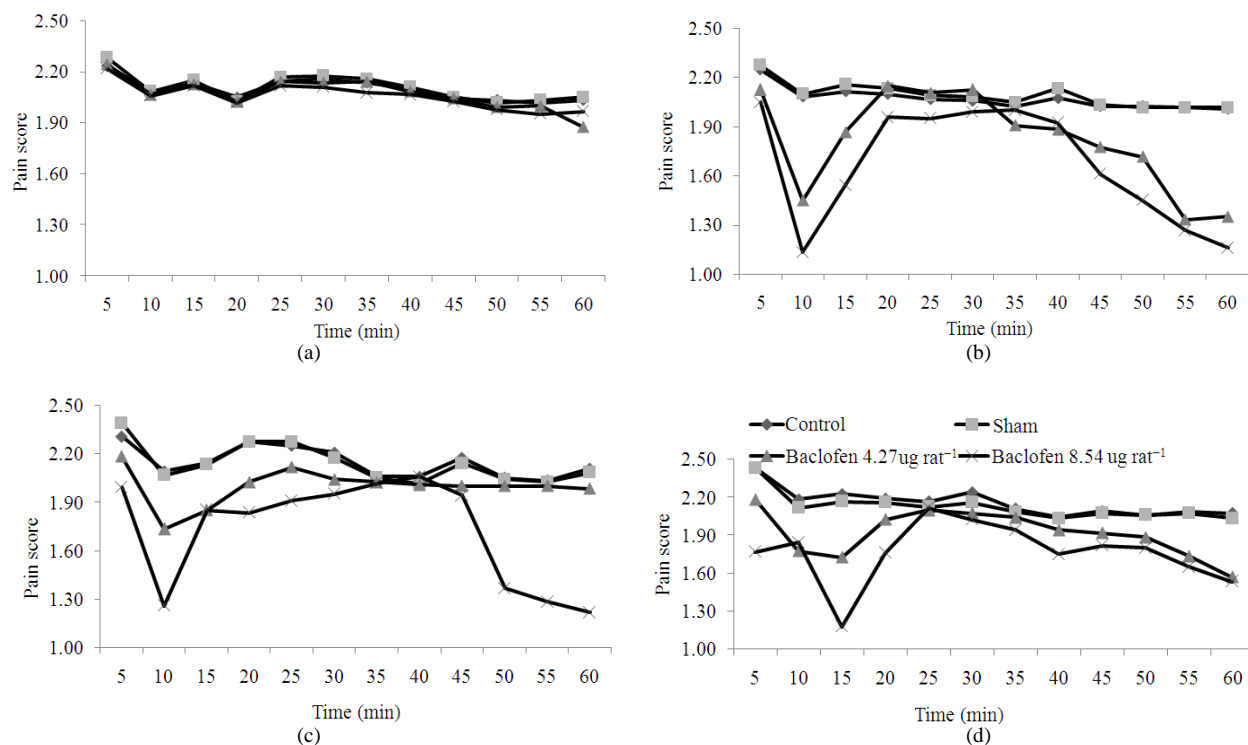


Fig. 1: Effect of intrahippocampal injection of baclofen on mean of pain score during estrous cycle (A); proestrus, (B) estrus, (C) metestrus and (D) diestrus, N = 6 in each group

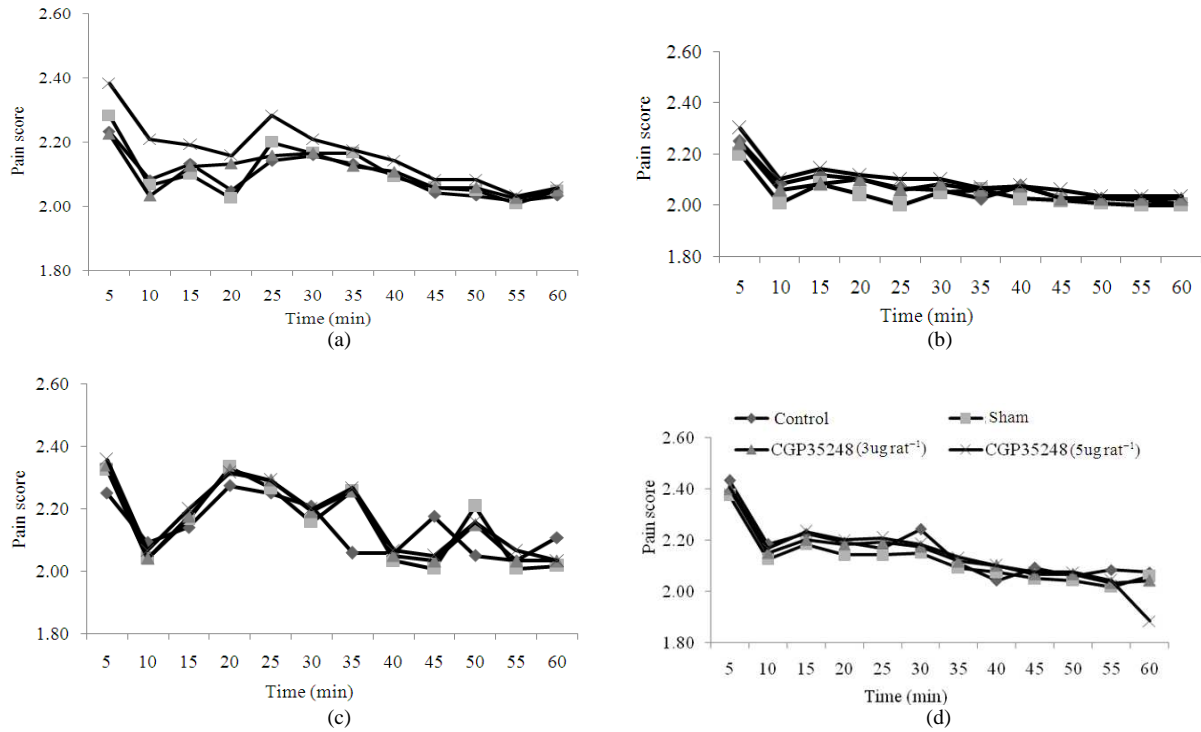


Fig. 2: Effect of intrahippocampal injection of CGP35348 on mean of pain score during estrous cycle; (A) proestrus, (B) estrus, (C) metestrus and (D) diestrus, N = 6 in each group

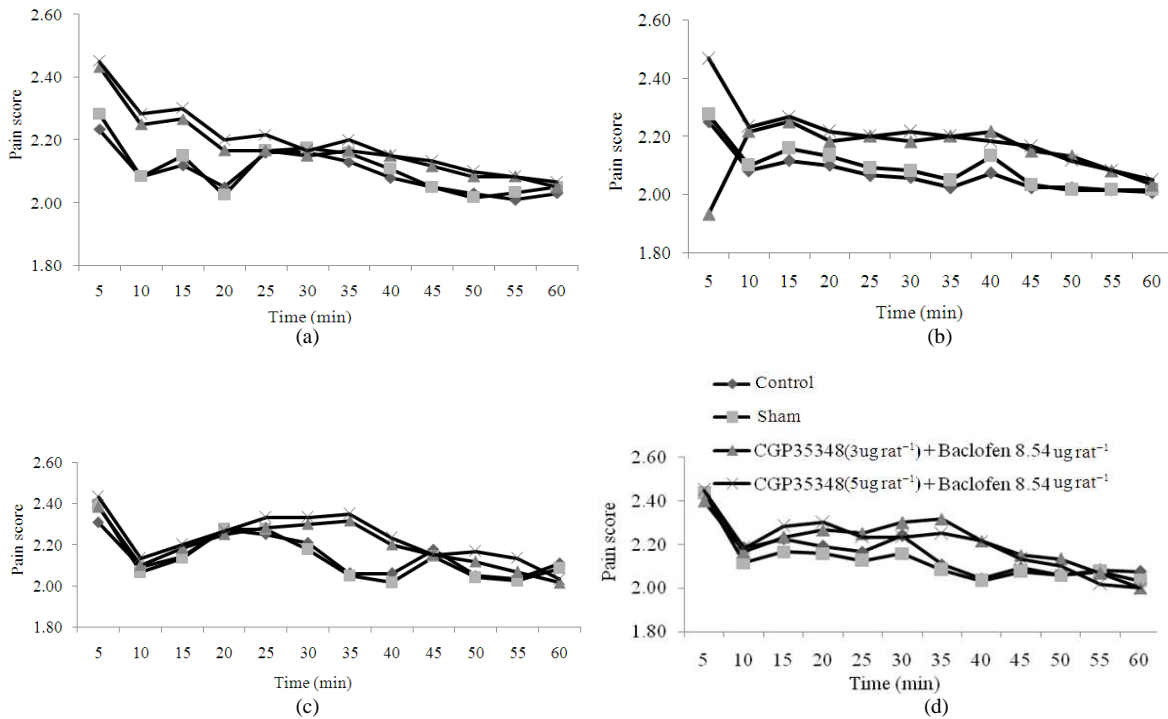


Fig. 3: Effect of intrahippocampal injection of baclofen pretreatment by CGP35348 on mean of pain score during estrous cycle; (A) proestrus, (B) estrus, (C) metestrus and (D) diestrus, N = 6 in each group

**Effect of intrahippocampal injection of CGP35348 + baclofen on pain sensitivity during estrous cycle:**

The simultaneous intrahippocampal injection of CGP35348 (3 and 5  $\mu\text{g rat}^{-1}$ ) + baclofen (8.54  $\mu\text{g rat}^{-1}$ ) in phase 1 of the formalin test, significantly ( $p < 0.05$ ) increased pain sensitivity only in the proestrus [F (3) = 36.6,  $p = 0.000$ ] stage of the estrous cycle, while simultaneous intrahippocampal injection of CGP35348 (3 and 5  $\mu\text{g rat}^{-1}$ ) + baclofen (8.54  $\mu\text{g rat}^{-1}$ ) in phase 1 of the formalin test, had no significant effect during estrus [F (3) = 1.75,  $p = 0.2$ ], metestrus [F (3) = 5.2,  $p = 0.078$ ] and diestrus [F (3) = 0.8,  $p = 0.5$ ] stage of estrous cycle. Simultaneous intrahippocampal injection of CGP35348 (3 and 5  $\mu\text{g rat}^{-1}$ ) + baclofen (8.54  $\mu\text{g rat}^{-1}$ ) in phase 2 of the formalin test, significantly ( $p < 0.05$ ) increased pain sensitivity in proestrus [F (3) = 9.76,  $p = 0.001$ ], estrus [F (3) = 7.17,  $P = 0.004$ ] and metestrus [F (3) = 14.3,  $p = 0.000$ ] and diestrus [F (3) = 29,  $p = 0.000$ ] stages of the estrous cycle (Fig. 3).

Simultaneous intrahippocampal injection of CGP35348 (3 and 5  $\mu\text{g rat}^{-1}$ ) + baclofen (8.54  $\mu\text{g rat}^{-1}$ ) induced hyperalgesia was greater than the administration of CGP35348 (3 and 5  $\mu\text{g rat}^{-1}$ ) alone.

The hyperalgesic effect of CGP35348 (3 and 5  $\mu\text{g rat}^{-1}$ ) after baclofen 8.54  $\mu\text{g rat}^{-1}$  was significantly higher during metestrus and diestrus than the proestrus and estrus [F (3) = 7.28,  $p = 0.011$ ] stage of the estrous cycle in phase 2 of formalin test. In phase 1 of formalin test, there is no significant difference between four phases of estrous cycle.

## DISCUSSION

The rat formalin test is a reliable animal model of pain induced by acute tissue injury (Tjolsen *et al.*, 1992). Moreover, the formalin-induced nociceptive behaviors elicited in animals are quantifiable and show two phases: an early phase (acute pain) that starts immediately after formalin injection and lasts approximately 5 min and a late phase which begins 15-20 min after the formalin injection lasting 20-40 min. This last phase, also named tonic or chronic phase, is dependent on local inflammation and/or prolonged changes in the CNS (Tjolsen *et al.*, 1992). Between these two phases there is a period, the interphase, in which the animals display hardly any pain-related behaviors (Tjolsen *et al.*, 1992). Both the first and the second phase of the formalin test are accompanied by an increase in the activity of A $\delta$  and C-fiber primary afferents.

The present study showed that baclofen injection in hippocampus ipsilateral to the formalin injected paw decreases pain sensitivity in all stages of

the estrous cycle, similar to the effect of intracerebroventricular injection of baclofen in our previous study (Taherianfard *et al.*, 2009). Potes *et al.* (2006) reported that baclofen injection in the ventrobasal complex of the thalamus contralateral to the formalin-injected paw decreased all nociceptive behaviors displayed by the rats in the formalin test in a dose-dependent manner, indicating an antinociceptive effect of this selective GABA<sub>B</sub> agonist (Potes *et al.*, 2006). Brusberg *et al.* (2009) demonstrated that systemic activation of GABA<sub>B</sub> receptors in conscious rats, either by the orthosteric agonist baclofen or, to a lesser extent, by the positive allosteric modulator CGP7930, reduces pseudo-affective responses to noxious colorectal distension in rats (Brusberg *et al.*, 2009). Intrathecal administration of the GABA<sub>B</sub> receptor agonist baclofen produced a dose-dependent decrease in response to formalin injected hind paw. This antinociceptive effect of baclofen was antagonized by pretreatment with GABA<sub>B</sub> receptor antagonist saclofen (Naderi *et al.*, 2005). Patel *et al.* (2001) reported that L-baclofen has marked antihyperalgesic activity in a model of neuropathic pain in the rat. The effect of baclofen was blocked by the selective GABA<sub>B</sub> receptor antagonist CGP56433A (Patel *et al.*, 2001). Yang *et al.* (2003) demonstrated that the activation of GABA<sub>B</sub> receptors by baclofen in the ventrolateral periaqueductal gray neurons has a threefold action: 1- hyperpolarization of periaqueductal gray neurons (postsynaptic inhibition), 2- decrease in the release of L-glutamate nerve terminals to periaqueductal gray neurons (presynaptic inhibition) and 3- decrease in the release of GABA from nerve terminals to periaqueductal gray neurons (presynaptic inhibition) (Yang *et al.*, 2003).

On the other hand, pain stimulus leads to changes in the expression of GABA<sub>B</sub> receptors. The results of Castro *et al.* (2006) point to differences in the expression of GABA<sub>B</sub> receptors in spinofugal pathways targeting pronociceptive and antinociceptive regions. McCarson *et al.* (2005) revealed that both thermal and mechanical withdrawal thresholds are lowered significantly in rats 7 days following a partial ligation of the sciatic nerve, while this nerve damage increases the gene expression of both the GABA<sub>B(1a)</sub> and GABA<sub>B(2)</sub> in the ipsilateral lumbar spinal cord (McCarson *et al.*, 2005). Formalin induced inflammatory pain increases the levels of mRNA and protein for GABA<sub>B(1a)</sub> and GABA<sub>B(2)</sub> in the dorsal horn of the rat spinal cord (Sands *et al.*, 2004).

In the present study, baclofen has the highest analgesic action in the estrus stage of the estrous cycle, whereas in the other study, the analgesic effect of

intracerebrospinal injection of the baclofen was higher during metestrus and diestrus (Taherianfard *et al.*, 2009). It seems that site of injection is important and this difference may be related to the estradiol receptor or GABA<sub>B</sub> receptor distribution in different brain locations. The metabotropic GABA<sub>B</sub> receptors mediate slow synaptic inhibition and consist of heterodimers of B1 and B2 subunits (Bettler *et al.*, 2004) which share structural homology with class-III G protein-coupled receptors (Binet *et al.*, 2004). The only known molecular diversity of the GABA<sub>B</sub> receptors arises from two GABA<sub>B1</sub> isoforms (Kaupmann *et al.*, 1998). Noriega *et al.* (2010) reported that 17 $\beta$ -estradiol and 17 $\beta$ -estradiol + progesterone led to the up regulation of the gene encoding the GABA<sub>B</sub> receptor subunit 1 in the amygdala (Noriega *et al.*, 2010).

Administration of CGP35348 has a paradoxical effect on pain response. In the present study, CGP35348 injection in hippocampus ipsilateral to the formalin injected paw alone in two doses induced hyperalgesia in the proestrus and estrus stage of the estrous cycle, but not in the metestrus and diestrus stage of the cycle, while pretreatment of CGP35348 in two doses before baclofen induced hyperalgesia in all stages of the estrous cycle. Poets *et al.* (2006) showed that administration of CGP35348 induced a decrease of pain related activities in both phases of the formalin test, as baclofen did (Potes *et al.*, 2006). However, there are other studies that have already reported similar effects of baclofen or of CGP35348 administration upon the nociceptive behavior of the animals. In fact, the intracerebrospinal administration of different doses of baclofen or of CGP35348 induced by themselves antinociception in both non-ligated and ligated mice subjected to the hot plate test (Zarrindast *et al.*, 2000). Ataka *et al.* (2000) revealed that baclofen (1 $\mu$ M) reduces the amplitude of EPSPs, which are monosynaptically evoked in substantia gelatinosa neurons of spinal cord slices by stimulating primary afferent fibers. This action of baclofen was more effective on EPSC than on A $\delta$  fiber EPSC, while CGP35348 antagonized both actions (Ataka *et al.*, 2000). There is evidence that the GABA<sub>B</sub> receptor antagonist CGP35024 reverses mechanical hyperalgesia in one model of neuropathic pain in the rat, with concomitant inhibitory activity on nociceptive transmission in the spinal cord (Patel *et al.*, 2001).

## CONCLUSION

In the present study, the hyperalgesic effect of CGP35348 alone is highest during the proestrus stage of the estrous cycle; while the hyperalgesic effect of

pretreatment of CGP35348 in two doses before baclofen in the metestrus and diestrus stages of the estrus cycle is more than in the proestrus and estrus stages of the estrus cycle. Intracerebrospinal injection of CGP35348 had a similar effect (Taherianfard *et al.*, 2009). There is evidence that GABAergic mediation of opioid effects is a widespread phenomenon and occurs throughout most regions of the CNS. Kalyuzhny and Wessendorf (1999) observed a colocalization between hippocampal  $\mu$ -opioid receptors in CA1, CA3 and dentate gyrus in rats. The localization of the  $\mu$ -opioid receptor on the GABAergic neuron suggests that these receptors, when activated, can directly control the hippocampal GABAergic neurons activity (Kalyuzhny and Wessendorf 1997; 1999). Torres-Reverone *et al.* (2009) demonstrated that ovarian steroids alter the trafficking of mu opioid receptors within subpopulations of hippocampal GABAergic interneuron (Torres-Reverone *et al.*, 2009). Al-Dahan and Thalmann (1996) results indicated that progesterone variation during the estrous cycle could be responsible for a component of the regulation of GABA<sub>B</sub> receptors that occurs in the neocortex during the estrous cycle of rat (Al-Dahan and Thalmann, 1996).

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**Conflict of interest:** All the authors can confirm that there is no financial or other relationship that would cause a conflict of interest.

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