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Reduction of inflammatory pain in female rats after NR2B NMDA cortical antagonism

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Studies have shown that N-methyl-D-aspartate (NMDA) receptors play a critical role in pain processing at different levels of the central nervous system. In this study, we used female adult Wistar rats to examine the effects of antagonizing the NR2B subunit of the NMDA receptor in phasic and tonic pain processes. All the rats underwent stereotaxic surgery for cortical cannula implantation and after at least one week of recovery, rats performed behavioral tests. For evaluating the effects of drugs on motor coordination rats were tested in the rotarod apparatus. Moreover, rats were evaluated in the paw withdrawal latency (PWL) to a noxious thermal stimulus. Furthermore, rats were tested in the formalin-pain test. Rats that received the NR2B antagonist Ro 25-6981 before and after formalin injection showed significantly reduced pain responses in the formalin test, as compared with female control rats ($p<0.05$). In contrast, no differences among groups were found in the phasic pain test (Hargreaves) and the rotarod test. Taken together, these results suggest that cortical antagonism of the NR2B subunit of NMDA receptors is able to reduce inflammatory pain levels not only before, but after the formalin injection in females at different phases of the estrous cycle.

Reducción del dolor inflamatorio en ratas hembras tras el antagonismo cortical de NR2B NMDA. Estudios han demostrado que los receptores de N-methyl-D-aspartato (NMDA) participan en el procesamiento del dolor en diferentes niveles del SNC. Este estudio empleó ratas hembras adultas Wistar para evaluar el antagonismo de la subunidad NR2B de NMDA en los procesos de dolor fásico y tónico. Se implantaron cánulas corticalmente con la cirugía estereotáxica y tras una semana de recuperación se realizaron pruebas conductuales. Se evaluaron los efectos del fármaco en la coordinación motora en el aparato de barra giratoria. Además, las ratas realizaron la prueba de latencia de retirada de la pata a un estímulo termal nocivo. Posteriormente, las ratas realizaron la prueba de formalina. Las ratas hembras que recibieron el antagonista de NR2B, Ro 25-6981, antes y después de la inyección de formalina denotaron respuestas de dolor significativamente menores en comparación con los controles ($p<0.05$). En contraste, no se encontraron diferencias significativas en la prueba de dolor fásico (Hargreaves) y la prueba de barra giratoria. En conjunto, estos resultados sugieren que el antagonismo cortical de la subunidad NR2B de los receptores NMDA es capaz de reducir los niveles de dolor inflamatorio, antes y después de la inyección de formalina en las distintas fases del ciclo estral.

Experts in the field of sex differences (Becker et al., 2005; Mogil, Davis, & Derbyshire, 2010; Young & Becker, 2009) emphasize the relevance of considering the sex cycle in the design and interpretation of neurobiological studies. Moreover, in the field of pain, different authors have reported sex differences mediated by NMDA receptors (Bryant, Eitan, Sinchak, Fanselow, & Evans, 2006; Kavaliers & Choleris, 1997; Mogil, Sternberg, Kest, Marek, & Liebeskind, 1993). In the present research work, it was explored the effects of NR2B antagonism at the level of the anterior cingulate cortex (ACC) on pain responses of female rats across different phases of the estrous cycle.

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N-methyl-D-aspartate (NMDA) receptors from different areas in the central nervous system, including the ACC (Li et al., 2010; Mogil et al., 2010; Wei et al., 2002) are essential in the generation of sustained pain responses. Nervous system areas explored in these studies included spinal cord (Coderre & Melzack, 1992), periaqueductal gray substance (PAG) (Vaccarino, Clemmons, Mader, & Magnusson, 1997) and thalamus (Eaton & Salt, 1990) and concluded that NMDA receptor agonism generates an increase in the inflammatory pain response. Reciprocally, a decrease in the activation of NMDA receptors by means of genetic or pharmacologic manipulations generates a subsequent decrease in inflammatory pain response (Quintero, Erzurumlu, & Vaccarino, 2007; South et al., 2003).

The long term potentiation (LTP) process in the ACC is proposed as the molecular mechanism underlying the sensitization of chronic pain conditions (Wu & Zhuo, 2009; Zhuo, 2006, 2007); the LTP mechanism involves mainly the excitatory neurotransmitter glutamate and its postsynaptic receptors AMPA and NMDA (Kim,

Descalzi, & Zhuo, 2010; Wei, Li, & Zhuo, 1999). In addition, some authors suggest the specific exploration of the NR2B subunit antagonism at the level of the ACC as strategy for reducing persistent pain (Wei et al., 2001; Wu & Zhuo, 2009) having the advantage of reduced side effects in contrast to other NMDA antagonists (Boyce et al., 1999). Furthermore, other studies report sex differences in the mechanisms of pain and analgesia that involved NMDA receptors (Juni, Klein, Kowalczyk, Ragnauth, & Kest, 2008; Kavaliers & Choleris, 1997; McRoberts, Li, Ennes, & Mayer, 2007; Mogil et al., 1993).

Previous study has shown that Ro 25-6981 is a very selective and potent antagonist and activity-dependent blocker of NMDA receptors that contain the NR2B subunit (Fischer et al., 1997). In the present study, the antinociceptive effect of NR2B antagonist Ro 25-6981, was evaluated in the phasic and inflammatory pain responses of female rats.

The objectives of the present study were to evaluate the antinociceptive effects of cortical antagonism of NR2B subunit of NMDA receptor on phasic and tonic pain responses of female adult rats. The present work shows that antagonism of NR2B subunit of NMDA receptor at the level of the ACC is able to reduce inflammatory pain in females at different phases of the estrous cycle (estrus and metestrus) if applied before or after inflammatory insult; these results confirm that supraspinal strategies for managing pain, specifically, antagonism of NMDA receptor at the level of ACC is worthy in female at estrus and metestrus phases of the estrous cycle. Studies focused on supraspinal treatment of pain responses in female populations are few in the literature.

Methods

Subjects

A total of 182 adult female Wistar rats were employed; female rats performed behavioral tests in diverse phases of the estrous cycle. Breeding pairs of rats were obtained from Harlan Company (Mexico City, Mexico) and bred, housed and maintained in the animal care facilities of the INDICASAT-AIP according to the Public Health Service (PHS) procedures. Animals were housed in light/dark cycles of 12 h × 12 h with water and food available ad libitum. All the experiments performed adhered to the guidelines of the Committee for Research and Ethical Issues of IASP published in PAIN® (Zimmermann, 1983).

Instruments

For measuring phasic pain responses, it was employed an analgesiometer equipment (IITC Life Sciences, Woodland Hills, CA, USA). For measuring motor coordination, it was employed the rotarod apparatus (IITC Life Sciences, Woodland Hills, CA, USA).

Procedure

Surgery. For stereotaxic surgery procedure, animals were anesthetized with an intramuscular (i.m.) injection of 75mg/kg ketamine + 10 mg/kg xylazine. Surgery was carried out using a stereotaxic apparatus. For microinfusion experiments, chronic guide cannula were implanted according to the following procedure: double stainless steel guide cannula (26GA, Plastics One Inc., Roanoke, VA, USA) were implanted 1mm over the ACC injection site (AP: +2.6, D/V: -1.6, M/L: + 0.6 from Bregma). After chronic guide cannula implantation, holes were closed with dummy cannulas of the same extension (.008"/.2MM, Plastics One Inc., Roanoke, VA, USA). One to three weeks later, rats performed behavioral experiments and received a treatment of drug (Ro 25-6981) or vehicle (saline) infusions through injector cannulas (33GA, Plastics One Inc., Roanoke, VA, USA) connected to a tubing (PE 20, Plastics One Inc., Roanoke, VA, USA) and syringe system. All the animals that were included in the study recovered from surgery as verified by post surgery weight measurements.

Drug Treatment. Ro 25-6981 (Sigma, St. Louis, MO, USA), an antagonist of the NR2B subunit of the NMDA receptor, was prepared in sterilized isotonic saline solution (0.9% NaCl). The drug was prepared at a 1 ug/0.5 uL concentration and administered at the rate of 0.5 uL/90 sec (Johansen & Fields, 2004) for a total of 0.5 uL of volume per side. The drug or vehicle was administered through a cannula system connected to a PE tubing (PE 20, Harvard Apparatus, Holliston, MA, USA) and Hamilton syringe (MODEL 7001 GAS TIGHT, Harvard Apparatus, Holliston, MA, USA). Before the injection of the drug or saline control, the injector cannulas were left for a period of 20 to 30 sec, and after injection of the drug or saline, these cannulas were left for a period of 2 min. For the Hargreaves experiments, behavioral measurements (latencies) were recorded at 5 min for naïve and experimental groups, and 30 min for experimental groups after drug or saline cortical infusion. Moreover, for the formalin test, drug or vehicle infusion was performed 15 min before and 25 min posterior to formalin injection. Finally, for the rotarod test, the trials were performed 30 min before and 30, 60 and 90 min after drug or vehicle infusion.

Pain tests. All the behavioral testing was performed by evaluators blinded to the drug treatment condition. In each of the pain tests, the rats were previously habituated to the behavioral apparatus (Hargreaves or formalin cages) during 40 min for 3 consecutive days. Moreover, rats were handled by the experimenter previously to the test. Behavioral tests, Hargreaves, formalin and rotarod test, were performed on light-cycle hours; furthermore, all the animals were behavioral assessed in groups to avoid any effects of isolation analgesia.

In the Hargreaves test, paw withdrawal latency (PWL) to thermal stimuli was measured according to the techniques previously described (Hargreaves, Dubner, Brown, Flores, & Joris, 1988), using an analgesiometer (IITC Life Sciences, Woodland Hills, CA, USA), and a 15-sec cutoff. The mean of two PWL (1 left and 1 right) measured at 3-5 min intervals as registered for each rat.

The formalin test was used as model of tonic inflammatory pain response (Alreja, Mutalik, Nayar, & Manchanda, 1984; Dubuisson & Melzack, 1977; Hunskaar, Fasmer, & Hole, 1985; Lee & Jeong, 2002); furthermore, the rats were injected s.c. into the plantar surface of the right hind paw with 60uL of 5% formalin.

After formalin injection, the time employed licking and/or biting the injected hind paw was selected as measure of more intense level of pain. The quantity of time consumed in the pain behavior was recorded for 1 hour, and quantified in time blocks of 5 min; later, the average score of two independent and blinded evaluators was calculated.

The rotarod test is a valid and widely test used for motor coordination (Carter, Morton, & Dunnett, 2001; van Dellen, Cordery, Spires, Blakemore, & Hannan, 2008). Rats were trained for two

days prior to testing. The rotarod apparatus (IITC Life Sciences, Woodland Hills, CA, USA) rotated accelerating 5 to 25 rpm, setting a cut off time of 60 secs. Rats were evaluated at a baseline (-30) and 30, 60 and 90 min following intracortical injection.

Histology. After finishing the behavioral experiments, rats were administered a lethal dose of ketamine/xylazine and brain extracted and processed for histology (Johansen & Fields, 2004). The female estrous cycle was determined by a fast method previously described (Marcondes, Bianchi, & Tanno, 2002) and at least 2 hours before experiments (Lariviere, Sattar, & Melzack, 2006).

Data analysis

The data of the Hargreaves test was analyzed by a Analysis of Variance (ANOVA) test. The data of the formalin test was analyzed by ANOVA test; significant differences were then evaluated by a LSD post hoc test, with a criterion of $p<0.05$. The data of the rotarod test was examined by ANOVA test.

Results

Analysis of Variance (ANOVA) revealed no significant differences in paw withdrawal latencies between female that received saline and drug (5 min and 30 min) in the different estrous cycle phases (figure not shown); estrus: ($F(3, 22)= 0.128$, $p= 0.94$, n.s.), metestrus ($F(3, 24)= 1.146$, $p= 0.35$, n.s.), proestrus ($F(3,24)= 1.174$, $p=$ n.s.) and diestrus ($F(3,26)= 3.045$, $p=$ n.s.).

The formalin test produces a biphasic profile of pain response that is characterized by an "early phase" during the first 5 min after formalin injection that is followed by a "late phase" pain response from 10 to 60 min (Dubuisson & Melzack, 1977). Figure 1 show the total time spent licking/biting the formalin- injected paw during the 25 first min of formalin test, when cortical infusion was applied 15 min before to formalin injection. Data are expressed as mean time (sec ± SEM) spent licking/biting the injected hind paw. ANOVA revealed significant differences during the early phase (0 to 5 min) ($F(3,25)= 5.260$, $p<0.05$). Specifically, the post hoc test show significant differences *(a)* between female in estrus that received saline and female in estrus that received drug ($p<0.001$), and between female in estrus that received saline and female in metestrus that received drug ($p<0.001$); moreover, post hoc test also revealed significant differences in this interval *(b)* between female in metestrus that received drug and female in metestrus that received saline ($p<0.05$). Furthermore, it was found marked differences in pain response during the interval of 20 to 25 min of late phase ($F(3,25)= 3.006$, $p= 0.05$). Specifically, post hoc test proved significant differences *(c)* between female in estrus that received drug and female in estrus that received saline ($p<0.01$) (more details in Figure 1).

Figure 2 illustrates the total time spent licking/biting the formalin-injected paw if cortical infusion applied after formalin injection (25 min later). Data are expressed as mean time (sec ± SEM) spent licking/biting the hind paw after formalin injection. ANOVA revealed significant differences during the 0 to 5 min interval after second cortical infusion ($F(3,22)= 3.647$, $p<0.05$). In particular, post hoc analysis show significant differences *(d)* between female in estrus that received saline and female in estrus that received drug ($p<0.05$), and between female in estrus that received saline and female in metestrus that received drug ($p<0.05$); moreover, it was found differences *(e)* between female in metestrus that received saline and female in metestrus that received drug ($p<0.05$), and between female in metestrus that received saline and female in estrus that received drug ($p<0.05$).

The rotarod test did not denoted effects of drug on motor coordination of rats during the phases of proestrus and diestrus (figure not shown). In effect, there were not significant differences

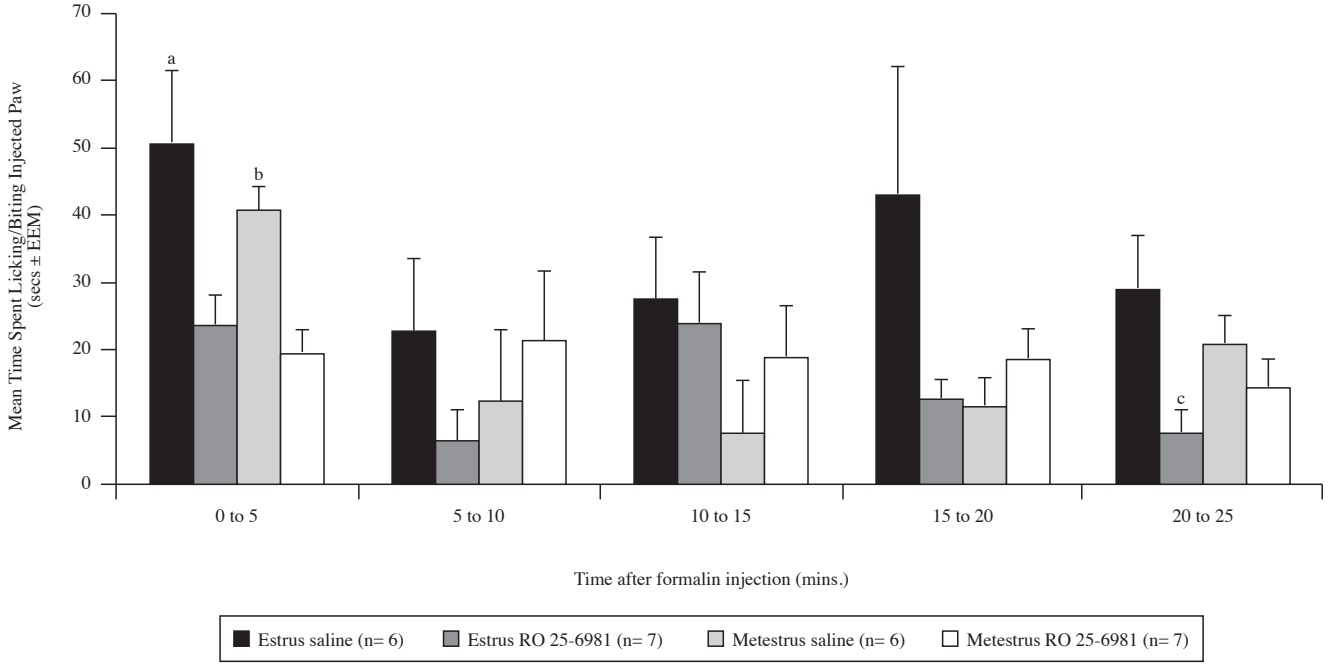

Figure 1. *Pain responses in female rats that received cortical infusion before the formalin injection; the figure represents the 25 min following to formalin injection*

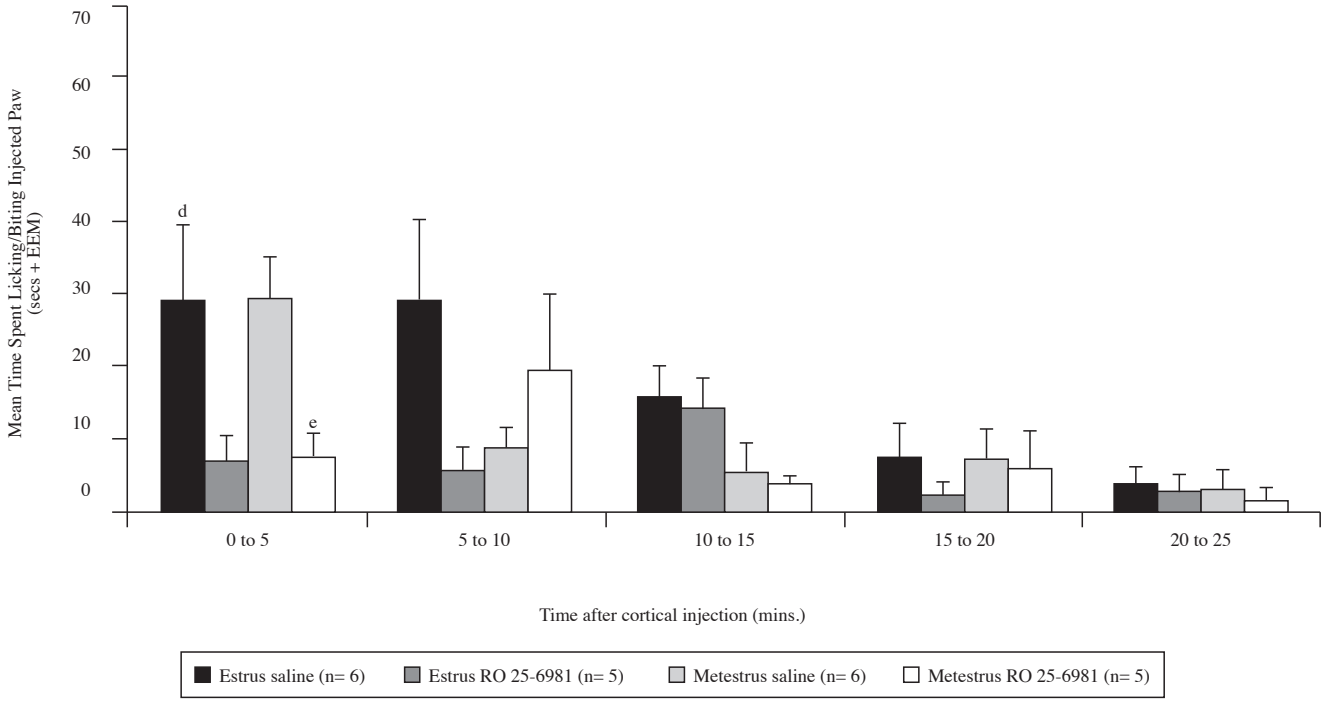

Figure 2. *Pain responses in female rats that received cortical infusion after formalin injection; the figure represent the 25 min subsequent to cortical infusion*

between experimental groups that received saline or drug at 30 min ($F(3,19)= 1.938$, $p>0.05$, n.s.), 60 min ($F(3,19)= 2.834$, $p>0.05$, n.s.), and 90 min ($F(3,19)= 0.685$, $p>0.05$, n.s.).

Discussion and conclusions

The results presented here demonstrate a dissociation of the role of anterior cingulate cortex NMDA receptors in mediating different types of pain. In particular, a decreased pain response was observed in rats that received drug in the formalin test but not in their sensitivity to a thermal stimulus. The PWL measures a phasic response to threshold level thermal stimulus in which major cortical structures would not be expected to play a critical role (Abbott & Melzack, 1982; Abbott, Melzack, & Samuel, 1982; Vaccarino & Melzack, 1989). By contrast, the formalin test produces inescapable pain due to tissue injury, which involves supraspinal structures, including the cortex and limbic system (Abbott & Melzack, 1982; Abbott et al., 1982; Ryan, Watkins, Mayer, & Maier, 1985; Vaccarino & Melzack, 1989). Indeed, the decrease in pain response in the formalin test observed in the rats that received cortical administration of Ro 25-6981 supports the critical role of cortical structures like ACC in mediating this type of pain. Furthermore, it is unlikely that non-specific effects could explain these results as no deficit was observed in the rotarod test, which evaluate motor coordination of the rats.

In this study, we observed a reduction in pain levels under conditions of cortical administration of the drug previous to, and posterior to formalin injection. Specifically, it was observed reduction of pain levels in the first phase of formalin test. However, previous studies have suggested that NMDA receptors are not involved in the first phase of the test (Hunter & Singh, 1994; South et al., 2003). This apparent contradiction could be explained by the inhibitory effect on ACC NMDA receptors, a condition which prevents the perception of the pain. Moreover, in those studies the NMDA cortical activity was intact.

The pharmacological model used in this study has an inhibition of the NR2B subunit of the NMDA receptor at the level of the ACC, which is an NMDA´s s subunit highly expressed in this brain area (Wei et al., 2001). As a consequence, these rats undergo inhibition of NMDA receptors in the ACC, whereas NMDA receptors in the rest of the brain are fully functional and intact. There is evidence that the cingulate cortex is involved in different aspects of pain processing (affective, sensory, integrative components of pain) and in tonic pain responses (Vaccarino & Melzack, 1989, 1992; Zhuo, 2007).

Furthermore, there is a consensus between the present results and previous imaging studies done in rodents; specifically, there is a relationship between the levels of excitatory activity at supra-spinal levels and the degree of pain behavior intensity (Chang & Shyu, 2001; Tuor et al., 2000). In addition, mutant studies of Quintero et al. (Quintero et al., 2007) also reported a dramatic reduction of tonic inflammatory pain in mice knockout of cortical NR1NMDA subunit. These studies, along with the results of the present report, suggest that cortical excitatory activity is involved in the perception of long lasting noxious stimulation. Moreover, the present results suggest that cortical inhibition of NMDA receptors is able to reduce tonic pain before and after formalin injection; in other words, NMDA cortical antagonism is able to reduce tonic pain after the initiation of spinal sensitization process (formalin injection) in female.

Previous studies have demonstrated sex-dependent differences in NMDA involvement in pain and analgesia (Juni et al., 2008; Kavaliers & Choleris, 1997; McRoberts et al., 2007; Mogil et al., 1993). Comparing the present results to similar study in male (Quintero preliminary results), sex similarities were found

in the effect of cortical NMDA receptor antagonism, as the pharmacological blockade of the NR2B subunit at the level of ACC reduced the formalin-induced pain response in both males and females (estrus and metestrus phases) after formalin injection. Moreover, previous studies on mutant mice lacking NR1 subunit at the level of the cortex showed dramatic reduction of inflammatory pain in both male and female (Quintero et al., 2007). The lack of difference may be explained by the lack of effect of hormonal correlates on cortical mechanism in the groups tested.

The data of the present study are in agreement with previous studies suggesting a critical role of NMDA receptors system in pain processes (Coderre & Melzack, 1992; South et al., 2003; Vaccarino et al., 1993). It is important to point out that in those studies different methods and levels of the nervous system were explored. For example, previous pharmacological (Coderre & Melzack, 1992) and mutant (South et al., 2003) studies, demonstrate the relevance of NMDA receptor system at the level of the spinal cord in the late phase of the formalin test. Moreover, a study by Vaccarino group (Vaccarino et al., 1993) probed that MK801, an NMDA channel blocker, applied interperitoneally was able to reduce tonic pain in the late phase but not in the early phase of formalin test, if applied prior to formalin injection. However, in the present study, the cortical application of the drug, previous to formalin injection, was able to reduce tonic pain, in both, the early and late phases of formalin test. Moreover, those studies did not include female groups at different estrous cycle phases. The results presented here are also in agreement with similar pain studies in related knockout mice models (Wei et al., 2002).

In conclusion, the present work proposes that NMDA receptors in the anterior cingulate cortex are an important element in the generation of the nociceptive response in tonic pain, but not in phasic pain of females at estrus and metestrus phases. In addition, NMDA receptors have been the center of studies related to the treatment of different suffering conditions including sustained pain (Smith, 2003). The present research suggests that ACC NMDA receptors have a significant influence in the development of inflammatory pain and that antagonists that target to ACC NR2B subunit of NMDA receptors could be an effective treatment for painful conditions in female.

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