Termination of Nociceptive Bahaviour at the End of Phase 2 of Formalin Test is Attributable to Endogenous Inhibitory Mechanisms, but not by Opioid Receptors Activation

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ABSTRACT

Introduction: Formalin injection induces nociceptive bahaviour in phase I and II, with a quiescent phase between them. While active inhibitory mechanisms are proposed to be responsible for initiation of interphase, the exact mechanisms which lead to termination of nociceptive response in phase II are not clear yet. Phase II is a consequence of peripheral and central sensitization processes, which can lead to termination of the noxious stimuli responses; 45-60 minutes after formalin injection via possible recruitment of active inhibitory mechanisms which we have investigated in this study.

Methods: To test our hypothesis, in the first set of experiments, we evaluated nociceptive response after two consecutive injection of formalin (50µL, 2%), with intervals of 5 or 60 minutes. In the next set, formalin tests were carried out in companion with injection of Naloxone Hydrochloride, a non-selective antagonist of opioid receptors, pre-formalin injection and 30 and 45 minutes post formalin injection.

Results: While normal nociceptive behaviour was observed in the group receiving one injection of formalin, a diminished response was observed in phases I and II of those receiving consequent injection of formalin, 60 minute after first injection. While second injection of formalin, 5 minute after first injection, had no effect. Administration of naloxone (1mg/kg) decreased nociception in phase 2A; but had no effect on delayed termination of formalin test.

Discussion: The results of this study suggest the existence of an active inhibitory mechanism, other than the endogenous opioids, that is responsible for termination of nociceptive behaviour at the end of formalin test.

1. Introduction

Formalin-test is commonly used in animal models of chronic inflammatory pain (Abbott, Franklin, & Westbrook, 1995; Dubuisson & Dennis, 1977) and also for investigating the analgesic effect of substances (Kim, Hong, Zhang, Ko, & Lee, 2012; Han et al., 2012). Formalin test has some advantages over acute nociceptive tests: it employs a painful stimulus and while animals show a spontaneous response, their nociceptive behaviour is lasted for more than 60 minutes, except for a period of quiescent interphase in which the animals show a decreased nociceptive response or no response at all (Abbott et al., 1995; Dubuisson et al., 1977). This test consists of two phases which recruit different modulation systems, thus it has more similarities to clinical pain rather than transient response observed in acute nociceptive tests (Staniland & McMahon, 2009; Hunskaar, Berge, & Hole, 1986; Hunskaar & Hole,

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1987). Sub plantar injection of different concentrations of formalin in the hind paw of rat or mice induces a series of nociceptive behaviours which lasts more than 60 minutes (Yashpal & Coderre, 1998; Sufka, Watson, Nothdurft, & Mogil, 1998; Abbott et al., 1995; Dubuisson et al., 1977). Formalin test consists of three phases: animals show a short period of nociceptive behaviours in phase I, which is followed by a quiescent or attenuation of nociceptive responses in interphase, and then a long lasting period of nociceptive behaviour for 60 minutes or even more (Hunskaar et al., 1986; Hunskaar et al., 1987). Finalization of formalin test is defined as the termination of nociceptive behaviour in the animals, but the underlying mechanism is still not clear. Despite the existence of local inflammation and edema 60 minutes after formalin injection, animals show a very few nociceptive behaviour. This point elicits an assumption for a possible active inhibitory mechanism that suppresses nociception 60 minutes after formalin injection. To our knowledge, no study has addressed the possible mechanisms which may be responsible for the attenuation and finalization of phase 2 nociceptive behaviours in formalin test. The existence of an active inhibitory mechanism responsible for interphase (Gaumond, Arsenault, & Marchand, 2002; Henry, Yashpal, Pitcher, & Coderre, 1999; Franklin & Abbott, 1993) arise the possibility of existence of another active mechanism responsible for the termination of phase 2. Therefore, in this study we evaluated the effect of consecutive injection of formalin into the same hind paw 60 minutes after the first injection and then measured the nociceptive response of animals for the second 60 minutes interval. The underlying hypothesis was that if the attenuation of the nociceptive behaviour at the end of phase 2 was due to an inactive mechanism, then an accumulation of nociceptive responses would occur after the second injection, due to pre-existing inflammation; but if the response was attenuated after the second injection, then an active mechanism would have been responsible for the finalization of painful responses in second phase of formalin test. In the next step, we used naloxone hydrochloride as a non-selective opioid antagonist to see whether opioid receptors are responsible for the finalization of nociceptive response at the end of the formalin test or not and to check for the possible activation of endogenous opioidergic inhibitory mechanism at the end of nociceptive responses in formalin test.

2. Methods

All Experiments were done in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, revised 1996) and were approved by the Research and Ethics Committee of Qazvin University of Medical Sciences, Qazvin, Iran. All efforts were made throughout the experiments to minimize the animal discomfort and to reduce the number of animals used. Adult male, Sprague–Dawley rats (220–300 g) purchased from Razi Institute (Hesarak Karj, Iran), were housed in groups of three in a temperature controlled room, under a 12 h light–dark cycle with lights on at 7:00 to 19:00. Food and water were provided ad libitum. During the experiments, attention was strictly paid to the regulations of local authorities for handling laboratory animals.

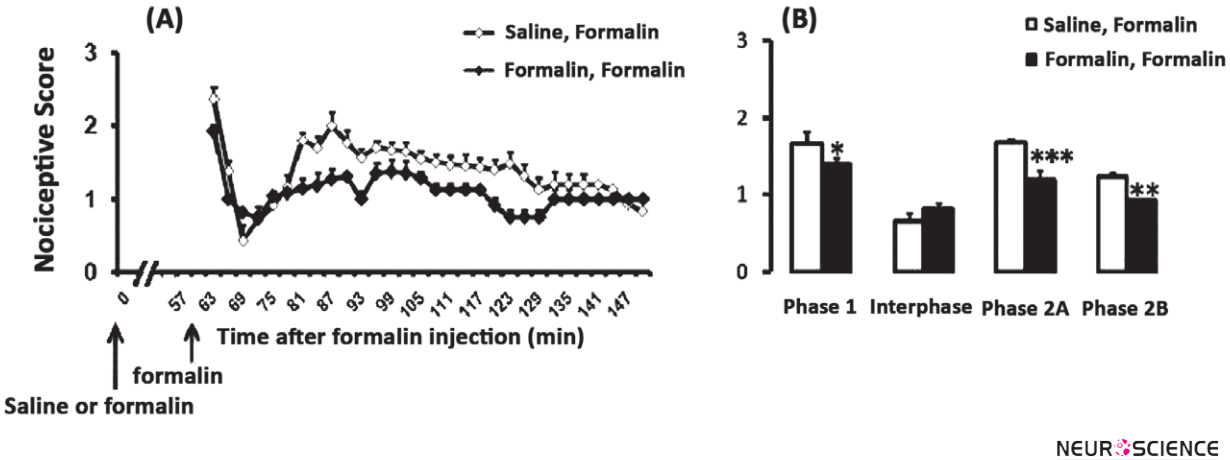

Figure 1. Time scores of formalin induced nociceptive behaviours (mean ±S.E.M. of 7–8 rats per group) following saline-formalin or formalin-formalin injection measured every 3 minutes for 150 minutes (A) and bar chart for them (B). The columns represent the mean of nociceptive score in each phase: phase 1 (1–7), interphase (8–14), phase 2A (15–60) and phase 2B (61–90), (B). Recording of the nociceptive behaviours began immediately after second injection (time 0) and was continued for the next 90 minutes. * P<0.05; ** P<0.01 and *** P<0.001 in comparison with saline-formalin group.

Rats were moved to the test room at least 1 hour before the beginning of the experiment. In the present study, rats were first acclimatized for 30 minutes in an acrylic observation chamber (30cm in diameter and in height) and then 50µL of 2% formalin was injected subcutaneously into the plantar surface of the right hind paw with a 30 gauge needle. Each rat was then immediately returned to the observation box, and behavioural recording was commenced. A mirror, placed at a 45° angle beneath the box, permitted the observation of behaviours without moving the box. Pain behaviours were scored as follows: 0, the injected paw was not favoured; 1, the injected paw had little or no weight placed on; 2, the injected paw was elevated and not in contact with any surface; and 3, the injected paw was licked or bit. Scores were continuously observed for the duration of the experiment (90 minutes). The nociceptive behaviour score for each 3-minutes interval was calculated as the weighted average of the number of seconds spent in each nociceptive behavioural condition, from the start of the experiment (Azhdari-Zarmehri, Semnanian, & Fathollahi, 2008; Azhdari-Zarmehri et al., 2011; Erami, Azhdari-Zarmehri, Ghasemi-Dashkhasan, Esmaeili, & Semnanian, 2012; Heidari-Oranjaghi, Azhdari-Zarmehri, Erami, & Haghparast, 2012). The scores were recorded in saline+ formalin group as well as in those who received formalin+ formalin injection. In each group, the second formalin injection (subcutaneously into the plantar surface of the right hind paw) was performed 60 minutes after the first one (saline in control and formalin in treated one) and behavioural responses of each rat during the first phase (1-7), inter-phase (8-14), and phase 2A (15-60) and 2B (61-90) were separately evaluated (Azhdari Zarmehri H. et al., 2011; Azhdari Zarmehri, Semnanian, & Fathollahi, 2008).

Rats were in seven groups. The 1st group was treated as described above with a saline-formalin injection of 2% formalin. The 2nd group was given a formalin-formalin injection of 2%. This second administration was given at 60 min interval. The 3rd and 4th groups were pre-treated with naloxone (1 and 3mg/kg) 5 min before the first formalin injection. The 5th and 6th groups received naloxone (s.c.; 1; 3mg/kg) 30 min after first formalin injection. To make a comparison with the 5th and 6th groups, the 7th group was treated with naloxone (s.c.; 3mg/kg) 45 min after first formalin injection.

Data are presented as Mean ±SEM. Painful scores over the 3 min time blocks, initiating upon the first injection, were analysed using repeated measures ANOVA, with comparisons between experimental groups and the control group at each time interval using Tukey's post-hoc test or planned comparisons. The defined level for statistical significance was $P<0.05$.

3. Results

Formalin at concentration of 2% produced typical biphasic pain responses. The first and second phases were separated by a brief inter-phase while a little to no nociceptive behaviour was observed in the control group. In saline-formalin injection group, formalin injection

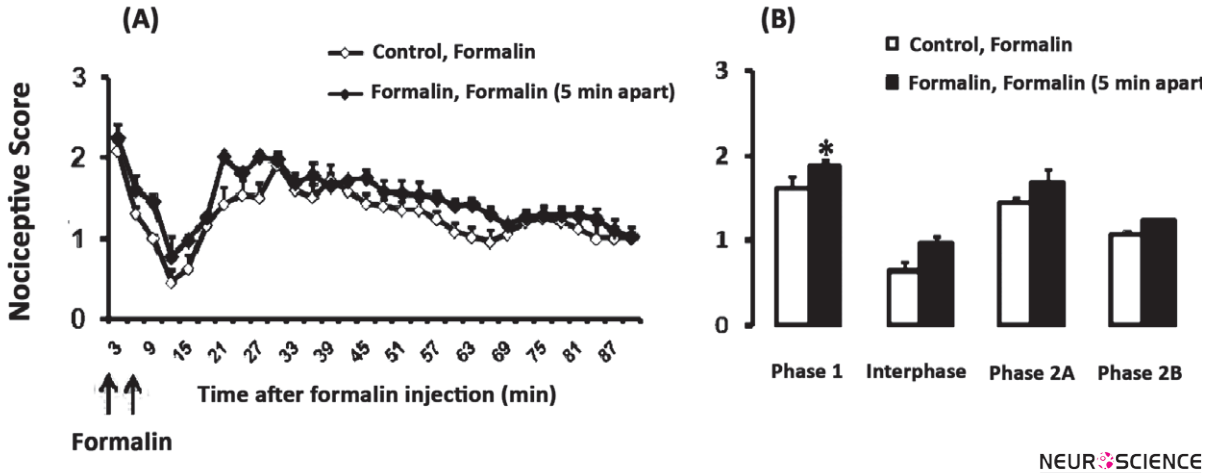

Figure 2. Time scores of formalin induced nociceptive behaviours (mean ±S.E.M. of 9 and 10 rats per group) following formalin-formalin injection (animals were given a second injection of 2% formalin 5min after the first) measured every 3 minutes for the next 90 minutes (A) and bar chart for them (B). The columns represent the mean of nociceptive score in each phase: phase 1 (1–7), interphase (8–14), phase 2A (15–60) and phase 2B (61–90), (B). Recording of the nociceptive behaviours began immediately after formalin injection (time 0) and was continued for 90 minutes. * $P<0.05$ in comparison with saline-formalin group.

induced nociceptive behaviours for less than 10 minutes, then attenuation or inhibition of nociceptive behaviours occurred which is considered as the interphase. Subsequently, the second phase (minutes 15–60) began approximately 15 min after the formalin injection. In formalin-formalin injection group, animals were given a second injection of 2% formalin 60 min after the first injection. A diminution of nociceptive responses happened during phases 1 and 2 for the second injection (for phase 1: [T(1,13)=2.261; p=0.042], for interphase: [T(1,13)=1.043; p=0.316], for phase 2A: [T(1,13)=4.455; p=0.001], for phase 2B: [T(1,13)=3.396; p=0.005]; Fig. 1).

In another experiment, animals were given the second injection of formalin 5 minutes after the first one. An increase of nociceptive responses happened during phases 1 (for phase 1: [T(1,17)=2.268; p=0.046], for interphase: [T(1,17)=1.837; p=0.093], for phase 2A: [T(1,17)=1.628; p=0.123], for phase 2B: [T(1,17)=1.207; p=0.244]; Fig. 2).

Both doses of naloxone (1 and 3mg/kg) which were pre-injected in control rats did not produce any significant change compared to saline (for phase 1: [F(2,19)=2.140; p=0.140], for interphase: [F(2,19)=1.844; p=0.185], for phase 2A: [F(2,19)=0.260; p=0.773], for phase 2B: [F(2,19)=1.038; p=0.373]; Fig. 3).

Naloxone (1 and 3mg/kg) administered 30 minutes after first formalin injection did not produce any significant changes in different phases, except for 1mg/kg of naloxone, which produced a decrease in nociceptive response in phase2A (for phase 1: [F(2,17)=0.273; p=0.764], for interphase: [F(2,17)=1.266; p=0.307], for phase 2A: [F(2,17)=4.954; p=0.020], for phase 2B: [F(2, 17)=0.030; p=0.971]; Fig. 4). Naloxone (3mg/kg) administered 45 minutes after formalin injection produced a slight but not significant increase in nociceptive response in phase2A (for phase 1: [F(2,17)=0.196; p=0.824], for interphase: [F(2,17)=1.409; p=0.271], for phase 2A: [F(2,17)=0.344; p=0.714], for phase 2B: [F(2,17)=0.257; p=0.776]; Fig. 5).

4. Discussion

In this study, a saline-formalin injection into the hind paw of the rats produced a typical biphasic response, while injection of saline did not produce any nociceptive behaviour. When a second dose of formalin was applied to the same hind paw, a decreased nociceptive response happened in phase 1 and 2 (1); and if the second injection was given 5 minutes after the first one, an increase in nociceptive behaviour was observed in phase 1. While pre-treatment with naloxone did not produce any significant changes in nociceptive behaviour during any of the phases, administration of this opioid non-selective antagonist in 1mg/kg dosage, 30 minutes after formalin injection, produced a significant decrease in phase 2A.

Although a number of studies recommend that the decrease in nociceptive behaviours after phase 1 of the formalin test is due to the active endogenous pain-suppressing mechanisms (Franklin et al., 1993; Gaumond et al., 2002; Gaumond, Arsenault, & Marchand, 2005; Henry et al., 1999), to our knowledge, there is no study

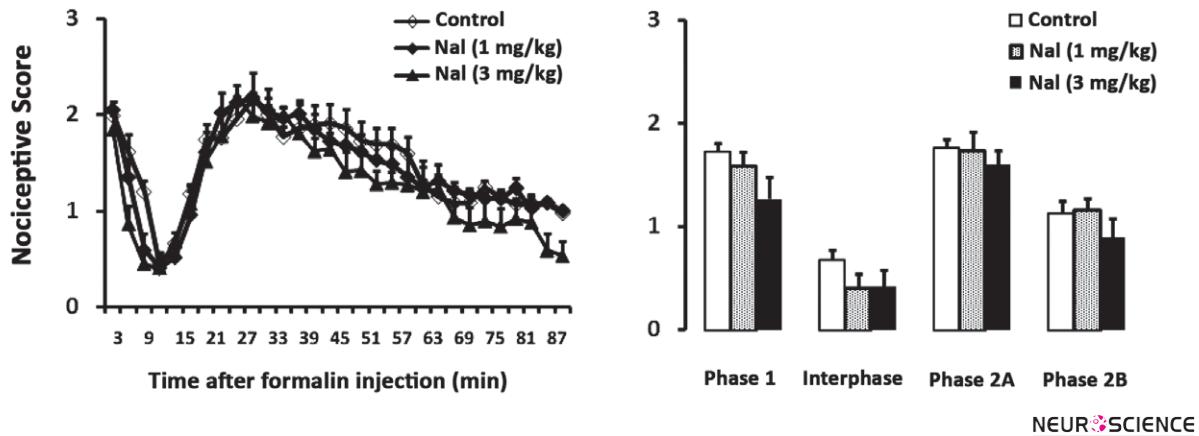

Figure 3. Time scores of formalin induced nociceptive behaviours (mean ±S.E.M. of 7 to 10 rats per group) following naloxone injection measured every 3 minutes for 90 minutes (A) and bar chart for them (B). The columns represent the mean of nociceptive score in each phase: phase 1 (1–7), interphase (8–14), phase 2A (15–60) and phase 2B (61–90), (B). Recording of the nociceptive behaviours began immediately after formalin injection (time 0) and was continued for 90 minutes.

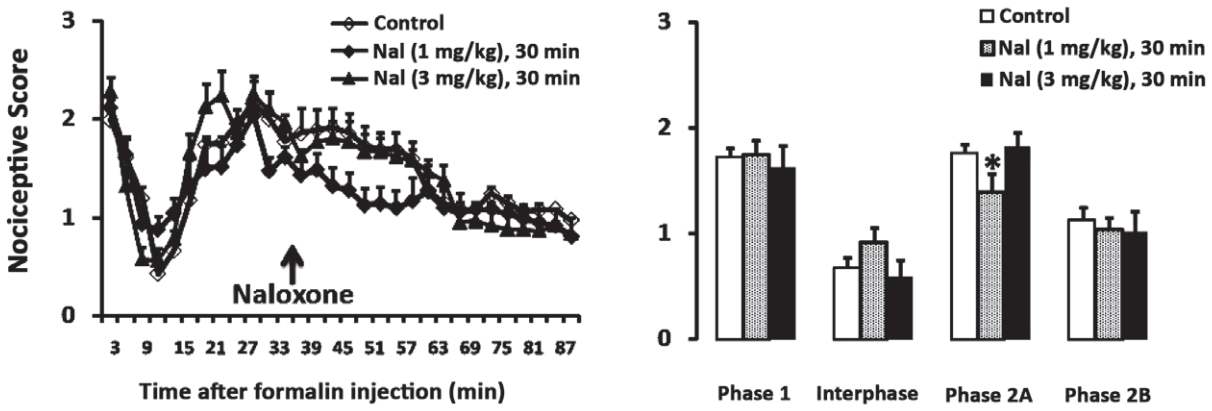

Figure 4. Time scores of formalin induced nociceptive behaviours (mean ±S.E.M. of 7 or 8 rats per group) following naloxone injected minute 30 measured every 3 minutes for 90 minutes (A) and bar chart for them (B). The columns represent the mean of nociceptive score in each phase: phase 1 (1–7), interphase (8–14), phase 2A (15–60) and phase 2B (61–90), (B). Recording of the nociceptive behaviours began immediately after formalin injection (time 0) and was continued for the next 90 minutes.

to indicate that finalization of phase 2 in the formalin test happens actively or passively. For example, it is reported that pentobarbital, diazepam and alcohol inhibit the 'inter-phase diminution' and suggest that these GABAA receptor agonists unmask the pain that is suppressed by some inhibitory mechanism (Franklin et al., 1993). In another study, Henry et al (1999) showed that after two injections of formalin (with 20 minutes interval), there was diminution of nociceptive scores after the second formalin injection, rather than an additive sum of the nociceptive responses to the two injections (Henry et al., 1999). These results propose that the attenuation in nociceptive behaviours during phase 1 and 2 which normally happens after the second injection of the formalin is due to an active inhibitory process, which can even prevent the increase in painful responses after second administration of formalin. It has been shown that the second phase of formalin test can be delayed and the nociceptive response can be manipulated by the researcher in this phase, these can suggest the existence of an active mechanism responsible for this phase. In the previous study, we demonstrated that chronic heterogeneous sequential stress increased the nociceptive behaviours in phase 2 of the formalin test in male rats compared to the control ones (data was presented in neuro2012, Japan). Also systemic administration of NMDA receptor an-

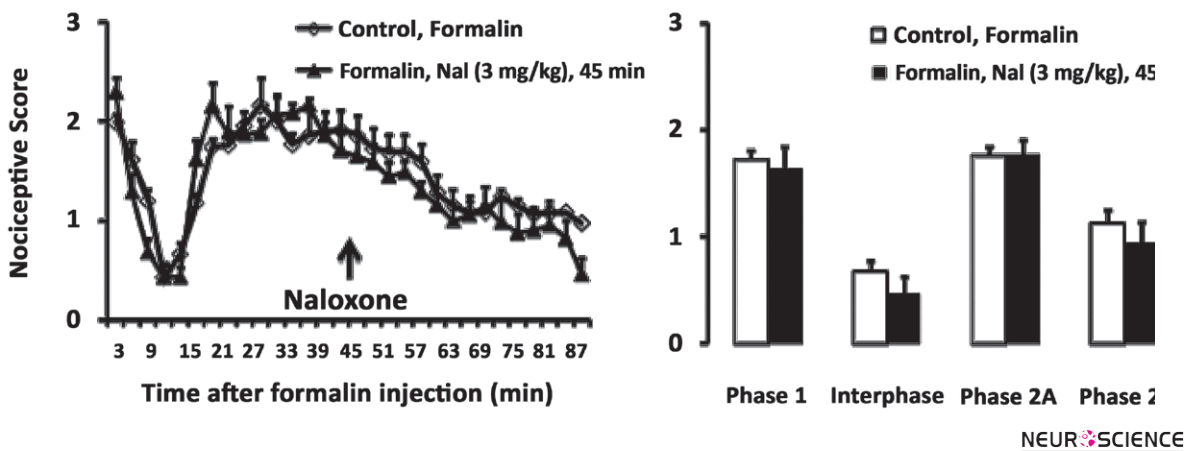

Figure 5. Time scores of formalin induced nociceptive behaviours (mean ±S.E.M. of 7 or 8 rats per group) following naloxone injection in 45 minutes after formalin injection measured every 3 minutes for 90 minutes (A) and bar chart for them (B). The columns represent the mean of nociceptive score in each phase: phase 1 (1–7), interphase (8–14), phase 2A (15–60) and phase 2B (61–90), (B). Recording of the nociceptive behaviours began immediately after formalin injection (time 0) and was continued for 90 minutes.

tagonists decreases the nociceptive responses observed during the late phase of the formalin test (Berrino et al., 2003; Sabetkasaie, Khansefid, & Ladgevardi, 2007).

Considering the fact that injection of formalin 60 minutes after the first injection was simultaneous with the decrease in nociceptive responses in phase one and two of the second test, we can suggest that attenuation of nociceptive responses in phases one and two is due to a previously activated mechanism started by the first injection of formalin. If we do not think of an active mechanism responsible for the termination of the second phase, then second injection of formalin would induce an increased response in the second experiment due to the pre-existing inflammation. But such an increase was not observed in our experiments, showing active inhibitory mechanisms responsible for finalization of nociceptive responses in formalin test.

Naloxone was ineffective in phase one and two, which is consistent with previous studies. Gaumond et al (2007) demonstrated that the inhibitory mechanism in interphase involves opioidergic system, especially in female rats (Gaumond, Spooner, & Marchand, 2007). Kocher et al. (1988) also demonstrated that systemic naloxone is not effective in altering nociceptive responses during phase one and two of formalin test, which is consistent with our findings (Kocher, 1988).

Naloxone (1mg/kg) administered 30 minutes after formalin injection was effective in reducing nociceptive behaviour in phase 2A, which is consistent with Foo and Westbrook study (1993), who demonstrated the hypoalgesic effect of naloxone (1mg/kg) on formalin test (Foo & Westbrook, 1993).

In conclusion, based on previous reports that an active inhibitory mechanism is responsible for the attenuation of nociceptive responses in interphase, we here report that the attenuation of nociceptive response at the end of the formalin test may involve an active mechanism. Since naloxone administered 45 minutes after formalin injection did not change nociceptive responses, another inhibitory mechanism seems to be responsible for the attenuation and finalization of nociceptive responses at the end of the formalin test. Further research would clarify the exact inhibitory mechanism involved.

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