PERIPHERAL INTERACTION OF OPIOID AND NMDA RECEPTORS IN INFLAMMATORY PAIN IN RATS

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Abstract : Both opioid and NMDA receptors have been known to be involved in pain processing in the central nervous system as well as in the periphery. The effect of drugs acting on opioid and NMDA receptors, and their role in modulation of pain response was observed in the formalin model of inflammatory pain in rats.

We have demonstrated that morphine has significant antinociceptive effect in the formalin model and this effect was enhanced when given in combination with ketamine. We have also reported modulation of pain response when naloxone or NMDA were co-administered with morphine or ketamine in various combinations. A noteworthy observation in our study is that low dose naloxone when co-administered with ketamine and morphine, or with ketamine and NMDA, caused decrease in the pain response.

These observations may suggest that low dose naloxone can cause modulation of opioid and NMDA receptors resulting in antinociceptive effect. Our study thus introduces a new concept of more than two drugs acting on opioid and NMDA receptors to modulate pain response.

Key words : morphine naloxone ketamine
   formalin test NMDA inflammatory pain

INTRODUCTION

The Evidence exists for the role of mu-opioid and N-methyl-D-aspartate (NMDA) receptors in pain processing (1–4). NMDA receptor antagonists have been known to cause potentiation of the opioid antinociception when given in combination (4–6). Therefore, attention has been focused on the concept of combining the properties of the mu-opioid and NMDA receptors to achieve maximum pain relief with minimum dose and side effects. We have attempted to explore the antinociception produced, using combination of various agonists and antagonists on opioid and NMDA receptors. Gillman et al (7) has proposed a dual system hypothesis suggesting the presence of two opposing opioid system. In addition, there have been reports of naloxone potentiating morphine-induced analgesia at high doses (7). However, the interactions of naloxone at a

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lower dose (0.3 mg/kg) with morphine, and with drugs acting on NMDA receptors have been less investigated. Combinations of more than two drugs acting on these receptors were also attempted to observe the modulation of the antinociceptive effect. Although the interaction between opioid and NMDA systems is a well-studied area, peripheral interactions have been less investigated. The present experiment was, therefore designed to examine the interaction of opioid and NMDA receptors following their peripheral administration using formalin-induced pain in rats, and to study the effectiveness of triple drug regimen to alleviate pain.

MATERIALS AND METHODS

Healthy male Wistar rats weighing 180–250 g were used for the study. The animals were procured from the Central Animal House, University College of Medical Sciences, Delhi. Animals were housed in groups of 4–5 per cage with free access to pellet diet and water in a temperature controlled facility. All the experiments were performed at daytime between 0930 and 1530 hr. Care of animals was as per the guidelines of Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA) (8). The study was duly approved by the Institutional Animal Ethics Committee, University College of Medical Sciences, Delhi.

Morphine Sulfate was obtained from Govt. Opium and Alkaloid Works, India. Ketamine Hydrochloride, Naloxone Hydrochloride, and N-Methyl-D-Aspartate (NMDA) were obtained from Sigma, U.S.A. Morphine, Ketamine, Naloxone and NMDA were used in doses of 5, 25, 0.3 and 10 mg/kg respectively. All the drugs were injected intraperitoneally prior

to the formalin injection, after dissolving them in 0.9% saline. The drug treated groups were injected formalin as per time of their peak effect, which is 30, 45, 15 and 30 min for Morphine, Ketamine, Naloxone and NMDA respectively (9–11). While groups involving administration of more than one drug, were scheduled such that there is no chemical interactions between the drugs at the injected site, and the peak effect of all the administered drugs appear at the same time.

Formalin test

The standard model of inducing inflammatory pain was used in the study (12). The animals were divided into 12 groups having 6 rats/group. The animals were placed in a plastic behavioral cage, where they were kept for 10 min for adaptation, and their spontaneous behavior was assessed over the 5 min period. The formalin test was carried out in a 30x30x30 cm clear plexiglass box. A mirror was mounted at a 45° angle below the transparent floor to allow an unobstructed view of the rat's paw. Then, 0.05 ml of 0.5% formalin was injected subcutaneously into the dorsal surface of the right hind paw. This amount of formalin, which is less than that used by Dubuisson and Dennis (13), produced less tissue damage without altering the sequence and duration of pain responses (14). Immediately, after the formalin injection, behavioral responses were recorded by the number of licks/bites in the early phase (0–7 min) and late phase (20–40 min). The initial 5 min period in the late phase (20–25 min) was selected on the basis of intense response seen in the preliminary studies.

Statistical analysis

The data showing number of licks/bites

were expressed as mean±S.E.M. (Standard Error of Mean) and the results were analyzed for significant differences between groups and within groups by ANOVA for repeated measures, followed by post-hoc Tukey's test. P-values less than 0.05 were considered significant.

RESULTS

Figs. 1 and 2 shows the number of licks/bites observed after formalin injection in the early and late phase respectively.

Control

On prior intraperitoneal injection of 0.9% saline and thereafter injection of 0.05 ml of 0.5% formalin produced vigorous licking and biting behavior, immediately after injection. Number of licks and bites were observed for a period of 0–7 min. After about 10 min following formalin injection, there was a period of 5–10 min during which no licking and biting behavior was seen. The late phase was observed between 20–25 min after formalin injection, and the pain response was observed as quantified by the number of licks and bites.

Morphine

Morphine administered in a dose of 5 mg/kg produced analgesic effect. The number of licks and bites were significantly reduced in both phases of pain response as compared to the control group (P<0.05) (Figs. 1 and 2).

Ketamine

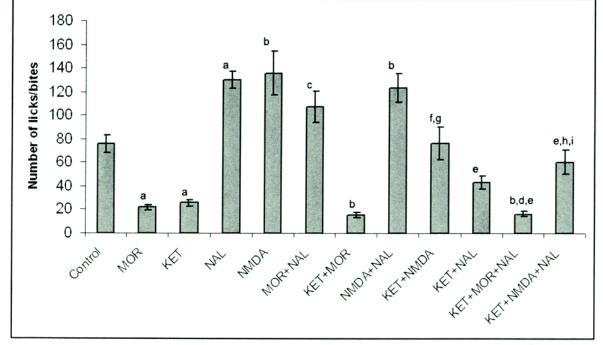At a dosage of 25 mg/kg, ketamine exhibited significant antinociceptive effect compared to control group (P<0.05). The

Fig. 1 : Effect of drugs acting on opioid and NMDA receptors on early phase (0–7 min) of formalin induced inflammatory pain response in rats (n=6). The drugs morphine (MOR), naloxone (NAL), N-methyl-D-aspartate (NMDA) and ketamine (KET) were administered intraperitoneally in doses of 5, 0.3, 10 and 25 mg/kg respectively. The data are expressed as mean±SEM. [a]P<0.05 vs control, [b]P<0.01 vs control, [c]P<0.001 vs MOR, [d]P<0.001 vs MOR+NAL, [e]P<0.001 vs NAL, [f]P<0.05 vs KET, [g]P<0.01 vs NMDA, [h]P<0.001 vs NMDA, [i]P<0.01 NMDA+NAL.
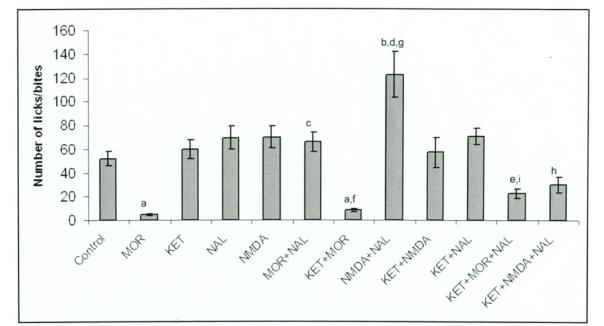
Fig. 2 : Effect of drugs acting on opioid and NMDA receptors on late phase (20–25 min) of formalin-induced inflammatory pain response in rats (n=6). The drugs morphine (MOR), naloxone (NAL), N-methyl-D-aspartate (NMDA) and ketamine (KET) were administered intraperitoneally in doses of 5, 0.3, 10 and 25 mg/kg respectively. The data are expressed as mean±SEM. [a]P<0.05 vs control, [b]P<0.001 vs control, [c]P<0.01 vs MOR, [d]P<0.01 vs NAL, [e]P<0.05 vs NAL, [f]P<0.01 vs KET, [g]P<0.01 vs NMDA, [h]P<0.001 vs NMDA+NAL, [i]P<0.05 vs KET+NAL.

effect was however, less than that observed after administration of morphine (5 mg/kg). It was also noted that the analgesic effect was found in the early phase but not in the late phase (Figs. 1, 2).

Naloxone

Administration of 0.3 mg/kg dose of naloxone produced extensive licking and biting behavior in the animals. This pain response was significantly greater (P<0.05) than that observed in the control group, for the early phase (Fig. 1). The late phase however, did not show any significant hyperalgesic effect as compared to control group (Fig. 2).

NMDA

NMDA (10 mg/kg) caused increased pain response in rats, in both phases of the formalin test, which was significant (P<0.01) for the early phase but not in the late phase compared to control group (Figs. 1, 2). The pain response in this group was quite similar to that observed with naloxone.

Morphine with naloxone

Administration of naloxone (0.3 mg/kg) was seen to antagonize the antinociceptive effect of morphine (5 mg/kg) in both the phases. This antagonism was however, more prominent in the early phase (Figs. 1, 2).

Ketamine with morphine

Ketamine in combination with morphine caused potent antinociceptive effect in both the phases. The P-values were less than 0.01 and 0.05 for the early phase and late phase respectively as compared to control group (Figs. 1, 2). The mean licking and biting

response in this group were lesser than both morphine (5 mg/kg) and ketamine (25 mg/kg) when administered alone. However, this effect was not statistically significant.

NMDA with naloxone

Administration of NMDA (10 mg/kg) and naloxone (0.3 mg/kg) caused significant increase in licking and biting response in both the phases as compared to control group (Figs. 1, 2). The P-values were less than 0.01 and 0.001 for the early phase and late phase, respectively as compared to control group. It is noteworthy that compared to pain response seen with NMDA or naloxone alone, the licking response was decreased with the combination of NMDA and naloxone in the early phase (Fig. 1). However, in the late phase, significant hyperalgesia was seen with this combination of NMDA and naloxone compared to either drug when administered alone (Fig. 2). Thus, these results suggest that there occurs some interaction between the opioid and NMDA receptors that causes analgesia in the early phase but hyperalgesia in the late phase.

Ketamine with NMDA

The results of this group show that NMDA has completely antagonized the analgesic effect of ketamine in the early phase (Fig. 1), while insignificant effect was observed in the late phase (Fig. 2).

Ketamine with naloxone

Naloxone (0.3 mg/kg) reversed the antinociceptive effect of ketamine (25 mg/kg) in the early phase (Fig. 2). This suggest that there is some interaction between the mu-opioid and NMDA receptors causing a reversal of analgesic effect of ketamine. The prominence of this effect in the early phase suggest that the efficacy of ketamine is more in the early phase, while the effect of

naloxone remained as such in the late phase (Fig. 2).

Ketamine with morphine and naloxone

In this group, the pain response in the early phase was significantly less (P<0.01) compared to control. The results were similar to that of ketamine with morphine group in the early phase, while hyperalgesic effect was seen in the late phase (Figs. 1, 2).

Ketamine with NMDA and naloxone

The results of this group reveal the potentiation of antinociception in both the phases compared with either ketamine with NMDA, or NMDA with naloxone group (Figs. 1, 2).

These results clearly indicate interaction between NMDA and opioid receptors. We have observed significant antinociceptive effect exhibited by morphine (5 mg/kg) in the formalin model, and this effect was markedly enhanced when given in combination with ketamine. When naloxone was co-administered with either ketamine and morphine, or with ketamine and NMDA, it caused some modulation of these receptors resulting in antinociceptive effect even at low dose (0.3 mg/kg).

DISCUSSION

Experimental models of pain include tests of response thresholds to high intensity stimuli (acute pain tests) and alteration in spontaneous or evoked behavioral responses in animals with peripheral injury or inflammation (persistent pain models). Formalin-induced pain response utilizes the long lasting stimulus which facilitates observation of feedback modulation and role of endogenous pain-regulatory systems such as opioid and monoaminergic systems (15–17). In rodents, two distinct phases of

the formalin test response may be used to address different aspects of nociception; since the early phase seems to be due to direct chemical stimulation of nociceptors, and the late phase is dependant on peripheral inflammation and changes in central processing due to that. This test has greater relevance for clinical situations (18).

The growing evidence of numerous adverse effects of opioids have led to the exploration of non-opioid treatment strategies like drugs acting on NMDA receptors, and also combination of opiates with antagonists of NMDA receptors for the excitatory amino acid glutamate (5, 19–20). The role of NMDA receptors in the mediation of sensitization of the interneurons following repetitive activation of nociceptors (wind up) is well proven (21). Opioid administration, even in a single dose has been shown to elicit adaptive changes in the nervous system, ultimately leading to attenuation of the effect of the opioid. These changes persist even when the antinociceptive or other depressive effects of opioid are reversed. These adaptive changes may be attributed to the activation of the NMDA receptors (19, 22). It has been demonstrated that the stimulation of NMDA receptors following opioid treatment reduces the magnitude and duration of opioid-induced antinociception, and NMDA receptor blockade acutely enhances opioid-induced antinociception (23–25). But, the significance of such an interaction has not been much emphasized. We, therefore, have examined this issue in detail and have found acute potentiation and prolongation of morphine-induced behavioral antinociception by NMDA antagonists in rats, and also some significant interactions between drugs acting on these two receptors. Such potentiation has been previously observed with both non-competitive NMDA antagonist dextromethorphan, ketamine, and

dizocilpin (MK-801) as well as competitive antagonist selfotel (CGS 19755) (26–28). In clinical practice, only ketamine and dextromethorphan have been used. Ketamine has been proven to be a good adjuvant to opioid for cancer pain (29). Ketamine combined with morphine for post-operative analgesia has generated inconsistent results (30–34). Among NMDA receptor antagonists, the most efficacious drug clinically is ketamine but produces marked sedation, dysphoria, hallucination and psychomotor effects. Thus, limiting its use to perioperative settings (35–37). Clinical data on other NMDA antagonists like dextromethorphan, memantine, amantadine are contradictory (38). Keeping these facts in mind, we selected ketamine at a dose 25 mg/kg to study the effect combination of mu-agonist and NMDA antagonist.

We have observed the effect of NMDA receptor antagonist by administration of 25 mg/kg ketamine in rats. Earlier studies using ketamine have used different doses i.e. 10 mg/kg, 40 mg/kg, 80 mg/kg (39–40). We have used a dose lesser than 40 mg/kg, since 40 mg/kg exhibited marked motor impairment, but higher than 10 mg/kg, which shows some antinociceptive activity only. Thus, though the interaction of mu-opioid receptors with NMDA receptors is known (4, 41–44), the dose of the drugs used in our study has not been much investigated. Thus, our study explores the interaction of mu-opioid receptors with NMDA receptors by their respective agonists and antagonists at these doses. Moreover, the formalin model of pain has not been given much importance for such studies as compared to hot-plate and tail-flick tests.

The antinociceptive effect of morphine (5 mg/kg) observed were different from previous studies (3) which demonstrated no

antinociception activity in formalin model at low dose (1–5 mg/kg) morphine. Previous studies using the hot-plate test and tail-flick test have shown antinociceptive property at lower doses but the same dose of morphine failed to elicit antinociceptive response in the formalin model. Our results however, show antinociception at 5 mg/kg morphine which is contrary to the earlier work done on this model at this dose (3). It is established that the antinociceptive property of morphine is variable, being influenced by factors such as species, strain, gender and age in addition to the test used for nociception (45–51).

Our study is quite different from the preclinical earlier studies performed on these receptors. Our results well demonstrated the effectiveness of 25 mg/kg ketamine in reducing the pain response in the early phase, but the response in the late phase was less satisfactory. However, the antinociceptive response in the early phase was lesser than that elicited by morphine 5 mg/kg. The combination of 25 mg/kg ketamine and 5 mg/kg morphine showed enhanced antinociception as compared to either morphine or ketamine when administered alone. This applied for both the phases of formalin-induced pain response. It has been discussed in earlier reports that certain opioid agonists and NMDA antagonists share a common receptor binding site under *in vitro* conditions (41). This provides the pharmacological basis for *in vivo* investigation of such interaction between these two groups of receptors as was done in our study, the results of which suggest the existence of direct or indirect interaction between opioid and NMDA receptors. It has been reported in *in vitro* studies that dynorphin A has been shown to potentiate the binding of a competitive NMDA receptor antagonist [$^3_1$H] CGP-39653 in rat brain

tissue probably via an interaction with glycine site of the NMDA receptor.

NMDA agonist (NMDA) and opioid antagonist (naloxone), increased pain as has been reported earlier (55, 62, 63). However, naloxone was seen to cause hyperalgesia more prominently in the late phase than the early phase of the formalin test. Moreover, naloxone, a selective mu-opioid antagonist at dose 0.3 mg/kg, partially reverses the antinociceptive effect of ketamine in the early phase, thus indicating an interaction between NMDA and opioid receptor systems. Reversal of ketamine-induced analgesia by naloxone has been reported earlier (52–54), but the dose of naloxone we have used is specific to antagonize mu-opioid receptors. Our results therefore, suggest specific interaction of ketamine at mu-opioid receptor, suggesting an interplay of NMDA and mu-opioid receptor modulation. It is noteworthy that naloxone, an opioid antagonist at lower doses (0.1 and 0.3 mg/kg) has been shown to antagonize morphine analgesia in formalin test (55). We have used 0.3 mg/kg naloxone and have met with similar results as this dose of naloxone completely antagonized analgesia induced by 5 mg/kg morphine in our experiment.

We too have observed potentiation of analgesic response when naloxone (0.3 mg/kg) is given in combination with morphine (5 mg/kg) and ketamine (25 mg/kg) together. This finding is important because lower doses of naloxone (0.3 mg/kg) is known to antagonize morphine-induced analgesia (55), but co-administration of ketamine (competitive NMDA antagonist) has probably caused receptor modulation resulting in potentiation. However, this interaction needs further study for better understanding.

It is established that at spinal level, opiates act by decreasing the release of substance P into the dorsal horn following noxious stimuli like formalin injection. This action of opiates is inhibited by naloxone (56). Naloxone also antagonized the endogenous opioid peptides which are released following a nociceptive stimulus (57). Contrary to these reports, there are also findings which show that naloxone at higher doses (10 mg/kg) produced significant potentiation of morphine-induced analgesia compared to morphine alone (55).

In previous reports, synergism between morphine and naloxone has been observed in humans (58–59), and partial opiate agonist, buprenorphine has also been reported to have similar effects when combined with naloxone (60). Furthermore, microinjections of morphine have been shown to produce analgesia as well as hyperalgesia in the rat, and both these effects were reversed by naloxone or naltrexone (61). Hence, a dual system hypothesis of pain perception as proposed by Gillman et al (7) suggest the presence of two opposing opioid systems, one enhancing and the other decreasing pain. Thus, the effect of naloxone and morphine was considered to be a net product of morphine analgesia and naloxone antagonism of morphine hyperalgesia. It is known that formalin-induced persistent inflammatory pain evokes the release of endogenous opioids (64). An earlier study showing hyperalgesia due to increased glutamate/ aspartate release due to endogenous opioids further strengthens the presence of two opposing opioid systems, one enhancing and the other decreasing pain (65). Naloxone could have possibly antagonized this hyperalgesic effect of endogenous opioids released following inflammatory conditions.

However, these interactions are subject to further studies.

NMDA receptors which have a role in the development of acute and chronic pain are stimulated by NMDA, and this effect is antagonized by its non-competitive antagonist ketamine. But in our study, this antagonism of NMDA-induced pain by ketamine is potentiated by the addition of 0.3 mg/kg naloxone. Thus, there is clear evidence of an interaction between NMDA and opioid receptors, so that naloxone can potentiate ketamine-induced analgesia even in the presence of an NMDA agonist. Naloxone when co-administered with NMDA, reduced the nociceptive effect of NMDA in the early phase, further indicating that naloxone has some modulatory action at the level of NMDA receptors as well, which is a new finding.

The combination of ketamine with NMDA and naloxone, significantly reduces the late phase of formalin-evoked nocifensive behavior as compared to control group, yet either of the drugs alone or in combination of two, do not have such effect. This suggests that triple drug combination is probably better as compared to single or two drug combination. The presence of naloxone in both the triple drug combinations studied in our study indicates the role of naloxone in modulating the antinociceptive effect of these drug combinations. These findings suggest that the hypothesis of Gillman et al (7) may hold true for triple drug combinations also involving NMDA receptors as well.

To conclude, our study clearly indicates interaction between NMDA and opioid receptors. We have demonstrated that low dose morphine (5 mg/kg) has significant antinociceptive effect in the formalin model,

and this effect was markedly augmented when given in combination with ketamine. The further decrease in pain response when naloxone was co-administered with either ketamine and morphine, or with ketamine and NMDA, suggest that naloxone can cause modulation of these receptors resulting in antinociceptive effect even at low dose (0.3 mg/kg).

Hence, this study introduces us to a whole new concept of combination of more than two drugs, to modulate the antinociceptive potential of morphine and ketamine, and thus may go a long way to understand the mechanism underlying these receptor modulations.

Using similar protocol and drug dose regimen, we are conducting study to validate this hypothesis of multiple receptor interactions during neuropathic pain.

ACKNOWLEDGEMENTS

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