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The Role of $GABA_A$ Receptor Inhibitor on Morphine Antinociception Action in Cuneiformis Nucleus

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Abstract: In order to investigate the role of cuneiformis (CnF) in pain modulation, we evaluated the effect of $GABA_A$ antagonist (bicuculline) on antinociceptive response of morphine in this nucleus. Eighty five male N-MRI rats, weighing 250-350 g were used, they were maintained on a 12 h light/dark cycle at 22-24°C. The animals were anesthetized with pentobarbital sodium (60 mg kg^{-1} ip) and were fixed on a stereotaxic apparatus. Then CnF was calculated and the animal was left to recovery for one week. The animals were divided into 4 groups; group I which received 0.5 μL of different doses of bicuculline dissolved in saline (12.5, 25, 50 and 100 ng), group II which received morphine (10 g/0.5 μL saline), group III which received a dose of bicuculline (50 ng/0.5 μL saline) plus morphine (10 g/0.5 μL saline) that followed with receive of naloxone (2 mg kg^{-1}, i.p.) and group IV; which received 0.5 μL saline and served as control. Tail Flick Latency (TFL) was measured as an index of pain during 2, 7, 12, 17, 22 and 27 min post micro-injection in CnF in all groups. Micro-injection of bicuculline in CnF increased TFL dose dependently. Morphine + bicuculline significantly increased maximum possible effect (%MPE) of antinociceptive response compared with morphine or bicuculline alone. Naloxone decreased the response to morphine. The results of this study indicates that CnF has a significant role in pain modulation, gabaergic system and opioid analogs have also play an important role on its antinociception.

Key words: $GABA_A$, morphine, cuneiformis, antinociception

INTRODUCTION

Descending inhibitory system from Rostral Ventromedial Medulla (RVM), Nucleus Raphe Magnus (NRM), Nucleus Magnucellularis (NMC) and Periequictal Gray (PAG) have regulatory action on spinal cord (Pomeroy and Behbahani, 1979).

The main source of projections to RVM and NMC are PAG and its lateral nucleus cuneiformis (CnF) (Bernard *et al*., 1989), there be electrical excitation of CnF will increase tail flick latency (TFL) via acting on NRM and NMC (Behbehani and Zemlan, 1986; Bernard *et al*., 1989).

Gamma amino butyric acid (GABA) is the main inhibitory neurotransmitter, in central nervous system (CNS), which is found in most areas of the human brain. It can interact with three found type of GABA receptor, namely $GABA_A$, $GABA_B$ and $GABA_C$ (Drew *et al*., 1984).

CnF that is located in ventro lateral region of PAG is an inhibitory descending system, as PAG and RVM it has opioid receptors and is sensitive to their antinociceptive effects (Pan and Fields, 1996; Zemlan and Behbehani, 1988).

Increasing of GABAergic inputs to off-cells of RVM, facilitate pain reflex and micro-injection of CGP_{35348} ($GABA_B$ antagonist) to RVM activate off-cells and so antinociception effects overcome (Drew *et al*., 1984; Heinricher and Kaplan, 1991).

Increasing of action of off-cells due to morphine injection to RVM, are through of a disinhibition of a GABAergic interneuron (Heinricher *et al*., 1992). Baclofen as a $GABA_B$ agonist, causes antinociception via effecting on spinal and supra spinal areas (Cutting and Forban, 1975; Levy and Proudfit, 1997; Sabetkasai *et al*., 1999).

Micro-injection of morphine in to PAG causes antinociception with activation of RVM neurons (Behbehani and Pomeroy, 1978; Pomeroy and Behbahani, 1979). However, RVM is source of axons that come from brain stem through dorsolateral funiculus (DLF) to dorsal horn of spinal cord which is the destinations for primary pain afferents (Heinricher *et al*., 1992).

Neurons in CnF have excitatory receptors to glutamate (Sabetkasai *et al*., 1999) and acetycholine (Ach) (Behbehani and Zemlan, 1986) and inhibitory receptors to GABA (Gilbert and Franklin, 2001; Nemmani and Mogil, 2003). Electrical excitation of

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CnF activate pain modulating descending system (Bernard *et al*., 1989). Regarding effect of intra cerebroventricular injection of GABA receptor agents on morphine-induced antinociception (Mahmoudi and Zarrindast, 2002) and also CnF projections to RVM, central nucleus of amygdale, PAG and other forebrain regions (Bernard *et al*., 1989; Gilbert and Franklin, 2002), we can conclude that pain defending system begins from CnF may be GABAergic and reaction between opioids and GABA receptors occur in this nucleus.

Our earlier studies with micro-injection of $GABA_A$ (Rezvanipour *et al*., 2004) and $GABA_B$ (Rezvanipour *et al*., 2005) agents into CnF nucleus by formalin test showed that $GABA_A$ and $GABA_B$ receptors in CnF have antinociception effects in chronic pain and GABAergic system via opioid receptor induced antinociception in the formalin test.

In the present study, the antinociceprive effects of $GABA_A$ receptor antagonist in the presence or absence of morphine was investigated in rat by thail flick test with micro-injection of bicuculline in CnF.

MATERIALS AND METHODS

Animals: Ninety six male N-MRI rats (weighing 200-250 g) were used in these experiments. Animals were kept 3 per cage (45 × 30 × 15 cm) at an environmental temperature of 22±3°C and a 12 h light-dark cycle. The animals had free access to food and water except during the time of experiments. Each animal was used once only and was euthanized immediately after the experiment.

Surgical procedure: Animals were anesthetized with sodium thiopental (45-60 mg kg^{-1}) intraperitoneally (i.p.) and placed in a stereotaxic frame. A sagittal incision was made from anterior to posterior area of skull. The position of cuneiformis nucleus was estimated according to Paxinos and Watson atlas (Paxinos and Watson *et al*., 1987). A hole (2 mm in diameter) drilled in the skull above the CnF and the dura reflected to allow the placement of guide cannula into the region. A guide cannula (5.3 mm in length) was inserted stereotaxically into the area of CnF (AP = 8.3 mm, L = 1.7 mm and D = 6.3 mm with references to Bregma and the cortical surface, respectively) and secured to skull with dental cement. A stainless steel dummy cannula was inserted into guide cannula and remained there when the guide cannula was not in use.

The animals were allowed to recover from surgery for at least one weak prior to the initiation of experimental protocol, to accommodate for handling stress (Heinricher *et al*., 1987). Micro-injection of bicuculline and morphine were made through a 33 gauge injection cannula that extended 3 mm below the 25 gauge guide cannula tip. All micro infusions were administered in a volume of 0.5 μL saline at a rate of 0.1 μL every 10 sec through a stainless steel internal cannula (30 gauge, Supa Co, Iran). This cannula was connected to a 1 μL Hamilton syring by a 23 cm piece of polyethylene tubing (PE-20) filled with drug solution.

Morphine sulfate (Temad Co, Iran) was dissolved in normal saline and then micro injected slowly into the CnF. A stylet was inserted into the cannula to keep it patent prior to injection. The injection needle was retained in the guide cannula for an additional 60 sec after injection to facilitate the diffusion of the drug.

Analgesic test: Tail-flick apparatus was used to evaluate the antinociceptive activity in eight groups of rats. The intensity of the radiant lamp was adjusted to provide baseline levels of 3.0 to 3.5 sec. The same setting was used for all groups (Heinricher and Kaplan, 1991) .

Each part of the tail in control and treated groups tested only once after saline or drug treatment to avoid unnecessary pain associated with radiant heat. The end point was tail-flick response sufficient to interrupt the photoelectric beam or a cut-off point of 10 sec. Data were expressed as tail-flick latency (TFL), or as percentage of maximum possible effect (%MPE), which was calculated as (Urban and Smith, 1994):

$$\%MPE = \frac{\text{Post} - \text{drug latency (sec)} - \text{Baseline latency (sec)}}{\text{Cut} - \text{off value (10 sec)} - \text{Baseline latency (sec)}}$$

The tail-flick latency time in intact (without any manipulation) and sham-operated (after surgical operations and recovery without any drug micro-injection into the CnF) rats, was measured for 20 min at 5-min intervals (0, 5, 10, 15 and 20 min; 5 trials). Following determination of baseline TFL (mean of 5 TFL trials), 0.5 μL saline or morphine (10 μg/0.5 μL saline) was infused into the CnF over a period of approximately 1 min. Two min later, TFL was measured at 5 min intervals for the period from 2 to 27 min (6 trials) post micro-injection. In main experiments, 0.5 μL from different doses of bicuculline were micro injected into the CnF and 2 min later TFL was measured at 5 min intervals for the period from 2 to 27 min (6 trials) and finally in bicuculline + morphine group, one min after micro-injection of bicuculline (50 ng), 10μg morphine was injected into the CnF and TFL was measured in 6 trials as in previous group. In the bicuculline + morphine group after measuring TFL, animals received naloxone (2 mg kg^{-1}, i.p) to reverse the effect of morphine and TFL was measured again in 5 and 10 min post injection.

Animals groups:

Control groups: Including intact (n = 15), sham-operated (cannulated) (n = 11) and saline treated (n = 11).

Morphine group: (n = 10).

Bicuculline groups: 12.5 ng (n = 7), 25 ng (n = 10), 50 ng (n = 10) or 100 ng (n = 12) of bicuculline in 0.5 μL saline was injected into the CnF.

Bicuculline: (50 ng) + morphine (10 μg) (n = 10).

Statistical analysis: Data are expressed as mean±SEM (standard error of mean). TFL time and %MPE in treated groups before and after drug administration were compared by Student's paired t-test and repeated measures analysis of variance (ANOVA) followed by protected Tukeys test for multiple comparison. On the other hand, data in all groups were subjected to the One-way and/or two-way ANOVA followed by Post-hoc analysis, as needed. p-values less than 0.05 were considered to be statistically significant.

Histological verification: At the completion of the experiment, protamine sky blue dye micro-injection (0.5 μL) into the CnF was done through the guide cannula. Then the rats were sacrificed with a lethal dose of sodium thiopental (100 mg kg^{-1}, i.p.).

Brain was removed and fixed with 10% formalin solution for no less than three days and then the brain coronal slices (50-100 μm) were prepared and examined by light microscopy for the site of injection into the CnF according to the atlas of Paxinos and Watson (Paxinos and Watson, 1987). All data have been reported here are only from animals which cannula placements were confirmed for the CnF.

RESULTS

Figure 1 shows that TFL times were not significantly different in intact, sham-operated (canulated) and saline-treated groups, so the saline-treated group, which received 0.5 μL saline by micro-injection into the CnF, was regarded as control group. The TFL time was 4.11±0.16 sec in this group. Figure 2 indicates that bicuculline dose dependently increased maximum analgesic response (%MPE) in comparison with saline and this response decreased in last minutes.

The results of comparison TFL induced by morphine (10 μg), bicuculline (50 ng), bicuculline + morphine are

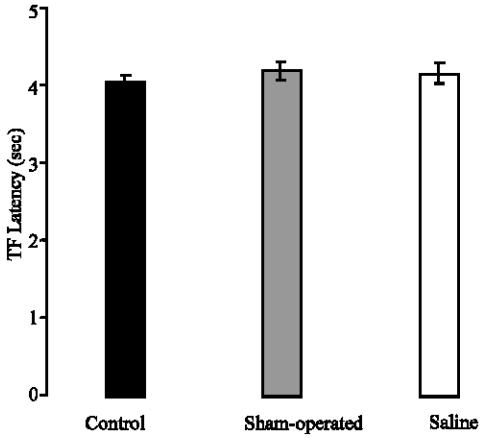

Fig. 1: Tail flick latency (TFL) time in control, sham-operated and saline micro injected groups (n = 11-15). No significant difference was seen between these groups. ($p<0.05$ was considered significant statistically)

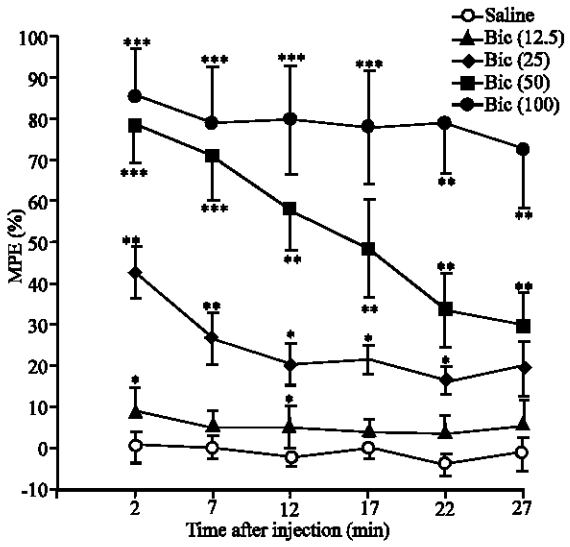

Fig. 2: MPE percentage due to injection of different doses of bicuculline compared to saline injection (n = 7-12) *** $p<0.001$, ** $p<0.01$, *$p<0.05$

shown in Fig. 3. The results indicates that analgesic response was started 2 min after micro-injection of morphine and bicuculline, but TFL was maximum in 27 and 2 min after injection in morphine and bicuculline treated groups, respectively. No difference was found between control and saline treated groups in all of times.

TFL in bicuculline + morphine is significantly more than morphine in 2,7 and 12 min after injection. The injection of naloxone (i.p) eliminated only the effect of morphine.

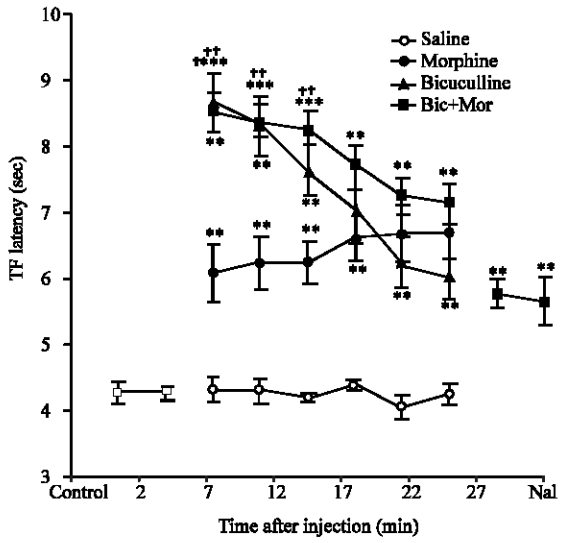

Fig. 3: Comparison of Tail flick latency (TFL) time after micro-injection of Saline (0.5 μL), morphine (10 μg/0.5 μL saline), bicuculline (50 ng/0.5 μL saline) and bicuculline + morphine (50 ng + 10 μg/0.5 μL saline) in CnF following injection of naloxone (2 mg kg^{-1} ip) (n = 10-15) *p<0.05, **p<0.01, ***p<0.001 compared to saline. p<0.01 between bicuculline+morphine and morphine

DISCUSSION

The CnF is considered as a part of the analgesia system which modulate the pain through its anatomical connections with other part of the pain modulating system, especially with periaqueductal gray (PAG) and rostral ventromedial medulla (RVM) which are the major targets for supraspinal analgesic actions of opioids (Edwards and de Olmos, 1976; Zemlan and Behbehani, 1984, 1988). Although the anatomical connections of CnF with other pain modulating systems has been studied (Bernard *et al*., 1989; Zemlan and Behbehani, 1984), but the neurophysiology of CnF has not been studied completely.

The opioid receptors are found in the pain modulating system in CNS, especially PAG and RVM (Heinricher *et al*., 1987, 1992 ; Heinricher and Tortorici, 1994). Other studies showed the presence of enkephalins on the CnF that could modulate pain (Siddall *et al*., 1994; Spinella *et al*., 1996). Others showed that there was a significant increase in TFL time 5 min after electrical stimulation of CnF and pretreatment with naloxone inhibited analgesic response of CnF to electrical stimulation (Behbehani and Zemlan, 1986; Zemlan and Behbehani, 1988).

In the present study, micro-injection of morphine into the CnF increased TFL time and analgesic response in rats, which was reversed by naloxone. These results indicate that CnF has opioid receptors, which are involved in pain modulation and the probably analgesic effect of morphine is through its binding with opioid receptors. These results are in agreement with the results of previous studies in other part of the CNS pain modulating system including PAG (Cheng *et al*., 1986) and our previous studies in CnF nucleus (Rezvanipour *et al*., 2004, 2005).

The other studies indicated that CnF through its connection with PAG could modulate pain indirectly via a powerful effect on NRM and nucleus reticularis gigantocellularis (NRGC) in RVM (Bernard *et al*., 1989; Reichling and Basbaum, 1990; Zemlan and Behbehani, 1988). About 75% of neurons in NRM respond to the electrical stimulation of CnF through DLF to inhibit nociceptive responses in the dorsal horn of spinal cord (Behbehani and Zemlan, 1986; Richter and Behbehani *et al*., 1991), which indicate that CnF plays a role in pain modulation via RVM. The analgesic response to electrical stimulation or opioid administration into the PAG mediates by on-cells and off-cells (Heinricher *et al*., 1987; Pan *et al*., 1990). Off-cells are inactivated by noxious stimuli, but analgesia causes the activation of off-cells that modulate pain control (Heinricher and Kaplan, 1991; Heinricher *et al*., 1992). Since the PAG and CnF have ultrastructural similarities it seems that CnF has also on and off-cells which modulate the analgesic effects of opioids, however further investigation is needed to prove this hypothesis. It suggests that CnF has inhibitory GABAergic output neurons (off-cells), which are connected to PAG and RVM nucleus and inhibition of off-cells in CnF by lidocaine micro-injection causes an increase in TFL time (Pan *et al*., 1990; Reichling and Basbaum, 1990). It seems bicuculline as a GABAA antagonist causes disinhibition of GABAergic inhibitory RVM off-cells neurons that originate from CnF.

In this study, micro-injection of morphine plus bicuculline in CnF caused maximum analgesic response but this effect was not reversed completely by naloxone. This results indicate that morphine and GABAA antagonist (bicuculline) induce independent analgesic effects. This finding is not completely in agreement with our studies with $GABA_B$ agents (Rezvanipour *et al*., 2005, 2004)

In conclusion, the results of this study showed that cuneiformis nucleus has a significant role in antinociception and GABAergic system and opioid analogues have important shares on this antinociception.

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