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MODIFICATION OF ANTIALLODYNIC AND ANTINOCICEPTIVE EFFECTS OF MORPHINE BY PERIPHERAL AND CENTRAL ACTION OF FLUOXETINE IN A NEUROPATHIC MICE MODEL

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We have previously reported that serotonin concentration was reduced in the brain of mice with neuropathic pain and that it may be related to reduction of morphine analgesic effects. To further prove this pharmacological action, we applied fluoxetine, a selective serotonin reuptake inhibitor, to determine whether it suppressed neuropathic pain and examined how its different administration routes would affect antinociceptive and antiallodynic effects of morphine in diabetic (DM) and sciatic nerve ligation (SL) mice, as models of neuropathic pain. Antiallodynia and antinociceptive effect of drugs were measured by using von Frey filament and tail pinch tests, respectively. Fluoxetine given alone, intracerebroventicularly (i.c.v., 15 μg/mouse) or intraperitoneally (i.p., 5 and 10 mg/kg) did not produce any effect in either model. However, fluoxetine given i.p. enhanced both antiallodynic and antinociceptive effects of morphine. Administration of fluoxetine i.c.v., slightly enhanced only the antiallodynic effect of morphine in SL mice. Ketanserine, a serotonin 2A receptor antagonist (i.p., 1 mg/kg) and naloxone, an opioid receptor antagonist (i.p., 3 mg/kg), blocked the combined antinociceptive effect of fluoxetine and morphine. Our data show that fluoxetine itself lacks antinociceptive properties in the two neuropathy models, but it enhances the analgesic effect of morphine in the periphery and suggests that co-administration of morphine with fluoxetine may have therapeutic potential in treatment of neuropathic pain.

Keywords: Neuropathic pain – serotonin – morphine – fluoxetine

INTRODUCTION

Patients with peripheral nerve injury often have persistent pain, which usually consists of allodynia (pain responses to non-noxious stimuli) and hyperalgesia (exaggerated pain responses to noxious stimuli). The management of neuropathic pain is one of the most difficult challenges facing pain specialists because of the lack of effi-

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cacy of available therapeutic modalities. Morphine, as the most robust painkiller drug is reported to be resistant in the management of neuropathic pain [2, 9].

Antinociceptive effects of morphine related to the serotoninergic system have been reported and numerous studies have revealed that the forebrain and spinal serotonin (5-hydroxytryptamine, 5-HT) pathways are involved in pain perception and also in morphine analgesia [12]. Some reports have revealed that tricyclic antidepressants relieve neuropathic pain [5, 6]. In a previous study, we reported that concentration of 5-HT was reduced in the raphe magnus nucleus of neuropathic animals [15], suggesting this was an active site where morphine induced analgesic effect. Another study also reported that 5-HT concentrations were reduced in the forebrain of neuropathic pain animals [21]. Therefore, the reduction of serotonin concentration may be involved in the attenuated analgesic effect of morphine.

Although, 5-HT is known to be an important mediator in pain modulation, its pharmacological action mechanism in morphine analgesia remains unclear. Peripheral and central actions of 5-HT which are implicated in antinociceptive effects of opioids still await elucidation. The pathway by which sensory information enters into the central nervous system, relates to the action of the serotonergic system, which induces direct stimuli information from the peripheral to the central axons to the brain. There are suggestions that the supraspinal opioid mechanisms are implicated in this stimulation of descending serotonergic transmission by noxious stimuli [22–24]. Thus, it is interesting to know whether there is contribution from 5-HT to peripheral and central action in neuropathic pain.

Referring to the basal pharmacological mechanism of these drugs, we hypothesized that fluoxetine, a selective inhibitor of presynaptic reuptake of serotonin should have an analgesic effect in the management of neuropathic pain. The aim of this study is to prove the antiallodynic and antinociceptive effects of fluoxetine given i.c.v or i.p., alone or co-administered with morphine in diabetic (DM) and sciatic nerve ligated (SL) mice as models of neuropathic pain. We examined whether there are differences in antinociceptive and antiallodynic effects of morphine depending on route of administration of fluoxetine in these models. Furthermore, the effects of ketanserin, a 5-HT_{2A} receptor antagonist and naloxone, on antinociceptive effects caused by co-administration of fluoxetine with morphine were also examined.

MATERIALS AND METHODS

Animals

Male ddY strain mice (16–18 g weight, Otsubo Experimental Animals, Nagasaki, Japan) were housed in groups of 6–8 animals. Mice were maintained in an ambient temperature (22 ± 1 °C) and relative humidity (55 ± 5%) in a control room with free access to laboratory diet (MF, Oriental Yeast, Tokyo) and tap water. After reaching

23 to 28 g, they were used for experiments. All procedures used in this study were approved by the Nagasaki University Animal Care and Use Committee.

Drugs and chemicals

Streptozotocin (STZ, Wako Pure Chemical Industries, Ltd, Tokyo, Japan), naloxone HCl (Sigma Co. Ltd., USA) and morphine HCl (Takeda Pharmaceutical Co. Ltd., Osaka, Japan) were dissolved in 0.9% saline. Ketanserin and fluoxetine (Sigma Co., USA) were dissolved in Ringer's solution, which consisted of 125 mM NaCl, 2.5 mM KCl, 0.5 mM NaH_2PO_4-H_2O, 5 mM Na_2HPO_4-$2H_2O$, 1.0 mM $MgCl_2$-$6H_2O$, 1.2 mM $CaCl_2$-$2H_2O$ and 5 mM D-glucose.

Induction of neuropathic pain

Sciatic nerve ligation

The neuropathic pain model animals were prepared by sciatic nerve ligation according to the methods of Malmberg and Basbaum [10]. Briefly, all animals were anesthetized by intravenous injection (i.v.) of pentobarbital sodium (Nembutal, 50 mg/kg) and the common sciatic nerve was exposed unilaterally (right side) at the mid-thigh, taking care to avoid interruption of epineural circulation. Two ligatures of silk (No. 8) were placed around the nerve and tied until they elicited a brief twitch in the high limb, the muscle layers and incision were then closed and sutured with silk (No. 3). Most of the experiments were conducted 10 days postligation and the mice were classified by the application of the von Frey filament (VFF), according to the method previously described by Chaplan et al. [4]; only the mice responding to von Frey filament stimuli under 1 g were considered to be neuropathic pain mice and used in the experiments.

Diabetic neuropathy

The diabetic mice were produced by a single i.v. injection of STZ at 200 mg/kg of body weight. STZ was dissolved in saline, adjusted to pH 4.5 with 1.5 mM citric acid as vehicle, and injected immediately into the tail vein. The control group of age matched non-diabetic mice (NM) received injections of vehicle only. After treatment for 2 weeks, the mice with serum glucose levels above 400 mg/dL were considered to be diabetic and used in the experiments.

Intracerebral injection of drugs

The i.c.v. injection was carried out into the left lateral ventricle of mice. Injection was performed using a Hamilton microsyringe fitted with a 27-gauge × 1/2″ i.c.v. needle according to the method of Haley and McCormick [8]. The site of injection was 2 mm caudal and 2 mm lateral to the bregma and 3 mm in depth from the skull surface.

Measurement of nociceptive response by the tail-pinch test

The tail-pinch test was conducted according to modified Haffner's method as previously reported by Takagi et al. [17]. Briefly, mice were pre-tested by pinching their tail base with forceps and only mice showing a nociceptive response such as vocalizing or biting the clip within 2 sec were used for experiments. The pressure stimuli were applied for less than 10 sec, following morphine administration, to prevent tissue damage and the test was conducted at 15 min intervals for a period of 90 min after morphine administration.

Measurement of mechanical allodynia

The mechanical allodynia test was conducted in a quiet room. The mice were individually placed on wire mesh (1 cm^2 perforations) and covered with a clear plastic cage. The VFF (North Coast Medical, Inc.) was applied in ascending weight order (0.04, 0.07, 0.16, 0.4, 0.6, 1.0, 1.4, 2.0 and 4.0 g). The VFFs were applied to the plantar surface of each hind paw from below the mesh floor and the filament was pressed against the surface at a 90° angle until it bent. Each stimulus was applied at random locations on the plantar surface for 5 times with duration of approximately 5 sec. The paw withdrawal threshold was defined as the minimum evaluator size eliciting a withdrawal reflex of the paw, at least once in 5 trials. Only robust and immediate withdrawal responses produced by the stimulus were counted. VFF of 2 g was found to be the mean evaluator size to elicit withdrawal response in normal mice, but after induction of neuropathy this was reduced to less than 1 g.

Measurement of mechanical allodynia was performed 3 times as follows: before surgery for initial cut off score, at 10 days after surgery for baseline score, and after drug administration for post-treatment score. The anti allodynic effects of drugs were measured at 15 min intervals for a period of 90 min after s.c. administration of drugs.

Data were converted to the individual percent maximum possible effect (%MPE) using the following formula:

$$\%MPE = \frac{(\text{post-treament score}) - (\text{baseline score})}{(\text{cut off score}) - (\text{baseline score})} \times 100$$

Statistical analysis

All the values are expressed as mean ± SEM. Data obtained from a single administration were analyzed by Student's *t*-test, and those from co-administration were analyzed by one-way analysis of variance (ANOVA), followed by a post hoc multiple modified *t*-test (Dunnett's method). Differences between the test and the control groups were regarded as significant if $P < 0.05$.

RESULTS

Antinociceptive effects by i.c.v. or i.p. administration of fluoxetine in SL and DM mice

Animals were administered with fluoxetine at 15 μg/mouse (i.c.v.), and at 5 or 10 mg/kg (i.p.), with the control groups receiving vehicle only. Administration of fluoxetine i.c.v. or i.p. failed to produce any antinoceceptive effects in either SL or DM mice (data not shown).

Antinociceptive and antiallodynic effects by co-administration; i.c.v. injection of fluoxetine and s.c. injection of morphine in SL mice

Animals were co-administered with fluoxetine at 15 μg/mouse (i.c.v.) and morphine by s.c. injection at 5 mg/kg, with control mice receiving vehicle and saline only. The co-administration of i.c.v. injection of fluoxetine with the s.c. injection of morphine failed to produce any antinociceptive effect in SL mice as shown in Fig. 1A. However, the antiallodynic effects of morphine were significantly enhanced from 28.3 ± 16.1 to 74.6 ± 16% MPE and gradually decreased to 52.8 ± 21.2% MPE at 90 min, as shown in Fig. 1B.

Antinociceptive and antiallodynic effects by co-administration of fluoxetine (i.p.) with morphine (s.c.) in SL mice

Animals were divided into 4 groups; the first group were treated with vehicle (i.p.) and saline (s.c.) as a control, the second group were treated with fluoxetine (i.p.) at 5 mg/kg and saline (s.c.), the third group were treated with vehicle (i.p.) and morphine (s.c.) at 5 mg/kg, and the last group were treated with fluoxetine (i.p.) at 5 mg/kg and morphine (s.c.) at 5 mg/kg. The co-administration of fluoxetine (i.p.) and morphine (s.c.) enhanced antinociceptive effects in SL mice, while the single administration of fluoxetine (i.p.) or morphine failed to produce the antinociceptive effect in SL mice. As shown in Fig. 1C, 15 min after co-administration of both compounds,

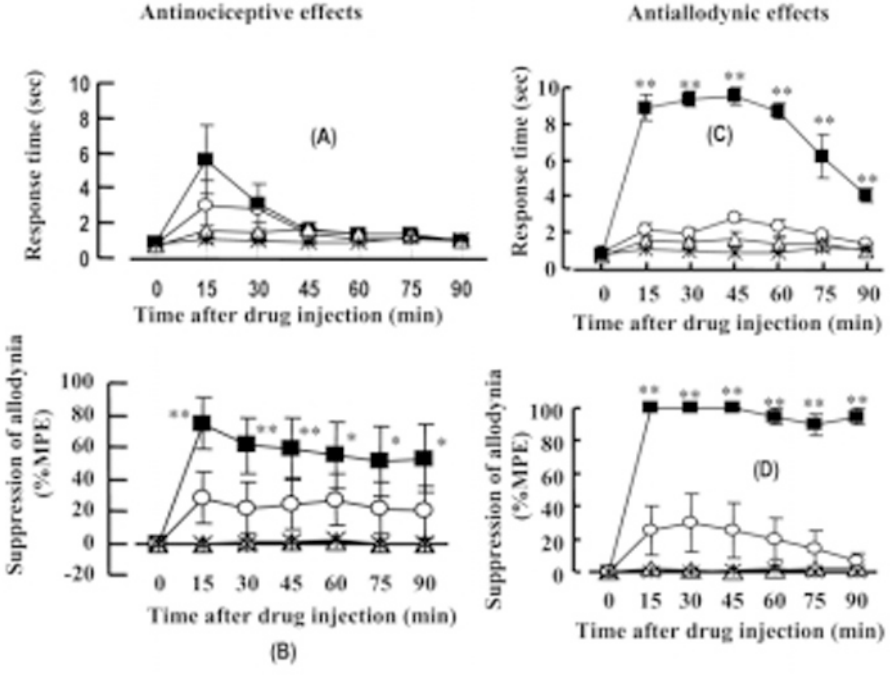

Fig. 1. Antinociceptive (A) and antiallodynic (B) effect by co-administration of morphine (s.c.) with fluoxetine (i.c.v.) or with fluoxetine [i.p., (C) and (D)] in sciatic nerve ligation mice

(A), (B): SL mice were treated with vehicle (i.c.v.) and morphine (s.c.) at 5 mg/kg (○); fluoxetine (i.c.v.) at 15 μg/mouse and saline (s.c., △); fluoxetine (i.c.v.) at 15 μg/mouse and morphine (s.c.) at 5 mg/kg (■); control group mice were treated with vehicle (i.c.v.) and saline (s.c., ✳)

(C), (D): SL mice were treated with vehicle (i.p.) and morphine (s.c.) at 5 mg/kg (○); fluoxetine (i.p.) at 5 mg/kg and saline (s.c., △); fluoxetine (i.p.) at 5 mg/kg and morphine (s.c.) at 5 mg/kg (■); control group mice were treated with vehicle (i.p.) and saline (s.c., ✳). Data present as the mean ± SEM (n = 6). Statistical significance compared with control group is represented as an asterisk (* $P < 0.05$, ** $P < 0.01$)

the latency time required to elicit pain response significantly increased to 8.8 ± 0.6 sec and the level was maintained for 60 min, decreasing gradually to 4.0 ± 0.3 sec at 90 min.

Co-administration of these compounds also produced antiallodynic effects in SL mice. As shown in Fig. 1D, 15 min after injection, the % MPE was increased to 100 and maintained at that level.

Antinociceptive and antiallodynic effects by co-administration of fluoxetine (i.c.v.) and morphine (s.c.) in DM mice

Animals were co-administered with fluoxetine at 15 mg/mouse (i.c.v.) with morphine at 5 mg/kg (s.c.), and the control group treated with vehicle (i.c.v.) and saline (s.c.)

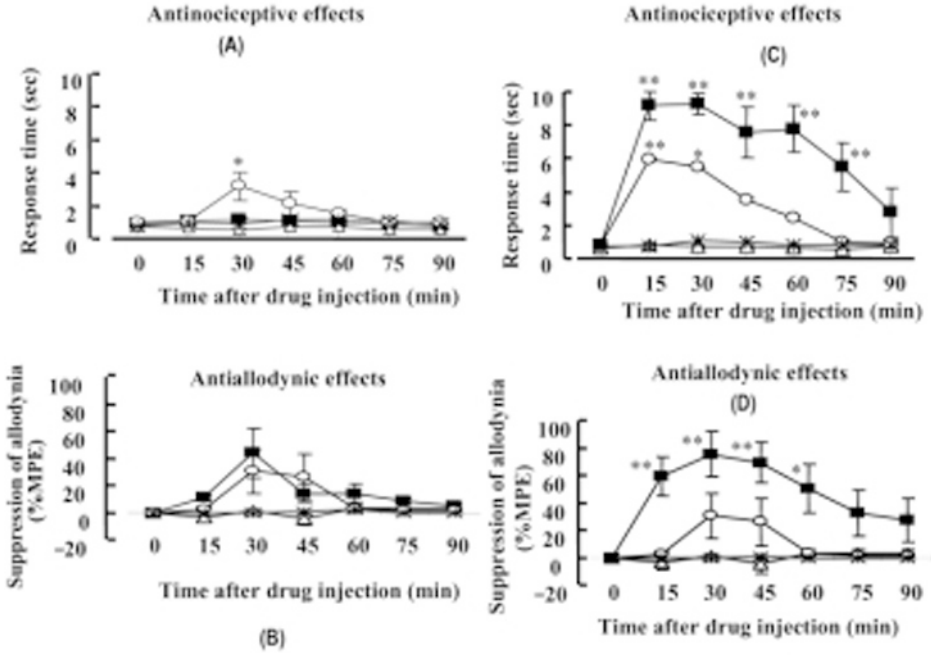

Fig. 2. Antinociceptive (A) and antiallodynic (B) effect by co-administration of morphine (s.c.) with fluoxetine (i.c.v.) or with fluoxetine (i.p.) in diabetic mice

(A), (B): DM mice were treated with vehicle (i.c.v.) and morphine (s.c.) at 5 mg/kg (○); fluoxetine (i.c.v.) at 15 μg/mouse and saline (s.c., △); fluoxetine (i.c.v.) at 15 μg/mouse and morphine (s.c.) at 5 mg/kg (■); control group mice were treated with vehicle (i.c.v.) and saline (s.c., ✳)

(C), (D): DM mice were treated with vehicle (i.p.) and morphine (s.c.) at 5 mg/kg (○); fluoxetine (i.p.) at 5 mg/kg and saline (s.c., △); fluoxetine (i.p.) at 5 mg/kg and morphine (s.c.) at 5 mg/kg (■); control group mice were treated with vehicle (i.p.) and saline (s.c., ✳). Data present as the mean ± SEM (n = 6). Statistical significance compared with control group and represented as an asterisk (* $P < 0.05$, ** $P < 0.01$)

alone. The co-administration of fluoxetine (i.c.v.) with morphine (s.c.) failed to produce either antinociceptive or antiallodynic effect in DM mice, as shown in Fig. 2A, B.

Antinociceptive and antiallodynic effects by co-administration of fluoxetine (i.p.) and morphine (s.c.) in DM mice

Unlike SL mice, DM mice receiving vehicle (i.p.) and morphine (s.c.) showed antinociceptive effects 15 min after administration, where the latency time increased to 5.9 ± 1.2 sec. After 30 min, antinociceptive effects started to decrease and continued until 90 min (from 5.4 ± 1.9 sec to 0.9 ± 0.05 sec). The co-administration of fluoxetine (i.p.) and morphine (s.c.) also significantly enhanced the antinociceptive

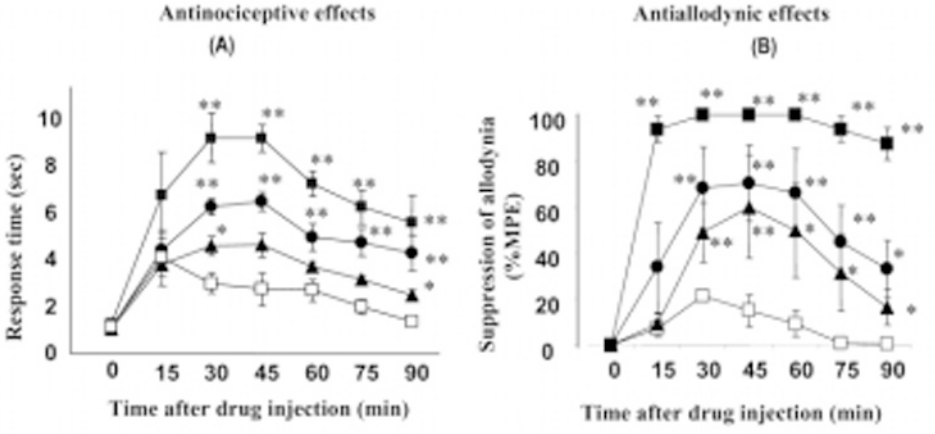

Fig. 3. Antinociceptive (A) and antiallodynic (B) effect by co-administration of morphine (s.c.) with fluoxetine (i.c.v.) or with fluoxetine (i.p.) in diabetic mice

(A), (B): SL mice were treated with vehicle (i.p.) and morphine (s.c.) at 5 mg/kg (□) as a control group; fluoxetine (i.p.) at 2 mg/kg and morphine s.c. at 5 mg/kg (▲); fluoxetine (i.p.) at 3 mg/kg and morphine s.c. at 5 mg/kg (●); fluoxetine (i.p.) at 5 mg/kg and morphine s.c. at 5 mg/kg (■). Data present as the mean ± SEM (n = 6). Statistical significance compared with control group and represented as an asterisk (* $P < 0.05$, ** $P < 0.01$)

effects in DM mice; the response time increased to 9.1 ± 0.8 sec at 15 min, 5.5 ± 1.4 sec at 75 min and 2.7 ± 1.4 sec at 90 min, as shown in Fig. 2C.

Co-administration of the compounds also enhanced the antiallodynic effect of morphine; %MPE increased to 59.2 ± 14.4 at 15 min after injection, reached a peak of 75.4 ± 16.4% after 30 min and started to decrease until 90 min from 68.9 ± 14.6 to 27.0 ± 16 as shown in Fig. 2D. Co-administration of the compounds induced antinoxiceptive and antiallodynic effects in a dose dependent manner as shown in Fig. 3A, B.

Effects of ketanserin and naloxone on antinociceptive effects by co-administration of fluoxetine (i.p.) and morphine (s.c.) in SL mice

The fluoxetine-enhanced antinociceptive effects of morphine were reversed when animals were pretreated with ketanserine at a dose of 1 mg/kg, i.p. as shown in Fig. 4A. Naloxone also reversed the fluoxetine-induced antinociceptive effects of morphine Fig. 4B.

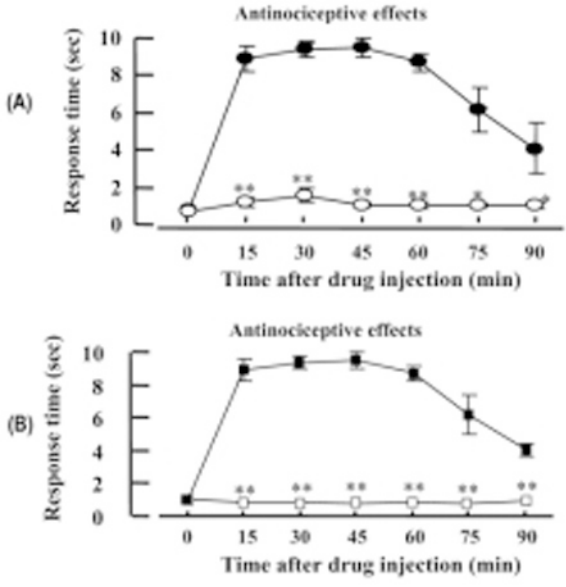

Fig. 4. Effect of ketanserin [i.p., (A)] and naloxone [s.c., (B)] on antinociceptive and antiallodynic effect by co-administration of fluoxetine (i.p.) and morphine (s.c.) in sciatic nerve ligation mice

(A): SL mice were treated with vehicle (i.p.) fluoxetine (i.p.) at 5 mg/kg and morphine (s.c.) at 5 mg/kg (●); ketanserin (i.p.) at 0.1 mg/kg and fluoxetine (i.p.) at 5 mg/kg and morphine (s.c., ○). Data present as the mean ± SEM (n = 6); The data were presented as mean ± SEM Statistical significance compared with vehicle (i.p.), fluoxetine (i.p.) at 5 mg/kg and morphine (s.c.) at 5 mg/kg group (●) are represented as an asterisk (* $P < 0.05$, ** $P < 0.01$)

(B): SL mice were treated with saline (s.c.) and fluoxetine (i.p.) at 5 mg/kg and morphine (s.c.) at 5 mg/kg (■); s.c. naloxone at 3 mg/kg and fluoxetine (i.p.) at 5 mg/kg and morphine at 5 mg/kg (s.c., □). Data present as the mean ± SEM (n = 6). Statistical significance are represented as an asterisk (** $P < 0.01$)

DISCUSSION

The mechanism by which fluoxetine exerts its analgesic effect is still open to discussion. Our data revealed that i.p. or i.c.v. administration of fluoxetine alone failed to suppress either nociception or allodynia. Another report has also revealed that fluoxetine failed to produce antinoceptive effect in a hot plate assay, but it produced antinociceptive effect in both a tail flick test and an abdominal constriction test [20]. This suggests that the antinociceptive effect of fluoxetine depends on a nociceptive stimulus. Although these reports were performed in normal animals, our study was performed in neuropathic pain model animals. In a previous study, we reported that serotonin concentration was reduced in raphe magnus nucleus of SL and DM mice as neuropathic pain models [15]. This suggests that fluoxetine fails to produce any antinociceptive effect in 5-HT-depleted animals. The reduction in 5-HT concentration in neuropathy animals may lead to the lack of analgesic properties of fluoxetine.

Further, it has been suggested that some side-effects of morphine such as dependence and addiction, are associated with the modulation of the synthesis of monoamine in brain [13]. In the previous study we found that lack of development of tolerance to morphine in a neuropathy animal model might be due to depletion of 5-HT in the brain of these model animals [16].

Our findings show that fluoxetine given i.p. not i.c.v., enhanced antinociceptive and antiallodynic effects of morphine, which were reversed by pre-administration with ketanserin, a 5-HT_{2A} receptor antagonist, suggesting that 5-HT_{2A} plays a significant role in pain modulation, with peripheral more than central action. Another report also revealed that 5-HT_{2A} relates to hyperalgesia in the peripheral site [19]. Also some studies have reported that there are several 5-HT_{2A} receptors which sensitize peripheral terminals of peripheral afferent fibers via the suppression of K^+ currents [3, 18] and suggest that 5-HT_{2A} receptors exert an antinociceptive action and trigger the GABAergic/glycinergic inhibitory potentials in the spinal cord [1]. Although it has been suggested that serotonergic neurons project to the dorsal horn, there is no evidence to prove that those neurons are involved in opioid responses [11].

Our results show that naloxone reversed the antinociceptive effect of morphine, which was enhanced by co-administration with fluoxetine. There are discrepant suggestions that a complex mechanism of serotonergic systems relate to the analgesic effect of opioids. Our findings support the suggestion that antagonism displayed by naloxone is due to the potential effect of morphine on fluoxetine-induced antinociception. This could be clarified by the fact that fluoxetine increases the release of some opioid peptides, which interact as agonists at both μ- and δ-opioid receptors to inhibit nociceptive transmission [7, 14]. Meanwhile, opiates enhance serotonergic activity in the brain by increasing the turnover rate of serotonin without altering overall concentration of the neurotransmitter [6].

In conclusion, our findings show that fluoxetine alone fails to suppress antinociception in neuropathy model mice, but it enhances the analgesic effects of opioid agonists. These actions are prominently revealed in peripheral sites, suggesting that different roles of active sites of serotonin are involved in the opioidermic system to induce antinociceptive effects in the neuropathy model. Furthermore, no sedative action of fluoxetine during its antinociceptive effects was found in our study. Thus, this mechanism may become a candidate for the development of novel therapeutic agents against neuropathic pain.

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