ISSN 1671-4083
Shanghai Institute of Materia Medica
Chinese Academy of Sciences
http://www.ChinaPhar.com

Effect of morphine-induced antinociception is altered by AF64A-induced lesions on cholinergic neurons in rat nucleus raphe magnus

Kenji ABE, Kota ISHIDA, Masatoshi KATO, Toshiro SHIGENAGA, Kyoji TAGUCHI[1], Tadashi MIYATAKE

Department of Neuroscience, Showa Pharmaceutical University,
3-3165 Higashitamagawagakuen, Machida, Tokyo 194-8543, Japan

KEY WORDS cholinergic fibers; acetylcholine; raphe nuclei; morphine; rats

ABSTRACT

AIM: To examine the role of cholinergic neurons in the nucleus raphe magnus (NRM) in noxious heat stimulation and in the effects of morphine-induced antinociception by rats. **METHODS:** After the cholinergic neuron selective toxin, AF64A, was microinjected into the NRM, we examined changes in the antinociceptive threshold and effects of morphine (5 mg/kg, ip) using the hot-plate (HP) and tail-flick (TF) tests. **RESULTS:** Systemic administration of morphine inhibited HP and TF responses in control rats. Microinjection of AF64A (2 nmol/site) into the NRM significantly decreased the threshold of HP response after 14 d, whereas the TF response was not affected. Morphine-induced antinociception was significantly attenuated in rats administered AF64A. Extracellular acetylcholine was attenuated after 14 d to below detectable levels in rats given AF64A. Naloxone (1 μg/site) microinjected into control rat NRM also antagonized the antinociceptive effect of systemic morphine. **CONCLUSION:** These findings suggest that cholinergic neuron activation in the NRM modulates the antinociceptive effect of morphine simultaneously with the opiate system.

INTRODUCTION

The nucleus raphe magnus (NRM) and adjoining structures located in the rostral ventral medulla modulate the transmission of nociceptive information. The NRM contains a high density of serotonergic neurons that mediate morphine-induced analgesia[1]. In addition to serotonergic neurons, cholinergic terminal markers such as choline acetyltransferase[2], acetylcholinesterase[3] and cholinergic receptors[4] are present in the NRM.

Morphine interacts with cholinergic neurons in the central nervous system. Systemic morphine decreases the release of acetylcholine (ACh) in the rat striatum[5]. In contrast, intravenous morphine produces a dose-dependent increase in the ACh level of human cerebrospinal fluid[6]. Furthermore, the cholinergic system in the brainstem is thought to play a part in the endogenous pain control system, a disruption of which can produce antinociception and analgesia in many animals, including humans[7]. Pharmacological studies provide evidence that either ACh or carbachol administered into NRM can produce pronounced antinociception that is reversed by muscarinic antagonists[8]. Evidence indicates that the descending cholinergic system, spinal cholinergic receptors, and anticholinesterase are involved in the

[1] Correspondence to Kyoji TAGUCHI. Phn 81-042-721-1415. Fax 81-042-721-1588. E-mail taguchi@ac.shoyaku.ac.jp
Received 2002-04-02 Accepted 2002-08-05

mechanisms of opioid analgesia[9,10]. We reported that the systemic administration of morphine seems to exert a direct stimulatory effect on ACh release in the rostral ventrolateral medulla and that morphine-induced antinociception is, in part, activated by ACh release[11]. These observations support the notion that activation of cholinergic neurons in the NRM mediates systemic morphine-induced antinociception.

The aziridinium ion of ethylcholine (AF64A) is a selective cholinergic neurotoxin that might be a powerful tool in unraveling the functional role of the cholinergic system in the NRM. The present study investigated the role of cholinergic neurons in the NRM on antinociception by microinjecting AF64A into the rat NRM and examined changes in antinociceptive responses and systemic morphine-induced antinociception.

MATERIALS AND METHODS

Treatment of AF64A Male Wistar rats weighing 250 to 350 g were acclimatized then anesthetized with sodium pentobarbital (50 mg/kg, ip) and placed in a stereotaxic apparatus. Experiments proceeded according to the guidelines for animal care and use published by the National Institutes of Health. A midline incision was made on the scalp, and a burr hole was drilled through the skull. The coordinates of the NRM were determined according to the atlas of Paxinos and Watson[12] as follows; bregma -11.0 mm, lateral 0 mm, depth -8.5 mm. A 30-gauge stainless steel injection cannula connected to a microsyringe by PE-10 polyethylene tubing was filled with either saline or a solution of AF64A (2 nmol/site). Either solution was injected in a volume of 0.5 μL over 60 s into the NRM. After infusion of saline or AF64A, the injection cannula was carefully withdrawn. The burr hole was filled with acrylic dental cement and the scalp was sutured. To confirm that no abnormalities resulted from the procedure, animals were observed for one week after surgery and prior to drug administration.

Measurement of antinociception Antinociceptive behavior were measured at 7 and 14 d after AF64A microinjection using the hot-plate (HP) and tail-flick (TF) tests to examine threshold changes. The antinociceptive effect of morphine (5 mg/kg, ip) was assessed 14 d after AF64A microinjection. Animals were placed onto a heated surface (55 °C) and the latency of paw licking or jumping was measured (HP test). A cut-off time (30 s) was established to avoid tissue injury. For the TF test, animals were held in the polycarbonate apparatus with the tail protruding. The tail was blackened with ink on the ventral surface at about 3 cm from the tip. Three spots were marked on this surface at intervals of 1 cm and high-intensity light heat was applied to blacken spots at random. The time interval between the onset of tail heating and the withdrawal response was electronically measured. We established a cut-off time of 10 s to minimize the likelihood of tissue damage. The mean of HP and TF response latency value from 3 consecutive trials before AF64A administration was taken as the baseline for the noxious threshold examination, and the value at 14 d after AF64A administration but before morphine administration, was taken as the baseline of morphine-induced antinociception. The HP and TF response of each rat in the morphine-induced antinociception study was converted to percent of maximal possible effect (%MPE), according to the following formula:

$$\%\text{MPE} = \frac{\text{Post-drug latency} - \text{Pre-drug latency}}{\text{Cut-off time} - \text{Pre-drug latency}} \times 100\%$$

The positive control was a group in which naloxone (1 μg/site) was microinjected into the NRM 15 min before morphine administration.

***In vivo* microdialysis** We compared the amount of ACh in the NRM with or without AF64A using *in vivo* microdialysis assay. Animals were anesthetized with sodium pentobarbital (50 mg/kg, ip) and positioned in a stereotaxic apparatus. A guide cannula was implanted and either saline or AF64A was applied as described above. After 14 d, a microdialysis probe was implanted into the NRM through the guide cannula and connected by polyethylene inflow and outflow tubes to a syringe pump and collection vials. Animals were released in a Plexiglas cage. The NRM was perfused with NaCl 125 mmol/L, KCl 3 mmol/L, $CaCl_2$ 1.3 mmol/L, $MgCl_2$ 1 mmol/L, $NaHCO_3$ 23 mmol/L, and neostigmine 1 μmol/L in phosphate buffer 1 mmol/L (pH 7.4) through the dialysis probe at a rate of 2 μL/min. Levels of ACh were measured by high-performance liquid chromatography using electrochemical detection, as described[11]. The perfusate was collected at 15 min intervals, and the mean value of the ACh content was calculated in 3 consecutive perfusates.

After all experiments, the animals were deeply anesthetized with sodium pentobarbital. The sites of microinjections and dialysis cannula were verified in each

animal by injecting methylene blue into the NRM. Animals were then sacrificed using an overdose of sodium pentobarbital. The brain was removed and fixed overnight in 10 % formalin. Cryostat sections were then cut for examination under light microscopy.

Data analysis Data are expressed as mean ±standard error (SE) for each group. The statistical significance of differences was analyzed by the paired *t*-test to examine the effects of AF64A on the noxious threshold. Significant differences in morphine antinociception between groups were analyzed by repeated measurement two-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison test. A value of $P<0.05$ was regarded as statistically significant.

Drugs We used morphine hydrochloride (Sankyo), naloxone hydrochloride (Endo Laboratories) and acetylethylcholine mustard-HCl (acetyl-AF64, Sigma-RBI). Acetyl-AF64 was dissolved in sterile distilled water. The pH was adjusted to 11.5 with NaOH 10 mol/L, and the mixture was stirred for 20 min convert the ester to AF64. The pH was then adjusted to 7.4 with concentrated HCl to yield the choline mustard aziridinium ion (AF64A). AF64A solutions were prepared 1-2 h before use. Morphine and naloxone were dissolved in sterile saline.

RESULTS

Effects of AF64A on the nociceptive response AF64A (2 nmol/site, $n=6$) microinjected into the NRM induced no significant change in latency time in HP and TF test 7 d later [(6.7±0.4) s and (3.0±0.4) s], as compared with before administration [HP test; (6.2±0.6) s, TF test; (2.87±0.14) s]. Although latency time was not affected in the TF test at 14 d after AF64A microinjection [(3.10±0.12) s], latency of HP response was significantly reduced [(4.41±0.18) s, $P<0.05$, Fig 1]. The microinjection of saline (0.5 μL/site, $n=7$) did not affect HP or TF latency at 7 and 14 d after surgery.

Effects of AF64A on systemic morphine-induced antinociception Intraperitoneally administered morphine (5 mg/kg, $n=5$) inhibited control HP and TF responses. The effects peaked at 15 min (HP; %MPE=65±6, TF; %MPE=89±6, Fig 2). The anti-nociceptive effect of morphine in the HP test decreased with elapsed time and returned to the baseline value, although the TF response maintained a high latency until 60 min after morphine administration (%MPE =56±13).

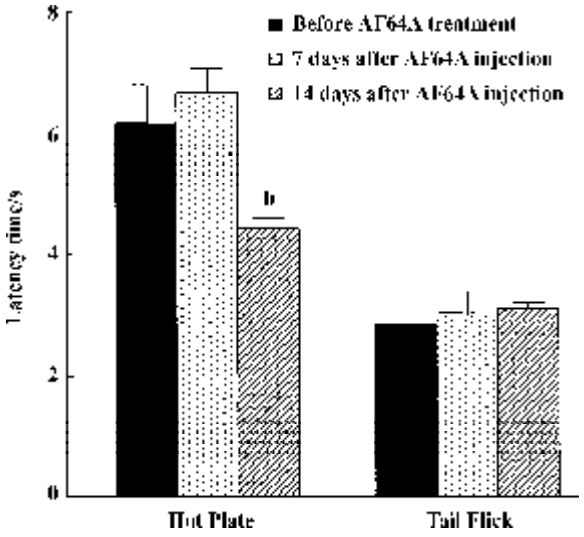

Fig 1. Effect of microinjection of AF64A into the NRM on noxious threshold. Fourteen days after AF64A administration, HP response was significantly decreased. HP and TF responses 7 d after AF64A microinjection, and TF response 14 d thereafter were not affected. Data were expressed as response latencies of 6-7 animals. Mean±SE. $^bP<0.05$ *vs* control group using paired *t*-test.

Microinjection of AF64A into the NRM significantly reduced the antinociceptive effect of morphine in both the HP ($n=5$) and TF ($n=5$) tests. The antinociceptive effect in the HP test was maximal at 15 min after morphine administration, and the response was significantly lowered in AF64A treated rats (%MPE=38 ±5, $P<0.01$, Fig 2). Reduced antinociceptive effect in AF64A treated group lasted for 40 min after morphine administration, demonstrating a statistically significant difference. Microinjection of AF64A also reduced TF latency. The maximal antinociceptive response of TF latency was at 10 min after morphine administration in AF64A treated rats (%MPE=56±15, Fig 2), after which latency tended to decrease. From 15 to 60 min, the inhibited antinociception of morphine lasted similarly to that in control rats assessed by the TF test. Naloxone (1 μg/site, $n=6$) microinjected into the NRM potently attenuated morphine-induced antinociception in the HP and TF response (Fig 2).

Histological verification of the microinjection site within NRM was shown in Fig 3. AF64A, naloxone or saline was microinjected into the NRM at the sites indicated by the circles in Fig 3.

Effects of AF64A on subseqent release of acetylcholine in the NRM The amount of extracellular ACh recovered from the NRM with an acute implanted

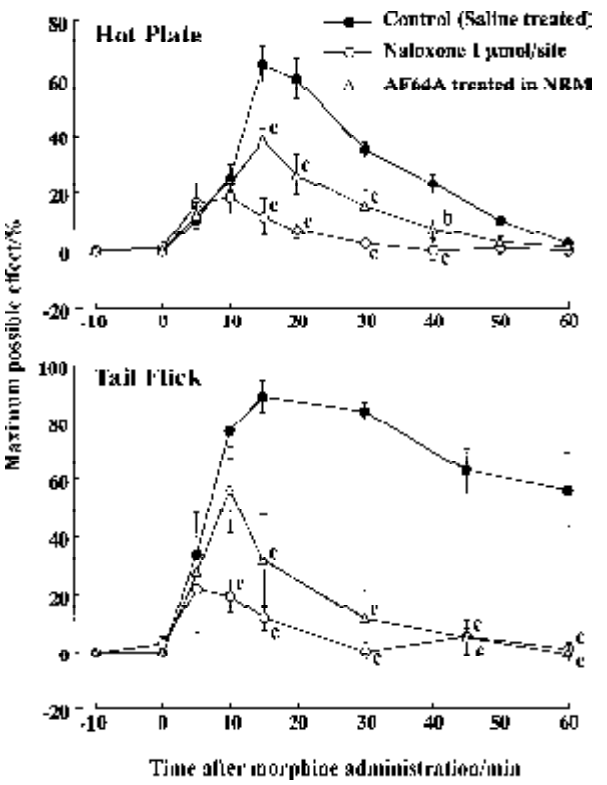

Fig 2. Effect of AF64A microinjected into the NRM on systemic morphine-induced antinociception. AF64A attenuates subsequent antinociceptive response to morphine in both HP and TF tests. Naloxone (1 μg/site) potently antagonized HP and TF responses. Data were expressed as response latencies of 5-6 animals. Mean±SE. $^{b}P<0.05$, $^{c}P<0.01$ *vs* control group using Dunnett's multiple comparison test.

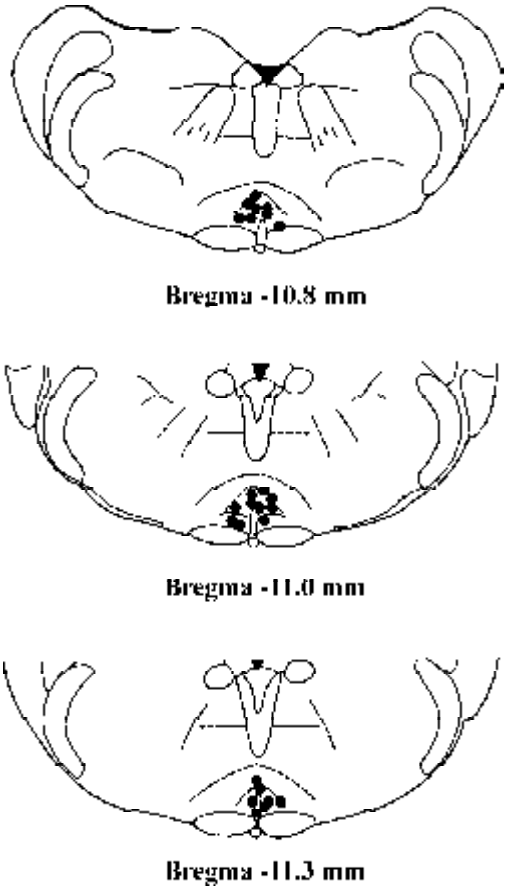

Fig 3. Location of microinjection site of AF64A in the NRM. All injection sites were located within NRM in the rostral ventromedial medulla.

microdialysis probe was (178±30) fmol per 30 μL sample (n=4) with a perfusion solution containing 1 μmol/L neostigmine in control group (Fig 4A, B). Fourteen days after AF64A microinjection into the NRM (2 nmol/site, n=4), ACh release in the dialysate was attenuated to undetectable levels (Fig 4C). The present data demonstrated that AF64A attenuates the release of extracellular ACh in the rostral ventromedial medulla, including the NRM. The track of the dialysis probe is shown in Fig 4D. These sites were confirmed within the rostral ventromedial medulla and including the NRM.

DISCUSSION

The present study evaluated the effects of microinjected AF64A, a selective cholinergic neurotoxin, on TF and HP antinociception. Microinjection of AF64A into the NRM of rats decreased the noxious threshold in the HP response, but not in the TF response. In general, the TF response is believed to be spinally mediated[13], whereas the HP response is supraspinally mediated[14]. Thus, the neurotoxin AF64A microinjected into the NRM may attenuate the noxious threshold in the supraspinal response by inactivating cholinergic pain control systems. In contrast, the TF response did not change the noxious threshold of the spinally mediated reflex with damaged cholinergic neurons in the NRM. These findings suggest that cholinergic neurons in the rat NRM play an important role at noxious threshold in the HP response.

The present findings demonstrated that microinjection of AF64A into the rat NRM significantly attenuates the efficacy of morphine (5 mg/kg, ip)-induced antinociception 14 d after administration. Our *in vivo* microdialysis study demonstrated that microinjection of AF64A completely attenuates extracellular ACh release in the NRM. Therefore, our results suggest that the antinociceptive effect attenuated by the systemic administration of morphine is affected by the activity of cholinergic neurons in the NRM. Anatomical data have

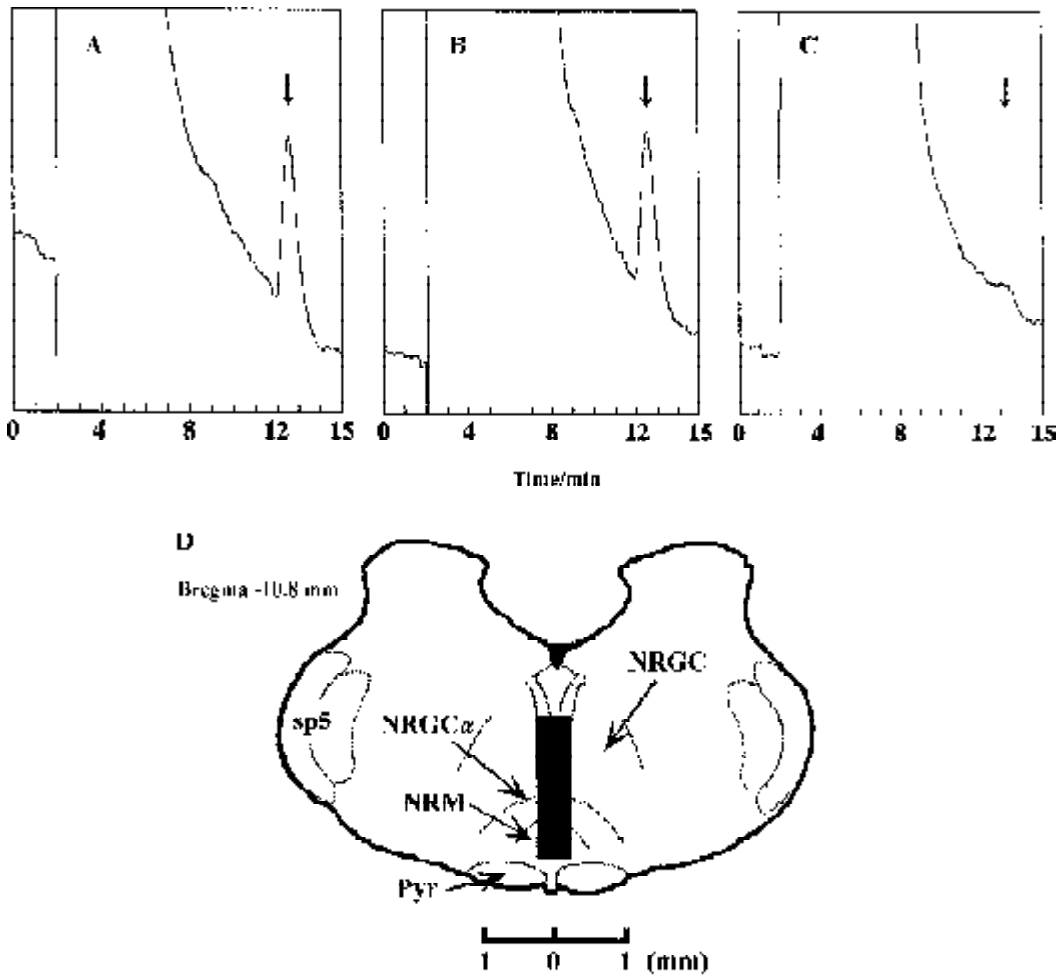

Fig 4. Typical peak of ACh in HPLC charts, A: Standard peak of ACh (injected 300 fmol in 30 μL); B: typical peak of control animal; C: 14 d after AF64A microinjection. D: Location of microdialysis probe. ACh content in dialysate decreased to undetectable levels in AF64A treated group.

demonstrated that the pedunculopontine tegmental nucleus in the brainstem is the major source of cholinergic afferents to the rostral ventrolateral medulla[15]. The presence of cholinergic terminal markers such as choline acetyltransferase[2], acetylcholinesterase[3], and cholinergic receptors[4] in the NRM suggests a functional cholinergic input to this region. Our data demonstrated that the microdialysis probe was used to measure the basal release of ACh from cholinergic terminals within the rostral ventromedial medulla including the NRM. Thus, these biochemical data indicate that cholinergic neurons are located in the NRM. However, the origin of cholinergic neurons in the NRM is unclear. Electrophysiological studies have shown that the iontophoretic application of ACh to the NRM produces both inhibitory and excitatory responses of pinch-excited cells and that pinch-inhibited cells are located in this area[16]. The NRM is generally throught to be composed of heterogeneous populations of neurons including ACh neurons. The cholinergic agonist, carbachol, administered into the NRM produces analgesia mediated by muscarinic receptors in the rat[8]. In our experiments, AF64A attenuated the morphine-induced antinociceptive responses, indicating that antinociception by systemic morphine is weak in the presence of endogenous ACh depletion. Our data and these reported findings indicate that the activation of cholinergic neurons in the NRM may, at least partially, modulate morphine-induced analgesia in rats.

Moreover, the opiate system of the NRM plays a role in morphine-induced antinociception. Microinjection of morphine into the NRM produces antinociception, and the systemic administration or microinjection of naloxone into the NRM attenuates the antinociceptive

effect of morphine administered by microinjection into the NRM[17]. The present data showed that naloxone microinjected into the NRM antagonizes morphine-induced antinociceptive responses. Thus, the antinociceptive effect of morphine may be regulated by synergistic interactions between the cholinergic and opiate systems in the NRM. On the other hand, the possibility cannot be ruled out that microinjection of AF64A influences serotonergic, GABAergic and glutaminergic neurons in the NRM.

In conclusion, lowered ACh content caused by AF64A in the NRM decreased the noxious threshold of the HP response, and significantly attenuated morphine-induced antinociception. Thus, the activation of cholinergic neurons in the NRM may modulate the antinociceptive effect of a systemic administration of morphine.

REFERENCES

1 Basbaum AI, Fields HL. Endogenous pain control systems: brainstem spinal pathways and endorphin circuitry. Ann Rev Neurosci 1984; 7: 309-38.
2 Kobayashi RM, Brownstein M, Saavedra JM, Palkovits M. Choline acetyltransferase content in discrete regions of the rat brain stem. J Neurochem 1975; 24: 637-40.
3 Palkovits M, Jacobowitz DM. Topographical atlas of catecholamine and acetylcholinesterase-containing neurons in the rat brain. II. Hindbrain (mesencephalon, rhombencephalon). J Comp Neurol 1974; 157: 29-42.
4 Rotter A, Birdsall NMJ, Field PM, Raisman G. Muscarinic receptors in the central nervous system of the rat. II. Distribution of binding of [^{3}H]propylbenzilyl-choline mustard in the midbrain and hindbrain. Brain Res Rev 1979; 1: 167-83.
5 Taguchi K, Hagiwara Y, Suzuki Y, Kubo T. Effects of morphine on release of acetylcholine in the rat striatum: an *in vivo* microdialysis study. Naunyn-Schmiedeberg's Arch Pharmacol 1993; 347: 9-13.
6 Bouaziz H, Tong C, Yoon Y, Hood DD, Eisenach JC. Intravenous opioids stimulate norephinephrine and acetylcholine release in the spinal cord dorsal horn. Anesthesiology 1996; 84: 143-54.
7 Hood DD, Eisenach JC, Tuttle R. Phase I safety assessment of intrathecal neostigmine methylsulfate in humans. Anesthesiology 1995; 82: 331-43.
8 Brodie M, Proudfit HK. Hypoalgesia induced by the local injection of carbachol into the nucleus raphe magnus. Brain Res 1984; 291: 337-42.
9 Hood DD, Mallak KA, James RL, Tuttle R, Eisenach JC. Enhancement of analgesia from systemic opioid in humans by spinal cholinesterase inhibition. J Pharmacol Exp Ther 1997; 282: 86-92.
10 Frang F, Proudfit HK. Spinal cholinergic and monoamine receptors mediate the antinociceptive effect of morphine microinjected in the periaqueductal gray on rat tail, but not the feet. Brain Res 1996; 722: 95-108.
11 Taguchi K, Kato M, Kikuta J, Abe K, Chuma T, Chikuma T, *et al*. The effects of morphine-induced increase in extracellular acetylcholine levels in the rostral ventrolateral medulla of rat. J Pharmacol Exp Ther 1999; 289: 1539-44.
12 Paxinos G, Watson C. The rat brain in stereotaxic coordinates. 2nd ed. New York: Academic Press; 1986.
13 Irwin S, Houde RW, Bennett DR, Hendershort LC, Seevers MH. The effects of morphine, methadone and meperidine on some reflex responses of spinal animals to nociceptive stimulation. J Pharmacol Exp Ther 1951; 101: 132-8.
14 Jensen TS, Yaksh TL. Spinal monoamines and opiate systems partly mediate the antinociceptive effects produced by glutamate at brainstem sites. Brain Res 1984; 321: 287-97.
15 Yasui Y, Cechetto DF, Clifford SB. Evidence for a cholinergic projection from the pedunculopontine tegmental nucleus to the rostral ventrolateral medulla in the rat. Brain Res 1990; 517: 19-24.
16 Hentall ID, Andresen MJ, Taguchi K. Serotonergic, cholinergic and nociceptive inhibition or excitation of raphe magnus neurons in barbiturate-anesthetized rats. Neuroscience 1993; 52: 303-10.
17 Dickenson AH, Oliveras JL, Besson JM. Role of the nucleus raphe magnus in opiate analgesia as studied by the microinjection technique in the rat. Brain Res 1979; 170: 95-111.