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# *Meta*-chlorophenylpiperazine attenuates formalin-induced nociceptive responses through 5-$HT_{1/2}$ receptors in both normal and diabetic mice

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**1** This study was designed to investigate the effect of *meta*-chlorophenylpiperazine (m-CPP; a 5-hydroxytryptamine (5-HT) receptor agonist) on the formalin-induced nociceptive responses in normal, insulin-dependent streptozotocin (STZ) diabetic and non-insulin dependent genetically diabetic (db/db) mice.

**2** A subcutaneous injection of diluted formalin (1% formaldehyde in 0.9% saline, 10 $\mu$l) under the plantar surface of the left hindpaw induced biphasic nociceptive responses, the first and second phases considered to represent acute and chronic pain, respectively. The former response in db/db mice was significantly lower than those in normal mice, and the latter responses in STZ and db/db mice were significantly lower than those in normal mice.

**3** In normal mice, m-CPP (0.32-3.2 mg $ml^{-1}$, p.o.) exhibited potent antinociceptive activity, dose-dependently attenuating the frst and second phase; the $ID_{50}$ value of the second phase was 0.4 mg $kg^{-1}$. m-CPP (0.32-3.2 mg $kg^{-1}$, p.o.) also dose-dependently attenuated the formalin-induced nociceptive responses in STZ-induced diabetic mice and genetically diabetic db/db mice, and the activities were comparable to those in normal mice.

**4** The antinociceptive activities of m-CPP (1 mg $kg^{-1}$, p.o.) were significantly inhibited by pretreatment with pindolol (a 5-$HT_1$-receptor antagonist, 1 mg $kg^{-1}$, i.p.) or ketanserin (a 5-$HT_2$ receptor antagonist, 1 mg $kg^{-1}$, i.p.) but were hardly affected by ICS205-930 (a 5-$HT_3$ receptor antagonist, 1 mg $kg^{-1}$, i.p.).

**5** These results suggest that m-CPP inhibits not only acute but also chronic pain transmission through 5-$HT_1$ and 5-$HT_2$ receptors, and that the 5-hydroxytryptaminergic antinociceptive pathways are little affected by diabetes.

**Keywords:** Formalin test; pain; antinociception; 5-hydroxytryptamine; diabetic mice

## Introduction

It has been postulated that both ascending and descending 5-hydroxytryptaminergic pathways are implicated in the central regulation of pain transmission. Lesioning of the descending 5-hydroxytryptaminergic systems or intrathecal administration of 5-hydroxytryptamine (5-HT) receptor antagonists elicits hyperalgesia (Berge *et al.*, 1983; Fasmer *et al.*, 1983), while systemic administration of 5-HT receptor agonists or injection of 5-HT itself into either the cerebral ventricles or the spinal subarachnoid space reduces behavioural and electrophysiological responses to noxious stimulation (Belcher *et al.*, 1978; Yaksh & Wilson, 1979; Lin *et al.*, 1980; Berge, 1982; Hylden & Wilcox, 1983). On the other hand, studies using radioligand binding assays and molecular biology techniques have revealed that at least four types of 5-HT receptors exist in the vertebrate CNS, i.e., 5-$HT_1$, 5-$HT_2$, 5-$HT_3$ and 5-$HT_4$ (Peroutka & Snyder, 1979; Kilpatrick *et al.*, 1987; Bockaert *et al.*, 1992). The 5-$HT_1$ receptor site has been further divided into six distinct receptor subtypes: 5-$HT_{1A}$, 5-$HT_{1B}$, 5-$HT_{1C}$, 5-$HT_{1D}$, 5-$HT_{1E}$ and 5-$HT_{1F}$ (Pedigo *et al.*, 1981; Pazos *et al.*, 1985; Heuring & Peroutka, 1987; Leonhardt *et al.*, 1989; Adham *et al.*, 1993). The 5-$HT_{1C}$ receptor shares homology with the 5-$HT_2$ receptor subtype and has recently been termed the 5-$HT_{2C}$ receptor while the 5-$HT_2$ receptor has been renamed the 5-$HT_{2A}$ receptor (Humphrey *et al.*, 1993). This enhanced understanding along with the development of a number of agonists and antagonists selective for the subtypes (Hoyer *et al.*, 1994) suggest that the 5-HT receptor subtypes play differential roles in the modulation of nociceptive responses.

Most behavioural studies concerning the role of 5-HT in pain transmission employ tests determining acute pain induced by thermal or mechanical stimulus. There is, however, evidence that the central processing may differ in acute and chronic pain (Fasmer *et al.*, 1985; Tjølsen *et al.*, 1991). The formalin test, an animal model showing hyperalgesia that is indicative of pain in some clinical states, was originally described in rats and cats by Dubuisson & Dennis (1977). In this test, subcutaneous injection of formalin produces a biphasic nociceptive response in rats. Several lines of evidence have indicated that the first phase represents an acute pain response to direct stimulation of the nerve endings, and the second phase represents a chronic pain response to subsequent inflammation (Dubuisson & Dennis, 1977; Shibata *et al.*, 1989). Recently, we have demonstrated that tiapride, a dopamine$_2$ receptor antagonist that relieves pain in patients with diabetic neuropathy (Ogata *et al.*, 1985), attenuates formalin-induced nociceptive responses, and that the activity was reversed by pretreatment with *p*-chlorophenylalanine (a 5-HT depletor), pindolol (a 5-$HT_1$ receptor antagonist) or ketanserin (a 5-$HT_2$ receptor antagonists) but not by ICS205-930 (a 5-$HT_3$ receptor antagonist) (Takeshita *et al.*, 1995). These findings suggest that 5-$HT_1$ and 5-$HT_2$ receptors play an attenuating role in acute as well as chronic pain transmission. Interestingly, the antinocicepitve effect of tiapride scarcely differed in diabetic and normal mice, although there has been ample evidence suggesting altered 5-HT metabolism in such animals (Curzon & Fernando, 1977; MacKenzie & Trulson, 1978a, b).

*m*-CPP (*meta*-chlorophenylpiperazine) is a direct 5-HT receptor agonist and binds to 5-$HT_{1A}$, 5-$HT_{1B}$, 5-$HT_{1C}$, 5-$HT_{1D}$,

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5-$HT_2$ and 5-$HT_3$ receptors in rat brain (Kilpatrick *et al.*, 1987; Hoyer, 1988; Hamik & Peroutka, 1989). The compound is a metabolite of trazodone (Caccia *et al.*, 1981) which crosses the blood-brain barrier (Rurak & Melzacka, 1983) and has been used as a useful probe of 5-HT function.

In the present paper, we examined the effects of m-CPP on formalin-induced pain responses in normal and streptozotocin (STZ)-induced diabetic and genetically diabetic (db/db) mice, as there are few data on analgesic effects of 5-HT in these diabetic animals. In addition, the effects of pindolol, ketanserin and ICS205-930 on the actions of *m*-CPP were studied in normal mice to elucidate which 5-HT subtypes are involved.

## Methods

### *Animals*

All the mice were housed at 22±1°C and 55±5% humidity under a 12 h light/12 h dark cycle and were given access to water and chow *ad libitum*.

*Normal mice:* Male ddY strain mice (5 weeks of age, SLC, Shizuoka, Japan) were purchased and used for the formalin test at the age of 7 weeks.

*STZ-induced diabetic mice:* STZ 200 mg $kg^{-1}$ or citrate buffer (pH 4.5) as vehicle was injected i.p. to the 5 week-old male ddY mice. Ten days later blood was obtained from the orbital sinus and plasma glucose levels were determined by a commercial kit (Glucose B-test Wako, Wako Pure Chemical Industries Ltd., Osaka, Japan). We used mice with blood glucose levels of >400 mg $dl^{-1}$. The formalin test was performed 2 weeks after administration of STZ.

*Genetically diabetic mice:* Female C57BL/KsJ-db/db mice (6 weeks of age, Jackson Labs., Bar Harbor, ME) were purchased. The formalin test was performed at the ages of 9–10 weeks because hyperglycemia became steady at 7–8 weeks of age. Blood was obtained from the orbital sinus and plasma glucose levels were determined by the commercial kit described above. We used db/db mice with blood glucose levels of >400 mg $dl^{-1}$.

### *Formalin test*

We used the formalin test previously described by Hunskaar *et al.* (1985) with modifications. Each mouse was placed in a observation chamber 10 min before the injection of diluted formalin to allow acclimatization to the new environment. The mice hardly licked their hindpaw although they did their body. Ten μl of 1% formaldehyde in saline was administered into the left hindpaw with an Ito microsyringe (Shizuoka, Japan). Each animal was then returned to the observation chamber and nociceptive response was recorded for a period of 30 min. The summation of time (in s) spent in licking and biting of the injected paw during each 5 min block was measured as an indicator of nociceptive response. The duration of responses in the first 10 min and that from 10 min to 30 min represent first and second phases, respectively. This test was performed in a temperature- and humidity-controlled (22±1°C, 55±5% respectively) room.

### *Statistical analysis*

The results are presented as the mean±s.e.mean, and statistical significance of differences between groups was analyzed by means of analysis of variance (ANOVA) followed by Dunnett's *t* test or by the unpaired *t* test where indicated. *P* values of less than 0.05 were considered significant. $ID_{50}$ values (i.e. the dose of drugs that reduced formalin-induced pain by 50% relative to control values) were estimated from individual experiments by using the linear regression methods in a computer program, produced in our laboratory.

### *Drugs*

3-Tropanyl-indole-3-carboxylate hydrochloride (ICS205-930) was synthesized in our laboratory. (±)- Pindolol hydrochloride and STZ were obtained from Sigma (St. Louis, MO). Ketanserin tartrate and m-CPP dihydrochloride were from Research Biochemical Inc. (Natick, MA). Formalin was from Wako Pure Chemical Co. Ltd. (Osaka, Japan). m-CPP was dissolved in saline and was administered p.o. 60 min before the test. Pindolol was dissolved in 1% tartaric acid, adjusted to pH 7 with 1 N NaOH and diluted in saline. Ketanserin and ICS205-930 were dissolved in saline. STZ was dissolved in 2 mM citrate buffer at pH 4.5. Formalin was diluted in saline. Pindolol, ketanserin and ICS205-930 were administered i.p. 30 min before the administration of m-CPP.

## Results

### *Differences in formalin-induced nociceptive responses between normal and diabetic mice*

A s.c. injection of diluted formalin to the hindpaw of the mice induced licking and biting of the injected paw. The nociceptive responses were biphasic: an acute, immediate response which peaked at 5 min and disappeared within 10 min (first phase), and a response which peaked at 15–25 min and lasted more than 30 min (second phase). Total response times for the first and second phases were 92±3 and 120±10 s, respectively. Formalin caused biphasic nociceptive responses also in the STZ mice as well as in the db/db mice. In the STZ-treated mice, the total response times for the first and second phases were 96±6 and 56±5 s, respectively, whereas in the db/db mice, the total response times were 48±3 and 77±8 s, respectively. When compared with normal mice, the second phase was significantly ($P<0.001$) reduced in the STZ-treated mice, whereas both phases were significantly (first phase; $P<0.001$, second phase; $P<0.01$) reduced in the db/db mice (Figure 1).

### *Effects of m-CPP on formalin-induced nociceptive response in normal and diabetic mice*

Figure 2 shows the effects of m-CPP, a 5-HT receptor agonist, on the formalin-induced nociceptive responses. m-CPP, dose-dependently inhibited both the first and second phases with a minimal effective dose of 1 mg $kg^{-1}$ (Figure 2a). The $ID_{50}$ value of the drug was calculated to be 0.4 mg $kg^{-1}$ for the second phase. Also, m-CPP dose-dependently attenuated the nociceptive responses in the STZ mice, with a more potent effect on the second phase ($ID_{50}$ = 1.1 mg $kg^{-1}$) than on the first (Figure 2b). A similar antinociceptive response was obtained in the genetically diabetic db/db mice, where the $ID_{50}$ values for the first and second phase were 3.5 and 0.4 mg $kg^{-1}$, respectively (Figure 2c).

### *Effects of 5-HT antagonists on the anti-nociceptive activity of m-CCP*

As shown in Figure 3a, m-CPP (1 mg $kg^{-1}$, p.o.) significantly inhibited the first phase of the formalin-induced nociceptive responses in the vehicle-treated mice, and the degree of inhibition was 49%, whereas in the mice treated with pindolol (a 5-$HT_1$ receptor antagonist; 1 mg $kg^{-1}$, i.p.), m-CPP inhibited the first phase only by 15% and the change was not statistically significant (pindolol-saline vs. pindolol-m-CPP). In the second phase, m-CPP caused statistically significant (77%) inhibition in the vehicle-treated mice, but only statistically insignificant (43%) inhibition in the pindolol-treated mice (Figure 3b). Pindolol treatment *per se* thus hardly affected the response

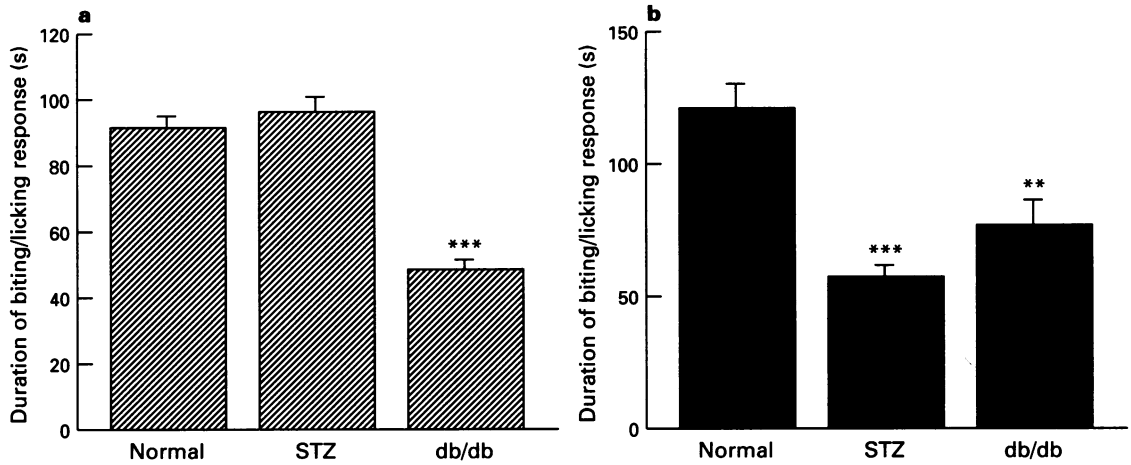


**Figure 1** Biphasic licking and biting responses induced by diluted formalin in normal, STZ and db/db mice. First phase (a) and second phase (b) represent the summation of nociceptive responses during 0–10 min and 10–30 min after injection of formalin, respectively. Each value represents mean ± s.e.mean ($n = 9-10$). $^{**}P<0.01$, $^{***}P<0.001$: Student's $t$ test.

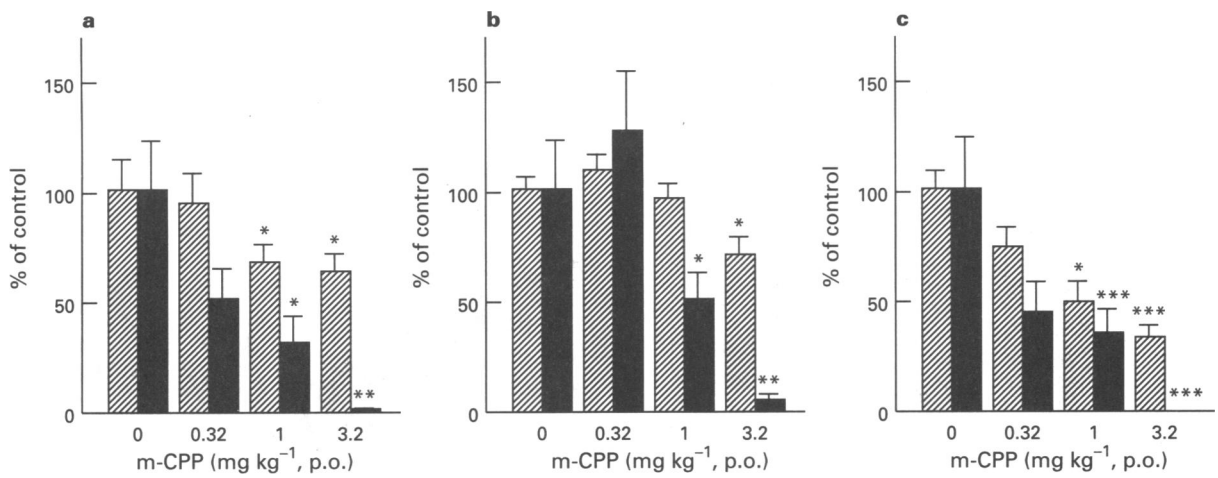


**Figure 2** Effects of *m*-chlorophenylpiperazine (m-CPP) on formalin-induced nociceptive responses in normal (a), STZ (b) and db/db mice (c). m-CPP was administered (p.o.) 60 min before the formalin injection. Each value represents % of control ($n = 8-10$). Hatched columns: first phase; solid columns: second phase. First phase and second phase represent the sum of nociceptive responses during 0–10 min and 10–30 min after injection of formalin, respectively. Dunnett's $t$ test for multiple comparisons subsequent to ANOVA was used; statistical significance at $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$.

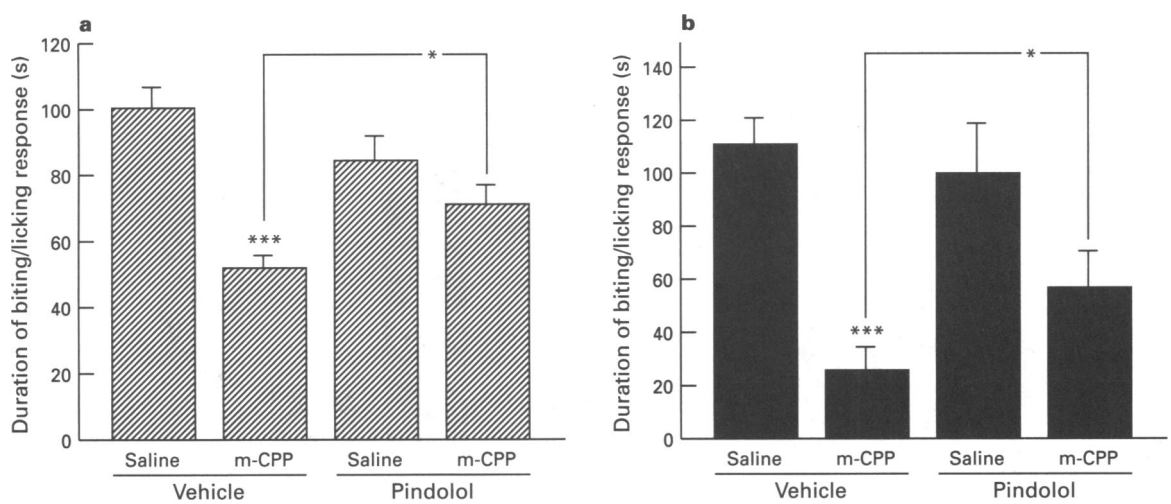


**Figure 3** Effects of pretreatment with pindolol on *m*-chlorophenylpiperazine (m-CPP; 1 mg kg$^{-1}$, p.o.) analgesia in formalin-induced nociceptive responses. (a) First phase, (b) second phase. Pindolol (1 mg kg$^{-1}$) was injected i.p. 30 min before administration of m-CPP. First phase and second phase represent the summation of nociceptive responses during 0–10 min and 10–30 min after injection of formalin, respectively. Each value represents mean ± s.e.mean ($n = 9$). $^{*}P<0.05$, $^{***}P<0.001$: Student's $t$ test.

time of the first and second phases in saline-treated mice (vehicle-saline vs. pindolol-saline), but significantly increased the response time in m-CPP-treated mice (m-CPP-vehicle vs. m-CPP-pindolol).

Similarly, m-CPP (1 mg $kg^{-1}$, p.o.) significantly reduced the first and second phases of the formalin test in the control mice treated with saline, and the respective degrees of inhibition were 39 and 72% (Figure 4a and b). The anti-nociceptive activity of m-CPP was greatly reduced in mice pretreated with ketanserin (a 5-$HT_2$ receptor antagonist; 1 mg $kg^{-1}$, i.p.); m-CPP attenuated the first phase by 11% and the second phase by 50%, and these changes were not statistically significant. Thus, ketanserin treatment *per se* hardly affected the response time of the first and second phases in saline-treated mice (saline-saline vs. ketanserin-saline), and tended to increase the response time in ketanserin treated mice (m-CPP-saline vs. m-CPP-ketanserin).

On the other hand, pretreatment with ICS205-930, a 5-$HT_3$ receptor antagonist, did not affect m-CPP analgesia in either phase of the formalin test (Figure 5a and b). ICS205-930 treatment *per se* did not affect the response time of the first and second phases in either the saline-treated mice (saline-saline vs. ICS205-930-saline) or the ICS205-930-treated mice (m-CPP-saline vs. m-CPP-ICS205-930).

## Discussion

While there has been ample evidence suggesting an inhibitory role of 5-HT receptors in thermal or mechanical nociceptive transmission, there have been only a few studies on their role in formalin-induced nociceptive transmission (Fasmer *et al.*, 1986; Giordano & Rogers, 1989; Giordano & Dyche, 1989; Giordano, 1991). An intraplantar injection of formalin induced biphasic nociceptive responses in mice and rats, and the first and second phases were considered to represent acute and chronic pain, respectively (Shibata *et al.*, 1989; Dubuisson & Dennis, 1977). We have recently found that tiapride, which relieves chronic pain in diabetic patients with peripheral neuropathy (Ogata *et al.*, 1985), attenuated the formalin-induced nociceptive responses in mice, and the effect was reversed by *p*-chlorophenylalanine (a 5-HT depletor) as well as by pindolol

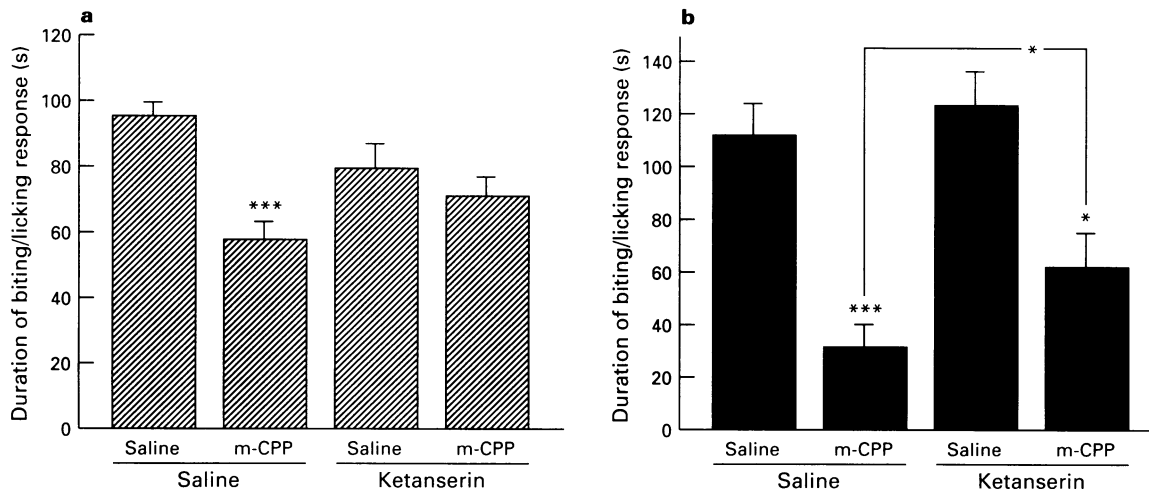


**Figure 4** Effects of pretreatment with ketanserin on *m*-chlorophenylpiperazine (m-CPP; 1 mg $kg^{-1}$, p.o.) analgesia in formalin-induced nociceptive responses. (a) First phase, (b) second phase. Ketanserin (1 mg $kg^{-1}$) was injected i.p. 30 min before administration of m-CPP. First phase and second phase represent the summation of nociceptive responses during 0–10 min and 10–30 min after injection of formalin, respectively. Each value represents mean ± s.e.mean ($n=10$). $^{*}P<0.05$, $^{***}P<0.001$: Student's *t* test.

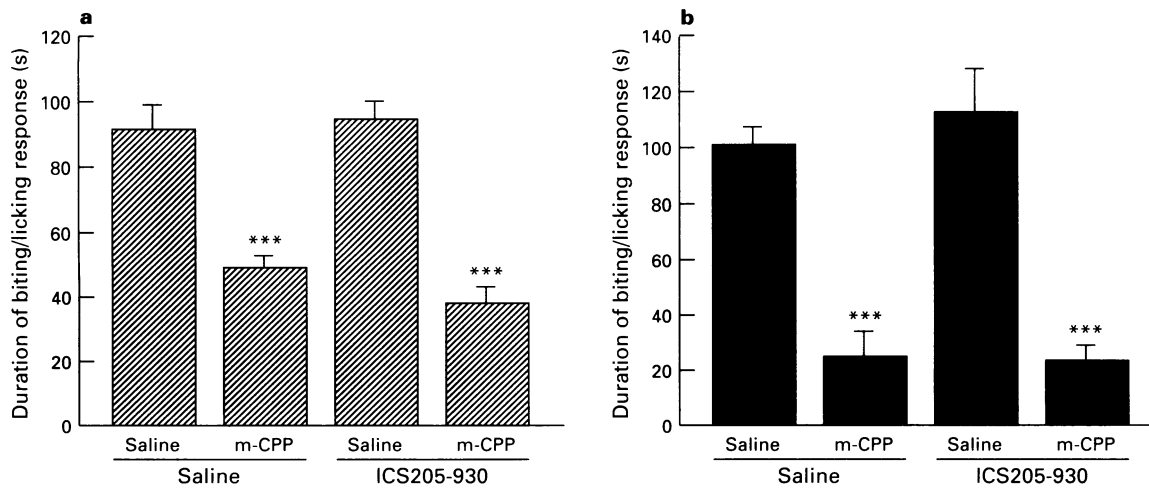


**Figure 5** Effects of pretreatment with ICS205-930 on *m*-chlorophenylpiperazine (m-CPP; 1 mg $kg^{-1}$, p. o.) analgesia in formalin-induced nociceptive responses. (a) First phase, (b) second phase. ICS205-930 (1 mg $kg^{-1}$) was injected i.p. 30 min before administration of m-CPP. First phase and second phase represent the summation of nociceptive responses during 0–10 min and 10–30 min after injection of formalin, respectively. Each value represents mean ± s.e.mean ($n=10$). $^{***}P<0.001$: Student's *t* test.

(a 5-$HT_1$ receptor antagonist) or ketanserin (a 5-$HT_2$ receptor antagonist) but not by ICS205-930 (a 5-$HT_3$ receptor antagonist) (Takeshita *et al.*, 1995). We thus hypothesized that the 5-hydroxytryptaminergic pathway involving 5-$HT_1$ and 5-$HT_2$ receptors plays an inhibitory role in not only acute but also chronic pain transmission. The former part of the speculation is in line with previous observations dealing with acute pain models (Zemlan *et al.*, 1983; Eide & Tjølsen, 1988; Murphy & Zemlan, 1990; Eide *et al.*, 1990; El-Yassir & Fleetwood-Walker, 1990; Crisp *et al.*, 1991; Alhaider, 1991; Eide & Hole, 1991; Alhaider & Wilcox, 1993; Xu *et al.*, 1994).

The most important finding of the present study is that a systemic administration of m-CPP (a 5-HT receptor agonist) dose-dependently attenuated both phases of the formalin-induced nociceptive responses. Further, the antinociceptive activities of m-CPP were reversed by pindolol (a 5-$HT_1$ receptor antagonist) or ketanserin (a 5-$HT_2$ receptor antagonist), but were not affected by ICS205-930 (a 5-$HT_3$ receptor antagonist). These results strongly support our hypothesis, mentioned above. As the 5-$HT_1$ and 5-$HT_2$ receptors have been further divided into subtypes, studies using specific antagonists, when they are available, are needed to clarify which subtypes are important for the antinociception. In this respect, it is interesting to note that the most evidence suggests a facilitatory role of the 5-$HT_{1A}$ receptor in pain transmission (Zemlan *et al.*, 1988). Although Fasmer *et al.* (1986) showed that 8-OH-DPAT 1 mg $kg^{-1}$ s.c. (8-hydroxy-2-(di-n-propylamino)-tetralin; a 5-$HT_{1A}$ receptor agonist) elicited hypoalgesia in the acute phase of the formalin test, a non-specific effect might be responsible for the weak activity of 8-OH-DPAT compared with its strong affinity to the 5-$HT_{1A}$ receptor (Middlemiss & Fozard, 1983). Whereas it has been demonstrated that intrathecal administration of m-CPP (selective for 5-$HT_{1B}$ receptors over 5-$HT_{1A}$ receptors, Murphy & Zemlan, 1990) induces antinociception (Eide *et al.*, 1990; Eide, 1992), and oral administration of the drug dose-dependently inhibited both phases of the formalin-induced nociceptive response, several lines of evidence suggest that there are multiple 5-$HT_1$ binding sites in the spinal cord, and about 35% are specific for 5-$HT_{1B}$, but the density of 5-$HT_2$ receptors is very low in the spinal cord (Leysen *et al.*, 1982; Monroe & Smith, 1983; Marlier *et al.*, 1991). 5-$HT_2$ binding sites have been demonstrated in the cortex (Hoyer *et al.*, 1986; Molineaux *et al.*, 1989), therefore activation of spinal 5-$HT_{1B}$ receptors and supraspinal 5-$HT_2$ receptors might be involved in the antinociception.

It was interesting to find that strong antinociceptive effects of m-CPP were observed in both types of diabetic model. As discussed above, indirect activation of 5-$HT_1$ and 5-$HT_2$ receptors by tiapride caused similar antinociception in normal and diabetic mice (Takeshita *et al.*, 1995). We thus speculate that the antinociceptive mechanism involving 5-HT receptors is little affected by diabetes. Interestingly, several lines of evidence suggest that brain tryptophan levels are decreased by 30–50% in streptozotocin diabetic rats but with no accompanying changes in the concentration of 5-HT or its major metabolite 5-HIAA, and that administration of insulin to the diabetic rats restored brain tryptophan concentrations to normal, but did not change 5-HT or 5-HIAA concentrations (Curzon & Fernando, 1977; MacKenzie & Trulson, 1978a, b; Trulson *et al.*, 1986). In addition, neither 5-HT receptor density in the forebrain (Trulson & MacKenzie, 1981) nor antidepressant effects of selective 5-HT uptake inhibitors (Massol *et al.*, 1989) were different between STZ-induced diabetic and normal rats.

In conclusion, we hypothesize that 5-$HT_{1/2}$ receptors play an inhibitory role in acute and chronic pain transmission, and are activated by m-CPP and that their role differs little between normal mice and those with diabetes mellitus.

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