ELSEVIER

European Journal of Pharmacology 308 (1996) 31–40

ejp

The antinociceptive action of paracetamol is associated with changes in the serotonergic system in the rat brain

Luigi A. Pini [a,*], Maurizio Sandrini [b], Giovanni Vitale [a]

[a] *Clinical Pharmacology Unit, Department of Internal Medicine, University of Modena, via del Pozzo 71, 41100 Modena, Italy*
[b] *Department of Biomedical Sciences, Section of Pharmacology, University of Modena, Modena, Italy*

Received 26 October 1995; revised 28 March 1996; accepted 2 April 1996

Abstract

The antinociceptive activity of paracetamol in the hot plate and formalin tests was studied to establish the relationship between antinociceptive activity and the central serotonergic system. Significant antinociceptive activity of paracetamol was observed in the formalin test at the dose of 300 mg/kg, while, at the dose of 400 mg/kg, the drug was active both in the formalin and in the hot-plate test. Serum paracetamol levels remained sub-toxic and the behavioral profile remained unchanged. Depletion of brain serotonin with *p*-chlorophenylalanine prevented the antinociceptive effect of paracetamol in the hot-plate test and in the first phase of the formalin response. Paracetamol significantly increased the serotonin content in the pontine and cortical areas (by 75 and 70%, respectively). The pretreatment with *p*-chlorophenylalanine reduced the 5-hydroxytryptamine (5-HT) content in cortical and pontine areas to 12 and 19% of baseline values, respectively, and prevented the enhancement induced by paracetamol. The maximum number of cortical 5-HT_2 receptors was reduced by paracetamol, while the number of 5-HT_{1A} receptors in both cortical and pontine areas was unchanged. Pre-treatment with *p*-chlorophenylalanine prevented the reduction in the number of 5-HT_2 receptors induced by paracetamol. These results provide evidence for the involvement of the central serotonergic system in the antinociceptive effect of paracetamol in the hot plate and formalin tests.

Keywords: Paracetamol; Antinociception; 5-HT (5-hydroxytryptamine, serotonin); 5-HT receptor, brain

1. Introduction

The mechanism of action of non-steroidal anti-inflammatory drugs (NSAIDs) is believed to result from suppression of prostaglandin synthesis due to cyclooxygenase inhibition. However, whereas the anti-inflammatory potency of NSAIDs can be correlated to their inhibition of cyclooxygenase, the relationship between the antinociceptive activity of these drugs and their potency for cyclooxygenase inhibition is controversial (Brune et al., 1991, 1992; Malmberg and Yaksh, 1994).

It has been observed that phenazone and paracetamol significantly inhibit prostaglandin synthesis in the central nervous system (CNS), and evidence suggests that paracetamol has a weak inhibitory activity on the synthesis of peripheral prostaglandin, but is a potent inhibitor of prostaglandin biosynthesis within the CNS (Clissold, 1986; McCormack, 1994).

Surprisingly, other authors have demonstrated that this compound has only a weak antinociceptive effect when injected intracerebroventricularly (i.c.v.) in rats (Okuyama and Aihara, 1984). This lack of activity may result from its inability to inhibit prostaglandin synthesis in rat CNS tissue (Abdel-Halim et al., 1978).

It has been suggested that central monoaminergic and serotonergic pathways may be involved in pain modulation (Warner et al., 1990) and that there may be a connection between analgesia induced by some NSAIDs and the increase in the turnover rate of dopamine, noradrenaline and serotonin (5-HT) in the rat CNS (Bensemana and Gascon, 1978; Groppetti et al., 1988). Moreover, prostaglandins seem to interact with a descending modulating system by inhibiting the release of neurotransmitters in the spinal cord (Taiwo and Levine, 1988).

Many types of analgesic drugs (morphine, NSAIDs) have been proposed to act through an increase in brain serotonin levels. The serotonergic system may regulate nociception in different ways, depending on the receptor subtypes involved and serotonin has been claimed to exert its central antinociceptive effect in defined brain areas

* Corresponding author. Tel.: +39-59 422098; fax: +39-59 422944.

PII S0014-2999(96)00261-0

through its receptor subtypes, notably 5-HT_{1A} (Eide and Hole, 1991), 5-HT_2 (Alhaider, 1991) and 5-HT_3 at the spinal level (Pelissier et al., 1995).

The bulk of the data suggests that stimulation of 5-HT_1 receptors reduces nociceptive sensitivity, whereas activation of 5-HT_2 receptors increases nociceptive responsiveness (Eide and Hole, 1993).

Our previous observations suggested that the antinociceptive effect of phenazone and acetylsalicylic acid in the hot-plate test in rats was associated with a decrease in the number of serotonin receptors in certain brain areas (Sandrini et al., 1993; Pini et al., 1995). We hypothesize that paracetamol exerts its antinociceptive effect through the 5-HT system, as shown with acetylsalicylic acid and phenazone, although it differs from other NSAIDs, in that it lacks anti-inflammatory effects.

The present study was thus aimed at investigating the effect of paracetamol: (a) in the hot plate and formalin tests, (b) on brain serotonin contents, and (c) on the characteristics of 5-HT_2 and 5-HT_{1A} receptors in two rat brain areas, namely, the pons and the cerebral cortex.

In order to further assess the relationship between serotonin and the antinociceptive effect of paracetamol, we pretreated with *p*-chlorophenylalanine, a tryptophan hydroxylase inhibitor, which causes marked depletion of brain serotonin. Brain serotonin measurements and receptor subtype evaluation were carried out to correlate the biochemical effects with the antinociception.

2. Materials and methods

2.1. Drug administration

Adult male Wistar rats (Morini, S. Polo d'Enza, Reggio Emilia, Italy), weighing 180–190 g at the beginning of the experiments, were housed in Plexiglas cages, four per cage, with free access to food and water, and were maintained on a 12 h dark/light cycle (light on at 07.00) under controlled environmental conditions (temperature 22 ± 1°C; humidity 60%). The ethical guidelines for investigation of experimental pain in conscious animals were followed in all tests (Zimmerman, 1983). All the procedures were carried out according to the EEC ethical regulations for animal research (EEC Council 86/609; D.L. 27/01/1992, no. 116).

Rats, divided into groups of eight animals, received intraperitoneally (i.p.) paracetamol, dissolved in vehicle, which consisted of 12.5% of 1,2-propanediol in sterile saline, 30 min before the formalin test or 90 min before the hot-plate test. These intervals were planned in order to synchronize the times of killing for biochemical evaluation. Three doses of paracetamol, 200, 300 and 400 mg/kg in a volume of 10 ml/kg were used for the formalin and hot-plate tests. Two additional groups of animals, tested with regard to their behavioral profile, received either paracetamol (400 mg/kg) or vehicle. To compare the analgesic potency of paracetamol with that of a reference compound in our experimental design, two other groups were subcutaneously injected with morphine (8 mg/kg, in a volume of 2 ml/kg of saline) 20 min before the formalin test or 30 min before the hot-plate test. *p*-Chlorophenylalanine (100 mg/kg i.p. in 2 ml kg of sterile saline) or saline was injected daily for four consecutive days, and paracetamol (400 mg/kg) or vehicle was administered two h after the last dose. The rats were then tested in the hot plate and in the formalin test with the above-mentioned experimental schedule.

In all experiments an equal volume of vehicle or saline was used as control for injections and the same procedure was followed as for treated groups.

Control experiments showed no significantly different response between saline and 1,2-propanediol in the concentration used; the data were therefore pooled.

2.2. Algesimetric tests

2.2.1. Hot-plate test

The hot plate consisted of an electrically heated surface (Socrel DS-35, Ugo Basile, Comerio, VA, Italy) kept at the constant temperature of 54 ± 0.4°C. The latencies for paw licking or jumping were recorded for each animal. The baseline latencies in the hot-plate test ranged from 5.7 ± 0.5 to 6.1 ± 0.5 s (analysis of variance (ANOVA), $P > 0.5$). The analgesic efficacy of the drug was evaluated as a percentage of the maximum possible effect (% MPE), according to the formula $(TL - BL)/(45 - BL) \times 100$, where TL = test latency, BL = baseline latency, 45 = cut-off time, in seconds.

Immediately after the last pain threshold measurement, 90 min after drug administration, the animals were anaesthetized with ethyl-ether and decapitated; the blood was collected and the serum was stored at −20°C; the brains were removed, weighed and stored at −80°C until required for the binding assay.

2.2.2. Formalin test

2 h before testing the animals were placed individually in standard cages and, after the adaptation period, 50 μl of 5% formalin solution was injected s.c. into the dorsal surface of the right hind paw using a microsyringe with a 26 gauge needle. Pain behaviour was monitored over a period of 60 min, the number of flinches/shakes of the injected paw being summed at 5 min intervals starting at time 0. Two phases of spontaneous flinching behaviour were observed: phase 1, immediately after formalin injection to 10 min thereafter; phase 2 began at time 10 min and a maximum response was observed around 25–35 min after the formalin injection. For the purpose of data analysis, the second phase was further divided into two phases: phase 2A (10–39 min) and phase 2B (40–60 min) (Malmberg and Yaksh, 1992). To avoid possible interference of

room temperature on skin temperature, all experiments were performed at a room temperature of 22 ± 1°C (Hole and Tjølsen, 1993).

After the observation period, the animals were immediately anaesthetized and decapitated as above, blood was collected and sera were stored at −20°C until assayed.

2.3. Behavioral profile

Given the possible appearance of false positives in the hot-plate test due to a potential aspecific drug-related activity, we decided to perform behavioral tests, using the dose of 400 mg/kg of paracetamol, to reveal any pain-independent drug-induced behaviour.

Behavioral tests were performed between 09.00 and 12.00 a.m. in a sound-proof room by experienced observers unaware of the treatments.

Motor activity was measured in an activity cage by means of an ultrasound apparatus (Cibertec, Barcelona, Spain) placed on the lid of the cage. The number of movements was recorded continuously for 1 h after an adaptation period of 30 min.

The sensorimotor test battery, described by Bjørklund et al. (1980), was carried out with minor modifications as soon as the recording of motor activity had finished: each animal was rated for general postural asymmetry and spontaneous rotation, tested for sensorimotor orientation (pin prick, whisker touch, snout probe) and examined for limb reflexes and coordinated limb use (forelimb placement, forelimb suspension, climbing grid, mouth probe). Checks were also made for signs of cortical and pyramidal impairment. Each test was scored on a 0–2 scale according to the intensity and duration of the sensory and motor deficit: 0 = absent; 1 = weak; 2 = strong.

2.4. Drug assay

Following the hot plate and formalin tests, paracetamol serum levels were measured 90 min after drug administration and were assayed by fluorescence polarization immunoassay (FPIA), which utilizes the concept of fluorescence detection with polarized light emission: fluorescein-tagged drug and unlabelled drug are incubated with antibody and then excited with polarized light; as the drug concentration rises, there is an increase in unbound fluorescein-labelled molecules which tumble free in solution and cause the light to be depolarized upon emission. A TDx analyzer was used for drug determination (Abbot Laboratories, Chicago IL, USA).

2.5. Serotonin determination

After the hot-plate test, brain areas were rapidly dissected, weighed and frozen; 24 h later the areas were homogenized in 0.1 N HCl (100 μl/mg of wet weight) and centrifuged for 10 min at 1500 × *g* at 4°C. Serotonin was measured in 25 μl of supernatant by means of radioimmunoassay (Manz et al., 1986). A commercial kit (IBL, Immunological Laboratories, Hamburg, Germany; intra-assay coefficient of variation = 10%, sensitivity = 12 pg/tube) was used. The data were expressed as nanograms of serotonin per gram of brain tissue.

2.6. Binding assay

The characteristics of 5-HT_{1A} and 5-HT_2 binding sites were evaluated according to Gulati and Bhargava (1990) and according to Leysen et al. (1989) with minor modifications. Brain regions were homogenized in 5 ml of ice-cold 0.25 M sucrose (12 strokes of a Teflon pestle at 120 rpm) and centrifuged at 1300 × *g* for 10 min at 4°C. This procedure was repeated, then the combination of sucrose supernatants was diluted with 10 ml of 50 mM Tris-HCl pH 7.7 and the suspension was centrifuged at 3500 × *g* for 10 min. The pellet was resuspended in 20 ml of Tris-HCl buffer and centrifuged once at 50000 × *g* for 10 min. The pellet was then homogenized and diluted in Tris-HCl (about 300 mg protein ml). Aliquots of membranes (800 μl) were placed in plastic test tubes containing [^{3}H]ketanserin (six increasing concentrations in 10% ethanol), methysergide (10 μM, dissolved in Tris-HCl buffer to define non-specific binding) or Tris buffer at 37°C for 15 min. The mixture was filtered under reduced pressure through Whatman GF/B glass fiber filters, previously soaked for 5 min in 0.5% polyethyleneimine, using a Millipore vacuum pump and rapidly rinsed twice with 5 ml ice-cold Tris buffer. The filters were transferred to plastic vials containing 6 ml of Packard Optifluor and shaken. The vials were stored for 20 h at 4°C in the dark.

The following concentration were used: 8-[^{3}H]hydroxy-2-(D-*n*-propyl-amino)tetralin ([^{3}H]8-OH-DPAT), a selective ligand for the 5-HT_{1A} subtype serotonin receptor, specific activity 142.9 Ci/mmol, 0.18–6 nM; [^{3}H]-ketanserin, a 5-HT_2 receptor ligand, specific activity 87.5 Ci/mmol, 0.05–4 nM.

2.7. Statistical analysis

The data were expressed as means ± S.E.M. The results obtained in behavioral tests were analyzed using Student's *t*-test and the Mann-Whitney *U*-test for motor activity and sensorimotor score, respectively.

The data obtained from the hot plate and formalin experiments as well as the values of 5-HT cerebral levels were evaluated by one-way ANOVA, followed by Student-Newman-Keuls' test when appropriate.

The results of binding experiments were analyzed according to the method of Rosenthal (1967). The equilibrium dissociation constant (K_D) and the maximum number of binding sites (B_{max}) were evaluated individually for each sample using six concentrations of labelled drug.

All assays were performed in duplicate; K_D and B_{max}

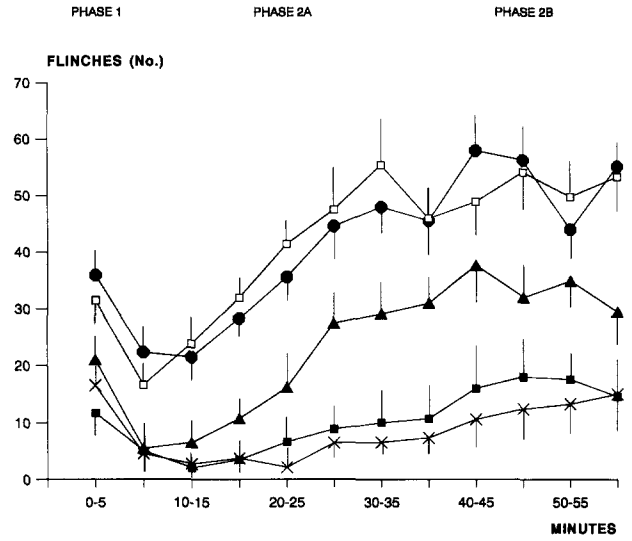

Fig. 1. Time-effect curve of i.p. paracetamol or s.c. morphine administered 30 and 20 min, respectively, before formalin test. The number of flinches per 5-min interval is plotted vs. time. Each line represents the mean ± S.E.M. for eight rats. Controls (□); paracetamol, 200 (●), 300 (▲) and 400 (■) mg/kg; morphine, 8 (×) mg/kg.

were summarized as arithmetic means and were correlated using ANOVA followed by Student-Newman-Keuls' test.

A two-way analysis of variance was used to analyse the effects of *p*-chlorophenylalanine pretreatment, paracetamol treatment and their interaction, followed by a 2 × 2 factorial analysis by means of orthogonal comparisons (Snedecor and Cochran, 1980).

Table 1
Effect of acute treatment with morphine, 8 mg/kg s.c, or increasing doses of paracetamol (Para), in the hot-plate test and on serum levels of paracetamol

Drugs (mg/kg)	Hot plate (% of MPE)	Serum levels (mM)
Control	3.1 ± 1.7	N.D.
Para 200	4.3 ± 2.1	0.43 ± 0.034
Para 300	6.0 ± 1.9	1.12 ± 0.046
Para 400	26.6 ± 5.6 [a]	1.67 ± 0.184
Morphine 8	48.5 ± 14.3 [a]	N.P.

%MPE represents the percentage of the maximum possible effect; serum levels of paracetamol are expressed as millimolar concentrations. The drug was i.p. injected, at the dose of 200, 300 and 400 mg/kg, 90 min before the test. The rats were then killed and brains were removed and stored at −80°C. Sera were obtained by rats submitted to both the formalin and the hot-plate tests and were stored at −20°C. Morphine was injected 30 min before the test. Data are expressed as means ± S.E.M. for eight rats for each group. N.D. = non-detectable; N.P. = not performed.
[a] $P < 0.05$ vs. control values (ANOVA followed by Student-Newman-Keuls' test).

The data were evaluated with the Student-Newman-Keuls' test when the evaluation concerned *p*-chlorophenylalanine or paracetamol single effects.

2.8. Drugs

Paracetamol, morphine sulphate and *p*-chlorophenylalanine were purchased from Sigma Chemical Co. (St. Louis, USA). [^{3}H]8-OH-DPAT and [^{3}H]ketanserin were from Du Pont NEN (Milan, Italy). Formalin was obtained through Bracco Chemical Co. (Milan, Italy).

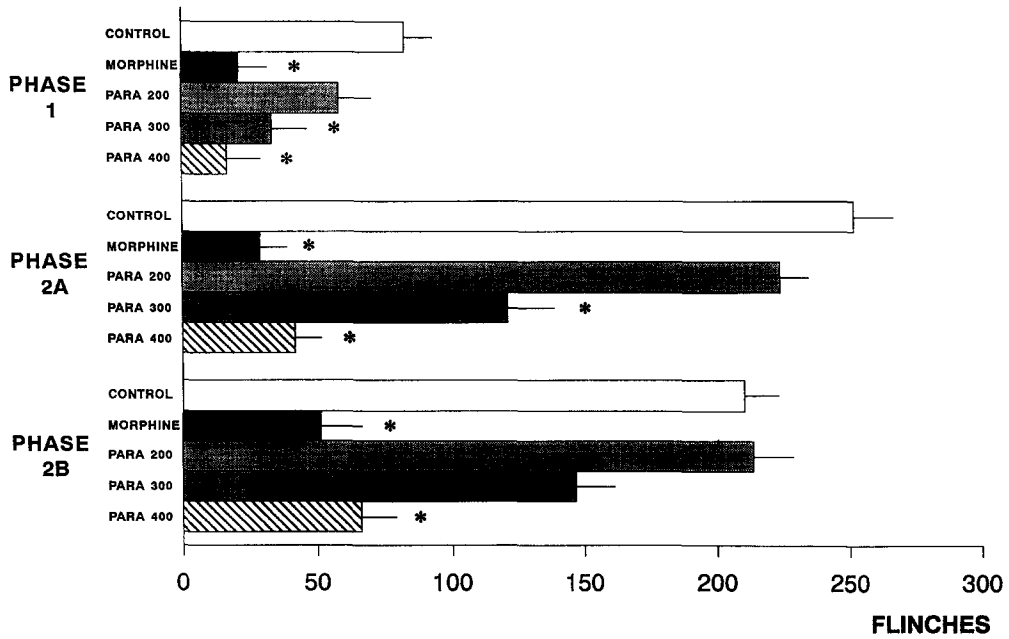

Fig. 2. Dose-response effect of acute treatment of i.p. paracetamol (PARA), 200, 300 and 400 mg/kg, or s.c. morphine, 8 mg/kg, administered 30 and 20 min, respectively, before formalin test. Each histogram represents the total number of flinches (mean ± S.E.M.) in phase 1, 2A and 2B (eight rats per group). * $P < 0.05$ vs. control values (ANOVA followed by Student-Newman-Keuls' test).

Table 2
Influence of *p*-chlorophenylalanine (PCPA) treatment on the antinociceptive action of paracetamol (Para) in the hot-plate test, on [^{3}H]ketanserin binding and on brain 5-HT levels

Treatment	Pain threshold	Cortical 5-HT$_2$ characteristics		5-HT levels	
	Hot-plate % of MPE	B_{max} fmol/mg prot.	K_D nM	Cortex ng/g	Pons ng/g
Saline + vehicle	5.9 ± 1.7	192.8 ± 5.4	1.17 ± 0.07	123.1 ± 8.4	271.3 ± 18.2
PCPA + vehicle	5.8 ± 3.0	209.8 ± 5.3	1.42 ± 0.04	23.4 ± 3.8 [a]	32.5 ± 8.3 [a]
Saline + Para	32.8 ± 9.7 [a]	134.5 ± 6.4 [a]	1.50 ± 0.13	209.2 ± 16.2 [a]	475.1 ± 40.2 [a]
PCPA + Para	11.0 ± 3.2 [b]	177.3 ± 6.9 [b]	1.30 ± 0.06	61.3 ± 9.6 [a,b]	125.7 ± 31.4 [a,b]

p-Chlorophenylalanine (100 mg/kg/day) or saline were i.p. injected for 4 consecutive days; paracetamol (400 mg/kg) or vehicle were i.p. administered 2 h after the last injection of *p*-chlorophenylalanine. Rat behaviour in the hot-plate was tested 90 min after paracetamol or vehicle administration. At the end of the experiments, the rats were killed and their brains were dissected for binding assay and for serotonin determination in pons and cerebral cortex. % MPE = percentage of the maximum possible effect; B_{max} (maximum binding capacity) and K_D (equilibrium dissociation constant) values were derived from a Rosenthal plot. Cortical and pontine serotonin contents were measured by means of radioimmunoassay. Values are means ± S.E.M. for eight rats. [a] $P < 0.05$ vs. saline + vehicle; [b] $P < 0.05$ vs. saline + Para (ANOVA followed by Student-Newman-Keuls' test).

Paracetamol was dissolved in 12.5% of 1,2-propanediol in sterile saline, morphine and *p*-chlorophenylalanine were dissolved in sterile saline.

3. Results

3.1. Antinociceptive activity

The % MPE of rats treated with increasing doses of paracetamol (200, 300 and 400 mg/kg) or morphine (8 mg/kg) are reported in Table 1. Only at the dose of 400 mg/kg did paracetamol produce a significant antinociceptive effect, while 200 and 300 mg/kg paracetamol produced no antinociceptive effect in the hot-plate test as compared to that in vehicle-treated rats (Table 1).

The time course of the flinching response of rats treated with paracetamol (200, 300 and 400 mg/kg) and morphine (8 mg/kg) is compared with control values in Fig. 1. As shown in Fig. 2, paracetamol induced a dose-dependent reduction in the total number of flinches in phase 1 and phase 2A. The minimal effective dose was 300 mg/kg, while inhibition of phase 2B occurred only at the highest dose (400 mg/kg). At the dose of 8 mg/kg s.c., morphine was effective in all phases of the formalin test, proving as potent as the highest dose of paracetamol.

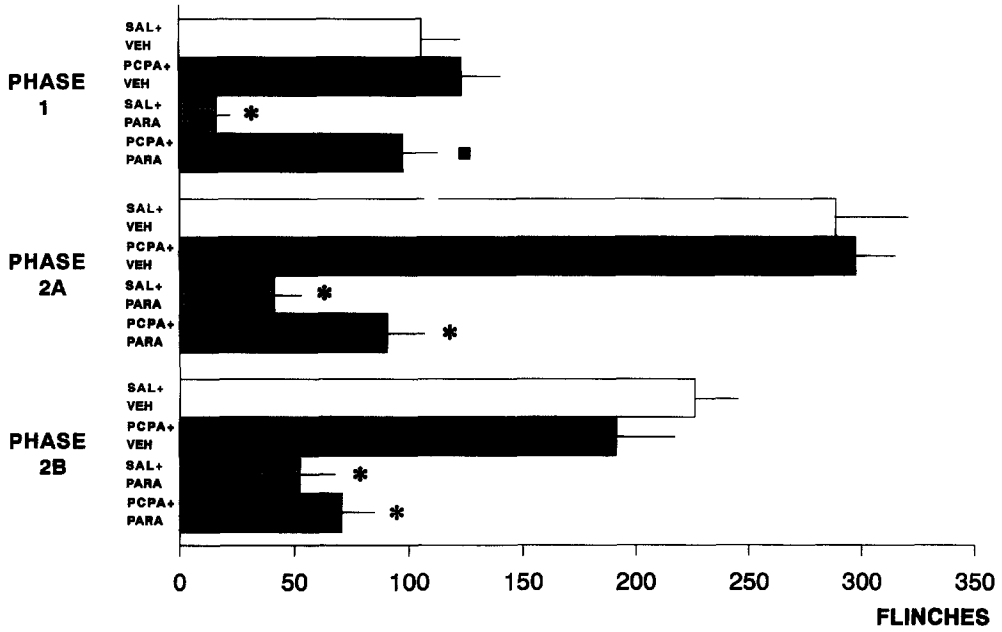

Fig. 3. Influence of parachlorophenylalanine (PCPA) treatment on the action of paracetamol in the formalin test. Paracetamol (PARA, 400 mg/kg i.p.) was administered 2 h after the last injection of PCPA (100 mg/kg/day i.p. for 4 days) and the rats were tested 30 min thereafter. Each histogram represents the total number of flinches (mean ± S.E.M.) of eight rats in phase 1, 2A and 2B. SAL = sterile saline; VEH = 12,5% of 1,2-propanediol in sterile saline (vehicle). * $P < 0.05$ vs. saline + vehicle; ■ $P < 0.05$ vs. saline + PARA (ANOVA followed by Student-Newman-Keuls' test).

Depletion of central serotonin by *p*-chlorophenylalanine treatment significantly reduced the antinociceptive effect of 300 and 400 mg/kg paracetamol in the hot-plate test and the first phase of the formalin test. There was no difference between the % values of MPE and total number of flinches in phase 1 for *p*-chlorophenylalanine + paracetamol-treated rats and saline + vehicle-treated rats ($P > 0.05$, Student-Newman-Keuls' test after ANOVA for both tests), (Table 2; Fig. 3). Indeed, a significant negative interaction occurred between the *p*-chlorophenylalanine and paracetamol treatment ($F(1,44) = 24.5$; $P < 0.001$ and $F(1,28) = 21.4$; $P < 0.001$ for % MPE and flinches in phase 1, respectively).

On the other hand, the effect of paracetamol on phase 2A and 2B was not affected by the depletion of 5-HT induced by *p*-chlorophenylalanine ($F(1,28) = 1.94$; $P > 0.05$ and $F(1,28) = 2.07$; $P > 0.05$ for *p*-chlorophenylalanine-paracetamol interaction in phase 2A and 2B, respectively).

Moreover, *p*-chlorophenylalanine per se had no activity in the algesimetric tests, % MPE and total number of flinches in all the phases being similar to those obtained in saline + vehicle-treated rats.

3.2. Behavioral profile

The motor activity of rats treated with paracetamol at the dose of 400 mg/kg was compared with that of vehicle-treated rats. No significant difference in the total number of movements was observed between the experimental and control groups (1378 ± 60 and 1332 ± 59 (mean ± S.E.M.), respectively; $n = 10$ $P > 0.05$, Student's *t*-test), nor did the experimental animals exhibit any significant impairment of the behavioral parameters examined (sensorimotor score: 1.03 ± 0.4 and 0.42 ± 0.3 (mean ± S.E.M.), respectively, in experimental versus control rats; $n = 10$ $P > 0.05$, Mann-Whitney *U*-test).

3.3. Drug assay

Table 1 shows the serum levels of paracetamol, 90 min after its administration and the levels obtained after either the formalin or the hot-plate test; since they were not significantly different they were pooled.

The effects of a 4-day pretreatment with *p*-chlorophenylalanine (100 mg/kg) were then investigated. Serum paracetamol levels in *p*-chlorophenylalanine + paracetamol-treated rats were first compared with those of rats treated with saline plus paracetamol (400 mg/kg), to exclude any pharmacokinetic interaction between *p*-chlorophenylalanine and paracetamol; 90 min after drug administration, the respective levels were similar (1.58 ± 0.165 and 1.64 ± 0.191 mM (mean ± S.E.M.); $n = 12$ $P > 0.05$, Student's *t*-test).

3.4. Serotonin determination

Fig. 4 shows the serotonin levels in the cortical and pontine areas. At the doses of 300 and 400 mg/kg, paracetamol significantly increased the levels of serotonin

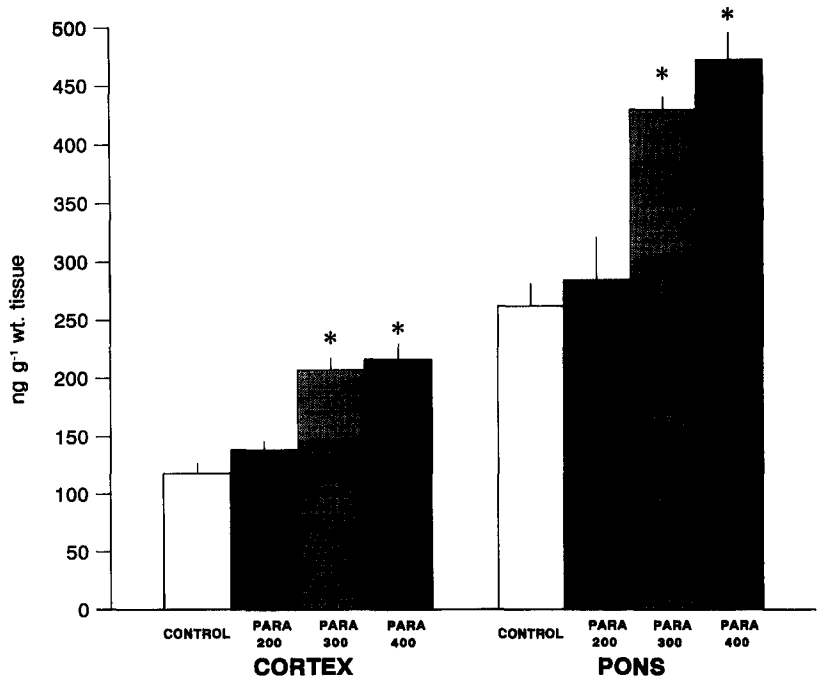

Fig. 4. Effect of i.p. administered paracetamol (PARA), 200, 300 and 400 mg/kg on serotonin content in cortical and pontine brain areas. Rats were killed immediately after the hot-plate test and brain areas were weighed and frozen at −80°C until assayed. Values are expressed as means ± S.E.M. for six rats for each group. * $P < 0.05$ vs. control values (ANOVA followed by Student-Newman-Keuls' test).

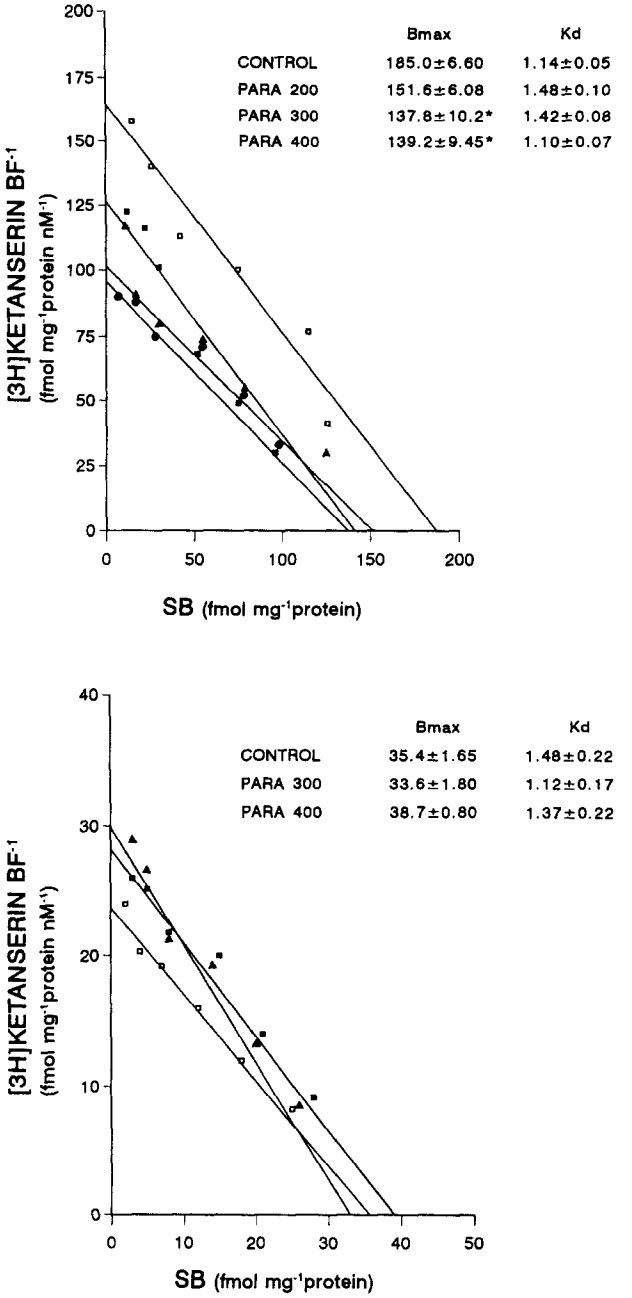

Fig. 5. Rosenthal plots of brain 5-HT_2 receptor binding labelled with [^{3}H]ketanserin for control rats (□), and the rats with paracetamol (PARA), 200 (●), 300 (▲) and 400 (■) mg/kg, i.p. injected. The hot-plate test was performed 90 min after the drug treatment. The animals were then killed and brains were stored until assayed. Each point represents the mean of six separate experiments performed with pooled tissues of 4–6 animals. Ordinate scale: bound over free (B F^{-1}). Abscissa scale: specific binding (SB). B_{max} = maximum binding capacity (fmol/mg/protein), K_D = equilibrium dissociation constant (nM). Data are expressed as means ± S.E.M. * $P < 0.05$ vs. control values (ANOVA followed by Student-Newman-Keuls' test).

in the cerebral cortex and pons.

The changes in 5-HT contents induced by *p*-chlorophenylalanine and paracetamol were in opposite directions, and the depletion of serotonergic neurons induced by *p*-chlorophenylalanine blocked the effect of paracetamol on 5-HT content both in the pons and in the cerebral cortex (Table 2). *p*-Chlorophenylalanine depleted the 5-HT levels in the pons and cerebral cortex by 88 and 81%, respectively, while the increase induced by paracetamol was 75 and 70%, respectively.

3.5. Binding assay

As shown in Fig. 5, the number of 5-HT_2 receptors decreased significantly in the cerebral cortex, but not in the pons, of the rats treated with the two highest doses of paracetamol, while the affinity constant (K_D) remained unchanged. At none of the doses used was paracetamol able to modify either the maximum number (B_{max}) or the affinity (K_D) of 5-HT_{1A} receptors in the two brain areas examined (Table 2).

At doses ranging between 10^{-8} and 10^{-4} M, paracetamol was unable to displace, in vitro, the binding of either 2 nM [^{3}H]8-OH-DPAT or 2 nM [^{3}H]ketanserin.

Pretreatment with *p*-chlorophenylalanine did not modify the number of 5-HT_2 receptors but abolished the decrease in the maximum number of 5-HT_2 receptors induced by paracetamol in the cerebral cortex (Table 3). The interaction test showed an antagonistic relationship between *p*-chlorophenylalanine treatment and paracetamol treatment ($F(1,20) = 4.54$; $P < 0.05$).

4. Discussion

The hot-plate test measures both a reflex and the complex response to an acute non-inflammatory nociceptive imput. In this test, paracetamol displayed an antinociceptive activity at the dose of 400 mg/kg, comparable with that of 8 mg/kg morphine, which is consistent with results obtained by Hunskaar et al. (1986). While this is the

Table 3
Effect of i.p. administration of paracetamol treatment on [^{3}H]8-hydroxy-2-(D-*n*-propylamino)tetralin ([^{3}H]8-OH-DPAT) binding to cerebral membranes

Treatment	Cortex		Pons	
(mg/kg i.p.)	B_{max} (fmol/mg prot.)	K_D (nM)	B_{max} (fmol/mg/prot.)	K_D (nM)
Control	264.2 ± 2.8	1.9 ± 0.3	193.7 ± 19.3	3.9 ± 1.2
Para 200	268.3 ± 9.9	1.7 ± 0.4	N.D.	N.D.
Para 300	265.0 ± 9.7	1.3 ± 0.6	205.2 ± 15.4	3.2 ± 0.9
Para 400	219.9 ± 9.1	1.8 ± 0.3	210.3 ± 15.2	3.6 ± 0.8

Rats were i.p. injected with vehicle, paracetamol (Para), 200, 300, 400 mg/kg, 90 min before the hot-plate test. They were then killed and the brains were stored until assayed. Each value represents the mean of six separate experiments performed with pooled tissues of 4–6 animals. B_{max} = maximum binding capacity and K_D = equilibrium dissociation constant were derived by Rosenthal plot. N.D. = not determined. Data are expressed as mean ± S.E.M. $P > 0.05$ (ANOVA).

maximum possible effect for paracetamol, higher doses of morphine have been shown to completely block the behavioral responses of the paw formalin injection.

The serotonin depletion induced in CNS by *p*-chlorophenylalanine was able to inhibit the antinociceptive activity of paracetamol in the hot-plate test, indicating that the integrity of the serotonergic system is crucial for this antinociceptive response.

In the formalin test, direct chemical stimulation of nociceptors results in an acute and intense activation of C fibres and, to a lesser extent, of A-delta fibres (phase 1) (Heapy et al., 1987). Experimental results indicated that substance P and bradykinin participate in the early phase (Shibata et al., 1989). There is intense neuronal activity both in the spinal cord and brain stem in the first few minutes, while no obvious inflammatory state during the first 5–10 min of formalin test has been observed (Hunskaar and Hole, 1987). The second, or late phase of the behavioral response in the formalin test is divided into two phases (2A and 2B), as a number of NSAIDs and paracetamol proved to be typically most active to reduce the response to formalin during the first period of the second phase of the formalin test (Malmberg and Yaksh, 1992). The second phase represents a prolonged tonic response in which inflammatory processes are involved and neurons in the dorsal horns of the spinal cord are activated (Tjølsen et al., 1992). Indeed, most A-delta and C-fibres displayed a second phase of increased activity 10–100 min after formalin injection. The highest discharge rates are usually observed 25–80 min after formalin application (Porro and Cavazzuti, 1993).

All the phases of the formalin test are suppressed by agonists of μ-opioid (as morphine, see Figs. 1 and 2), or α_2-adrenoceptors and adenosine A_1 receptors (Malmberg and Yaksh, 1993). The present results indicate that the highest doses of paracetamol reduce the number of flinches in all the phases, but, at doses of 300 mg/kg, the drug is active only in phases 1 and 2A. Narcotic analgesics, which are centrally acting agents, are able to suppress the response in phase 1 and in phase 2 when the inflammatory process occurs. The activity of paracetamol in both phases suggests a central mode of action comparable with that of opiates for phase 2. A possible explanation can be that, in the late phase, the response depends also on neural changes which are triggered by events occurring during the first phase as well as by inflammatory inputs from the periphery.

Interestingly, *p*-chlorophenylalanine prevents the action of paracetamol only in the first phase of the formalin test. The importance of 5-HT in the first phase is suggested by the results of Fasmer et al. (1985), who reported that the late formalin response was not altered by lesions of spinal serotonergic pathways. While 5-HT seems to be involved in the modulation of the first phase of the formalin test, the fall of 5-HT content provoked by *p*-chlorophenylalanine would reduce the effect of paracetamol mainly in the first phase of the test.

In our experimental model, the prevention of the paracetamol analgesic effect induced by *p*-chlorophenylalanine cannot be attributed to a hyperalgesic effect of *p*-chlorophenylalanine because this drug does not alter the reaction times in either hot-plate or formalin test. In addition, Taber and Lantranyi (1981) demonstrated that *p*-chlorophenylalanine did not affect yeast-induced hyperalgesia or the reaction times when pressure is applied to the non-inflamed rat paw.

It has been shown that 5-HT depletion in the brain plays an important part in decreasing the analgesic effect of morphine. Our data show a decrease of 81–88% (in cortical and pontine areas, respectively) in brain serotonin, following *p*-chlorophenylalanine, and are in line with the degree of serotonin depletion necessary to prevent analgesic effects (Bodnar et al., 1980).

The concentrations of paracetamol, evaluated 90 min after administration, are relatively high compared with those found in humans after therapeutic administration but are below the toxic levels in rats and mice (Granados-Soto et al., 1993; Hunskaar and Hole, 1987); other studies have also shown that such doses are necessary to induce antinociceptive effects in rodents (Hunskaar et al., 1986; Pelissier et al., 1995). Moreover, none of the behavioral parameters was significantly impaired by the drug treatment, so we can conclude that paracetamol exerted its antinociceptive effect without affecting gross behaviour.

Any pharmacokinetic interference between *p*-chlorophenylalanine and paracetamol has to be excluded since the serum paracetamol levels were not affected by *p*-chlorophenylalanine treatment, and direct interference of paracetamol with serotonin receptors must be excluded, as our 'in vitro' results demonstrate.

It has been suggested that 5-HT_{1A} and 5-HT_2 receptors are involved in the modulation of antinociception exerted by the central serotonergic system (Eide and Hole, 1991; Alhaider, 1991; Meller et al., 1991; Pelissier et al., 1995). The 5-HT_2 receptors are widely distributed in cortical areas and extrapyramidal nuclei, while 5-HT_{1A} receptors are found in the hippocampus, septum, raphe and spinal cord (Millan and Colpaert, 1991). The pons is particularly rich in cell bodies of serotonergic neurons, while the cerebral cortex contains a high density of axonal projections of these cells (Törk, 1990) as well as of 5-HT_2 receptors (Desamukh et al., 1983). Millan and Colpaert (1991) found that 5-HT_{1A} receptors play a role at the spinal level. In our study, paracetamol did not influence the number of 5-HT_{1A} receptors in the brain, suggesting that the antinociceptive effect of paracetamol, which significantly increases brain 5-HT levels, influences the change in 5-HT_2 receptors, as also proposed by Alhaider (1991). Enhancement of 5-HT can induce down-regulation of the 5-HT_2 receptor subtype in a relatively short period of time (Darmani et al., 1992).

5-HT_2 receptors are primarily involved in pain trans-

mission (Eide and Hole, 1993) and the reduction of these receptors might explain a decrease in pain perception as shown by the present data. The reduction in the number of 5-HT_2 receptors in the cortical but not in the pontine areas, could therefore depend on the different density of receptors in these areas, and would emphasize the role of the cortex as end-point for the serotonergic antinociceptive system.

Our data, therefore, confirm that paracetamol has an antinociceptive effect in non-inflammatory pain i.e. in the hot-plate test, in which inflammation does not occur, and in phase 1 of the formalin test and in phase 2 when pain is elicited by inflammation.

Evidence of a central antinociceptive effect of paracetamol involving spinal 5-HT receptors has been provided by Pelissier et al. (1995), and our data demonstrate that, when 5-HT brain levels fall after *p*-chlorophenylalanine treatment, both the behavioral and the biochemical effects of paracetamol disappear, as previously observed by our group for other NSAIDs (Pini et al., 1993, 1995). The data thus also point to a relationship between cortical 5-HT_2 receptors reduction and antinociceptive activity of this drug.

Indeed, the preventive effect of 5-HT lesions on the antinociceptive action of paracetamol has been demonstrated in the early phase of the formalin test (Tjølsen et al., 1991; Fasmer et al., 1985). Thus, the failure of both *p*-chlorophenylalanine pre-treatment (present paper) and central serotonergic system lesions (Tjølsen et al., 1991) to antagonize the antinociceptive effects of paracetamol in the second phase of the formalin test (phases 2A and 2B), when the inflammation process sets in, could be referred to a prevalent influence on peripheral inflammatory processes. However, we studied the ability of paracetamol to modify the perception of pain, using two tests with two different types of painful stimuli, whereas we did not explore the ability of this drug to modify the inflammation.

In conclusion, the present data support the hypothesis that 5-HT plays a pivotal role in the analgesic effect of paracetamol in the hot-plate and the formalin tests.

References

Abdel-Halim, M.S., B. Sjøquist and E. Anggard, 1978, Inhibition of prostaglandin synthesis in rat brain, Acta Pharmacol. Toxicol. 43, 266.

Alhaider, A.A., 1991, Antinociceptive effect of ketanserin in mice: involvement of supraspinal 5-HT_2 receptors in nociceptive transmission, Brain Res. 543, 335.

Bensemana, D. and A.L. Gascon, 1978, Relationship between analgesia and turnover of brain biogenic amines, Can. J. Physiol. Pharmacol. 56, 721.

Bjørklund, A., S.B. Dunnett, U. Stenevi, M.E. Lewis and S.D. Iversen, 1980, Reinnervation of the denerved striatum by substantia nigra transplants: function consequences as revealed by pharmacological and sensomotor testing. Brain Res. 199, 483.

Bodnar, R.J., J.H. Kordower, M.M. Wallace and H. Tamir, 1980, Stress and morphine analgesia: alterations following *p*-chlorophenylalanine, Pharmacol. Biochem. Behav. 14, 645.

Brune, K., W.S. Beck, G. Geisslinger, S. Menzel-Soglowek, B.M. Peskar and B.A. Peskar, 1991, Aspirin-like drugs may block pain independently of prostaglandin synthesis inhibition, Experientia 47, 257.

Brune, K., S. Menzel-Soglowek and H.U. Zeilhofer, 1992, Differential analgesic effect of aspirin-like drugs, Drugs 44(5s), 52.

Clissold, S.P., 1986, Paracetamol and Phenacetin, Drugs 32, 46.

Darmani, N.A., B.R. Martin and R.A. Glennon, 1992, Behavioral evidence for differential adaptation of the serotonergic system after acute and chronic treatment with (±)-1-(2,5-dimetoxy-4-iodophenyl)-2-aminopropane (DOI) or ketanserin, J. Pharmacol. Exp. Ther. 262, 692.

Desamukh, P.P., H.I. Yamamura, L. Woods and D. Nelson, 1983, Computer-assisted autoradiographic localization of subtypes of serotonin receptors in the rat brain, Brain Res. 288, 338.

Eide, P.K. and K. Hole, 1991, Different role of 5-HT_{1A} and 5-HT_2 receptors in spinal cord in the control of nociceptive responsiveness, Neuropharmacology 30, 727.

Eide, P.K. and K. Hole, 1993, The role of 5-HT receptor subtypes and plasticity in the 5-HT systems in the regulation of nociceptive sensitivity, Cephalalgia 13, 75.

Fasmer, O.B., O.G. Berge and K. Hole, 1985, Changes in nociception after lesions of descending serotonergic pathways induced with 5,6-dihydroxytryptamine, Neuropharmacology 24, 729.

Granados-Soto, V., F.J. Lopez-Muñoz, G. Castañeda-Hernandez, L.A. Salazar, J.E. Villareal and F.L. Flores-Murrieta, 1993, Characterization of the analgesic effects of paracetamol and caffeine combinations in the pain-induced functional impairment model in the rat, J. Pharm. Pharmacol. 45, 627.

Groppetti, A., P.C. Braga, G. Biella, M. Parenti, L. Rusconi and P. Mantegazza, 1988, Effect of aspirin on serotonin and met-enkephalin in brain: correlation with the antinociceptive activity of the drug, Neuropharmacology 27, 499.

Gulati, A. and H.N. Bhargava, 1990, Down-regulation of hypotalamic 5-HT_{1A} receptors in morphine-abstinent rats, Eur. J. Pharmacol. 182, 253.

Heapy, C.G., A. Jamieson and N.J.W. Russel, 1987, Afferent C-fiber and A-delta activity in models of inflammation, Br. J. Pharmacol. 90, 164p.

Hole, K., and A. Tjølsen, 1993, The tail-flick and formalin tests in rodents: changes in skin temperature as a confounding factor, Pain 53, 247.

Hunskaar, S., and K. Hole, 1987, The formalin test in mice: dissociation between inflammatory and non-inflammatory pain, Pain 30, 103.

Hunskaar, S., O-G. Berge and K. Hole, 1986, A modified hot-plate test sensitive to mild analgesics, Behav. Brain Res. 21, 101.

Leysen, E.J., C.J.E. Niemegeers, J.M. Van Neuten and P.M. Laduron, 1989, [^{3}H]Ketanserin (R 41 468) a selective [^{3}H]ligand for serotonin receptor binding sites. Mol. Pharmacol. 21, 301.

Malmberg, A.B. and T.L. Yaksh, 1992, Antinociceptive actions of spinal anti-inflammatory agents on the formalin test in the rat, J. Pharmacol. Exp. Ther. 263, 136.

Malmberg, A.B., and T.L. Yaksh, 1993, Pharmacology of spinal action of Ketorolac, Morphine, ST-91, U50488, and L-PIA on the formalin test and an isobolographic analysis of the NSAID interaction, Anesthesiology 79, 270.

Malmberg, A.B., and T.L. Yaksh, 1994, Antinociception produced by spinal delivery of the S and R enantiomers of flurbiprofen in the formalin test, Eur. J. Pharmacol. 256, 205.

Manz, B., H. Kosfele, O. Belovsky, H.J. Grill and K. Pollow, 1986, Direckte radioimmunologische Bestimmung des Serotonins in normalen und pathologischen Seren, Ärztl. Lab. 32, 135.

McCormack, K., 1994, The spinal actions of nonsteroidal anti-inflammatory-drugs and the dissociation between their anti-inflammatory and analgesic effects, Drugs 47 (5s), 28.

Meller, T., S.J. Lewis, M.J. Brody and G.F. Gebhart, 1991, The peripheral nociceptive actions of intravenously administered 5-HT in the rat requires dual activation of both 5-HT_2 and 5-HT_3 receptor subtypes, Brain Res. 561, 61.

Millan, M.J. and F.C. Colpaert, 1991, 5-Hydroxytryptamine $(HT)_{1A}$ receptors and the tail-flick response. II. High efficacy 5-HT_{1A} agonists attenuate morphine induced antinociception in mice in a competitive-like manner, J. Pharmacol. Exp. Ther. 256, 983.

Okuyama, S. and H. Aihara, 1984, The mode of action of analgesic drugs in adjuvant arthritic rats as an experimental model of chronic inflammatory pain: possible central analgesic action of acid non-steroidal anti-inflammatory drugs, Jpn. J. Pharmacol. 35, 95.

Pelissier, T., A. Alloui, C. Paeile and A. Eschalier, 1995, Evidence of a central antinociceptive effect of paracetamol involving spinal 5-HT_3 receptors. Neuroreport 6, 1546.

Pini, L.A., G. Vitale and M. Sandrini, 1993, The role of serotonin brain receptors in the analgesic effect of phenazone, Drugs Exp. Res. 19, 13.

Pini, L.A., M. Sandrini and G. Vitale, 1995, Involvement of brain serotonergic system in the antinociceptive action of acetylsalicylic acid in the rat, Inflamm. Res. 44, 30.

Porro, C.A. and M. Cavazzuti, 1993. Spatial and temporal aspects of spinal cord and brainstem activation in the formalin pain model, Prog. Neurobiol. 41, 565.

Rosenthal, H., 1967, A graphic method for the determination and presentation of binding parameters in a complex system, Anal. Biochem. 20, 520.

Sandrini, M, G. Vitale, E. Sternieri, A. Bertolini and L.A. Pini, 1993, Effect of chronic treatment of phenazone on the hot-plate test and [^{3}H]serotonin binding sites in pons and cortex membranes in the rat, Pharmacology 47, 84.

Shibata, M., T. Ohkubo, H. Takahashi and R. Inoki, 1989, Modified formalin test: characteristic biphasic pain response, Pain 38, 347.

Snedecor, G.W. and W.J. Cochran, 1980, Statistical Methods (Iowa State University Press, Ames, Iowa) p. 298.

Taber, R.I. and M.B. Lantranyi, 1981, Antagonism of the analgesic effect of opioid and non-opioid agents by *p*-chlorophenylalanine, Eur. J. Pharmacol. 75, 215.

Taiwo, Y.O. and J.D. Levine, 1988, Prostaglandins inhibit endogenous pain control mechanisms by blocking transmission at spinal noradrenergic synapses, J. Neurosci. 8, 1346.

Tjølsen, A., A. Lund and K. Hole, 1991, Antinociceptive effect of paracetamol in rats is partly dependent on spinal serotonergic systems, J. Pharmacol. 193, 193.

Tjølsen, A., O.-G. Berge, S. Hunskaar, J.H. Rosland and K Hole, 1992, The formalin test: an evaluation of the method, Pain 51, 5.

Törk, I., 1990, Anatomy of the serotonergic system, Ann. N.Y. Acad. Sci. 600, 9.

Warner, R., L. Hudson-Howard, C. Johnston and M. Skolnik, 1990, Serotonin involvement in analgesia induced by transcranial electrostimulation, Life Sci. 48, 1131.

Zimmerman, M., 1983, Ethical guidelines for investigation of experimental pain in conscious animals, Pain 16, 109.