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RESEARCH ARTICLE

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Effect of intrathecal serotonin on nociception in rats: influence of the pain test used

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Abstract The involvement of serotonin (5-HT) in the modulation of nociceptive impulse in the spinal cord has been widely studied. However, its activity, considering the nature of noxious stimuli and the type of 5-HT receptors involved, merits to be further elucidated. The present behavioural study was performed to compare the dose-antinociceptive effect relationship of 5-HT in rats, after intrathecal (i.t.) injection (10 µl/rat), using mechanical (paw pressure), thermal (tail immersion and tail-flick) and chemical (formalin) pain tests. In rats submitted to the paw pressure test, 5-HT was found to possess a dose-dependent antinociceptive activity (0.01, 0.1, 1, 10 and 20 µg/rat) when vocalization threshold was assessed as a pain parameter. A peak effect occurred 5 min after the injection and the effect was maintained for 45 min. The lowest active dose was 0.1 µg (maximum increase in vocalization thresholds, 23±3%) and a plateau was observed for 10 µg and 20 µg (maximum increase in vocalization thresholds, 72±7% and 71±6%, respectively). When paw withdrawal was assessed, 5-HT induced a weak hyperalgesic effect for the highest dose (60 µg), while other doses were ineffective. In the tail-immersion (warmth and cold) and tail-flick tests, different doses (0.01, 0.1, 1, 10, 30, 60 and 100 µg/rat) were studied. In the two immersion tests, only the highest doses (60 µg and 100 µg) significantly increased the withdrawal thresholds from 5 to 45 min after the injection. The maximum effect was observed at 5 min (23±4% and 21±6% for 60 µg; 27±3% and 30±6% for 100 µg in the warmth and cold immersion test, respectively). In the tail-flick test, the doses of 30, 60 and 100 µg/rat dose-dependently and significantly increased the withdrawal thresholds from 5 to 45 min after the injection, with a maximum effect at 5 min (30±5% for 30 µg; 37±6% for 60 µg; and 45±4% for 100 µg). In the formalin test, 5-HT (10, 25, 50, 75 and 100 µg/rat) produced dose-related antinociception. The nociceptive response (licking of the injected paw) was significantly reduced from 25 µg (–59±11%) in the early phase, whereas the lowest active dose in the late phase was 50 µg (–46±17%). For both phases, a total inhibition was obtained with 100 µg. It is concluded that the effect of 5-HT on pain tests may differ according to the applied stimulus and the parameter assessed; unspecific effects of 5-HT may modify motor reactions to noxious stimuli. Mechanical test (assessment of vocalization) was the most sensitive to 5-HT. These observations are of importance in order to further study the pharmacological mechanisms involved in 5-HT spinally induced antinociception.

Key words Serotonin · Nociception · Spinal cord · Pain tests · Rats

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Introduction

In spite of numerous studies showing the importance of the central serotonergic pathways in pain regulation, several points need to be clarified (Cesselin et al. 1994). For instance, the exact nature of the receptors involved in the serotonin (5-HT)-induced modulation of pain in the spinal cord remains unelucidated. Recent studies have revealed the presence of at least three families of 5-HT receptors in the spinal cord (5-HT_1, 5-HT_2 and 5-HT_3), with varying affinity for 5-HT (Hamon et al. 1990). Controversy exists about the possible role played by the three receptor subtypes in the modulation of pain. In the tail-flick test (TF), i.t. administered 5-HT_{1A} antagonists do not alter (Crisp et al. 1991) or produce a dose-related blockade (Xu et al. 1994) of 5-HT-induced antinociception. Intrathecal (i.t.) administration of 5-HT_{1A} agonists has been reported to facilitate (Crisp et al. 1991; Alhaider and Wilcox 1993), to inhibit (Eide and Hole 1991; Xu et al. 1994) or to not modify pain reactions (Solomon and Gebhart 1988; Mjellem et al. 1992; Millan 1994). A similar degree of controversy exists concerning the possible role played by the 5-HT_2 and 5-HT_3 receptors. Activation of the 5-HT_2 receptors has been reported to facil-

itate (Eide and Hole 1991) or to inhibit (Solomon and Gebhart 1988) the transmission of the nociceptive impulse. According to Glaum et al. (1988, 1990), spinal 5-HT_3 receptors mediate the antinociceptive effects of i.t. administered 5-HT. In contrast, the spinal 5-HT_3 receptors are not involved in this response, as demonstrated by Xu et al. (1994).

The marked inconsistency between the results reported by different groups may reflect the complexity of the serotonergic mechanisms but also give rise to questions regarding the influence of methodological aspects. Authors have shown that the nociceptive test and the quality of the noxious stimulus used for assessing animal sensitivity are of critical importance (Hole et al.1990; Murphy et al. 1992). A great majority of behavioural tests used in studies with 5-HT or 5-HT receptor agonists are thermal tests and mainly the tail-flick test. Other noxious stimuli (chemical and mechanical) are used rather less frequently (Le Bars 1988). Moreover, taking into account that opposite results have been obtained by using the same pain test and that 5-HT receptor subtypes exhibit differential affinity for 5-HT, it is important to compare the doses of ligands used in the different studies. Serotonin is generally used at high doses (25–200 µg/rat) (Glaum et al. 1988; Xu et al. 1994) as compared to the amount of 5-HT in the lumbar spinal cord (0.4 ng/mg tissue; Basbaum et al. 1987), which makes these experimental approaches widely dissimilar from physiological conditions. A wide dose range from 1 to 200 µg/rat (Solomon and Gebhart 1988; Giordano 1991) was also used in studies with 5-HT receptor agonists. Thus, the varying effects described above might be due to actions on different serotonergic or non-serotonergic sites according to the doses used (Crisp et al. 1991; Cesselin et al. 1994; Millan 1994). Another problem is that manipulations with serotonergic systems might alter motor responses and autonomic functions (Le Bars 1988). Such non-pain-related effects might influence the results in tests of nociception. Furthermore, concerning the often-used i.t. administration, the segmental level of the injection seems to be of importance. Larson (1985) reported that the antinociceptive activity of 5-HT was highly and differentially dependent on the segmental level at which it was injected.

Taking into account the discrepancies described in the literature and the methodological problems evoked, we have performed a study of the effects of 5-HT on spinal pain transmission. Several pain tests were used, involving either a thermal (tail immersion in warm or cold water, tail-flick induced by a radiant heat), a chemical (formalin) or a mechanical (paw pressure) stimulus and allowing the assessment of spinal or more integrated reactions to the pain stimulus in order to differentiate the influence of 5-HT on sensitive and motor phenomena. A wide dose-effect relationship (from 0.01 to 100 µg/rat i.t., according to the test) was investigated to compare the influence of low doses with that of higher doses generally described in the literature. Such a work is needed to optimise the design of experimental studies on the mechanism of 5-HT-induced spinal antinociception, which remains to be elucidated.

Materials and methods

Animals

Male Sprague-Dawley rats, weighing 250–300 g, were used. One week prior to the experiment, they were housed in standard laboratory conditions with free access to food and water. The experiments were performed blind in a quiet room, by a single experimenter, using the method of equal blocks, to study the effects of the different treatments in the same lapse of time, with randomization of treatments.

Pain tests and experimental procedures

Intrathecal injections

Intrathecal injections were performed as previously described (Mestre et al. 1994). Briefly, the rat was held in one hand by the pelvic girdle and a 25-gauge×1.6-cm needle connected to a 25-µl Hamilton syringe was always inserted between the spinous processes of L5 and L6 to the subarachnoidal space, until a tail flick was elicited. The syringe was held in position during a few seconds after the injection of 10 µl/rat.

Mechanical stimulus: paw pressure test

Nociceptive thresholds, expressed in grams, were measured with a Ugo Basile analgesimeter (Apelex; probe tip diameter 1 mm; weight 30 g; cut-off pressure 750 g) by applying increasing pressure to the left hind paw of rats until either a paw withdrawal (withdrawal threshold) or a squeak (vocalization threshold) was obtained. After obtaining two stable withdrawal or vocalization threshold values, the rats received an i.t. injection of saline (NaCl 0.9%) or 5-HT (0.01, 0.1, 1, 10, 20 µg/rat and 0.1, 1, 10, 30, 60 µg/rat for vocalization and paw withdrawal, respectively; n=12 rats per dose and for the control group). Then the thresholds were determined 5, 15, 30, 45 and 60 min after the injection.

Thermal stimuli

The tail immersion test. The tail of the rat was immersed in a water bath at noxious temperatures of 46°C or 5°C until the tail withdrawal or signs of struggle were observed (cut-off time 15 s). After obtaining two stable threshold values, the rats received an i.t. injection of saline or 5-HT (0.01, 0.1, 1, 10, 30, 60, 100 µg/rat and 10, 30, 60, 100 µg/rat for tests in warm and cold water, respectively; n=12 rats per dose and for the control group). Then the withdrawal thresholds were determined 5, 15, 30, 45 and 60 min after the injection.

The tail-flick test. The tail-flick test was performed using an automatic tail-flick apparatus (Apelex, LPB 255). Radiant heat was focused on the dorsal surface of the tail, 4–5 cm from the tip, and the latency from onset of stimulation to withdrawal of the tail was recorded. The intensity of the beam was adjusted to give a latency of 4–5 s for untreated animals. A cut-off time of 10 s was employed. After obtaining two stable threshold values, the rats received an i.t. injection of saline or 5-HT (1, 10, 30, 60 and 100 µg/rat; n=12 rats per dose and for the control group). Then the withdrawal thresholds were determined 5, 15, 30, 45 and 60 min after the injection.

Chemical stimulus: the formalin test

Chemo-inflammatory nociception was induced by injecting 50 µl of a 5% formalin solution into the plantar surface of the right hindpaw according to Dubuisson and Dennis (1977). The recording of the early response (early phase) started immediately after the injection of formalin and lasted for 5 min. The recording of the late response (late phase) started 20 min after formalin injection and lasted 10 min. In both phases, licking of the injected paw was used as the nociceptive response and the total duration of the response was measured. The i.t. administration of saline or 5-HT (10, 25, 50, 75 and 100 µg/rat; n=8 rats per dose and for the control group) was performed 5 min before the injection of formalin.

Drugs

5-HT, provided by Sigma Chemical (France), was dissolved in physiological saline (NaCl 0.9%). Solutions were prepared immediately prior to testing.

Expression of results and statistical analysis

Results were expressed as a variation (Δg) between scores obtained after treatment and control predug values for mechanical and thermal tests and as raw data (seconds) for the formalin test. To appreciate a global effect in the first two types of tests, estimated areas under the time-course curves (E. AUC) of the antinociceptive effects were calculated by summing the variation scores obtained for each individual time. Data were analyzed by a non-parametric test (Mann-Whitney's test).

Comparison of the effect of 5-HT in the different pain tests was performed by calculating, for each dose, the percentage of analgesia (maximum effect) as follows: [(maximum postdrug value)–(predrug value)]/[(cut-off value)–(predrug value)]×100.

"Predrug values" in the formalin test were values of the saline-treated group; because there is no cut-off value in this test, we use the maximal level of analgesia, i.e. 0 s licking. When hyperalgesia was observed, minimal postdrug value was used instead of "maximum postdrug value".

Results

Effect of 5-HT on the mechanical pain test

Before the injection, the vocalization and withdrawal thresholds were 287±4 g and 148±5 g, respectively. When vocalization thresholds were assessed (Fig. 1A), 5-HT produced a dose-dependent antinociception with a significant effect observed from 0.1 µg (maximum increase 23±3%). A maximum increase (72±7%) and a plateau were reached at 10 µg; no significant difference was observed between 10 and 20 µg (maximum increase 71±6%). Peak effects always appeared 5 min after the injection and 5-HT was totally inactive from 45 min. The determination of the E. AUC confirmed the dose-dependent antinociceptive effect from 0.1 to 10 µg/rat with a plateau from this last dose.

In contrast, when paw withdrawal was assessed, 5-HT (30 µg and 60 µg) induced a significant decrease (maximal variation: –23±4% and –27±4% for 30 µg and 60 µg, respectively; Fig. 1B). The determination of E. AUC confirmed this weak, significant hyperalgesic effect.

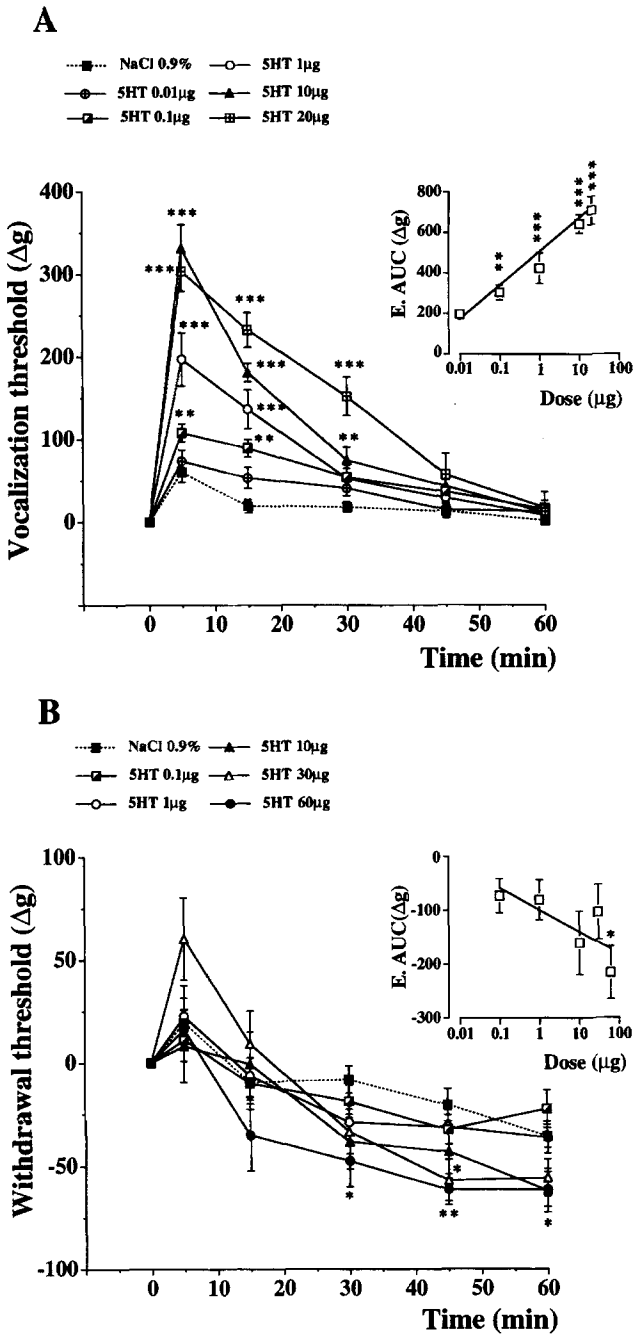

Fig. 1A, B Time-course of the antinociceptive effect of i.t. administered 5-HT on the paw pressure test. Vocalization thresholds (**A**) and withdrawal thresholds (**B**) were used as pain parameters. The values shown are the means±SEM of variations between post and predrug values (Δg; n=12 for each dose). Estimated area under the curves (*E. AUC*) were calculated by summing the variation scores obtained for each individual time. The dose-effect relationship was linear for vocalization thresholds in the 0.01–20 µg range (r^2=0.974, P<0.001). * P<0.05; ** P<0.01; *** P<0.001 as compared to corresponding saline-treated rats

Effect of 5-HT on thermal pain tests

The pre-drug latency for the tail withdrawal was not significantly different according to the test used: 4.7±0.1, 4.1±0.2 and 4.5±0.1 s, for the tail-immersion (warmth and cold) and the tail-flick tests, respectively.

The lowest doses of i.t. administered 5-HT (0.01, 0.1 µg) were ineffective in producing antinociception against tail immersion in warm water (data not shown). Accordingly, these doses were not used in the two other thermal pain tests. In the tail-flick test (Fig. 2A), 5-HT induced a significant dose-dependent effect from 30 to 100 µg. Maximum increases (5 min) were 30±5%, 37±6% and 45±4% for 30, 60 and 100 µg/rat, respectively. 5-HT was totally inactive from 45 min onwards. Determination of the E. AUC confirmed the dose-dependent antinociceptive effect of 5-HT in this test.

In the tail-immersion tests (warmth and cold; Fig. 2B,C) the minimal effective dose was 60 µg (23±4% and 21±6% in warmth and cold, respectively) with a weak increase in the maximum effect score for 100 µg (27±3% and 30±6% for the warmth and cold immersion test, respectively). Peak effects always appeared 5 min after the injection and 5-HT was totally inactive from 45 min except for 100 µg in the warmth immersion test (60 min). Using E. AUC, a linear dose-effect relationship was obtained in the warmth and the cold immersion test.

Effect of 5-HT on the chemical pain test

After formalin injection, the duration of the licking of the injected paw was 106±13 s and 139±22 s for the early and the late phase, respectively. The i.t. administration of 5-HT (10, 25, 50, 75 and 100 µg/rat) caused a dose-dependent reduction in the duration of the licking of the injected paw both in the early and the late phase (Fig. 3). The duration of licking was significantly reduced from 25 µg (–59±11%) for the early phase, whereas the first significantly active dose in the late phase was 50 µg (–46±17%). No early or late pain-induced behaviour was observed after 100 µg.

Global comparison of the results obtained in the different tests

The best sensitivity to the antinociceptive effect of 5-HT was obtained when the mechanical stimulus (with as-

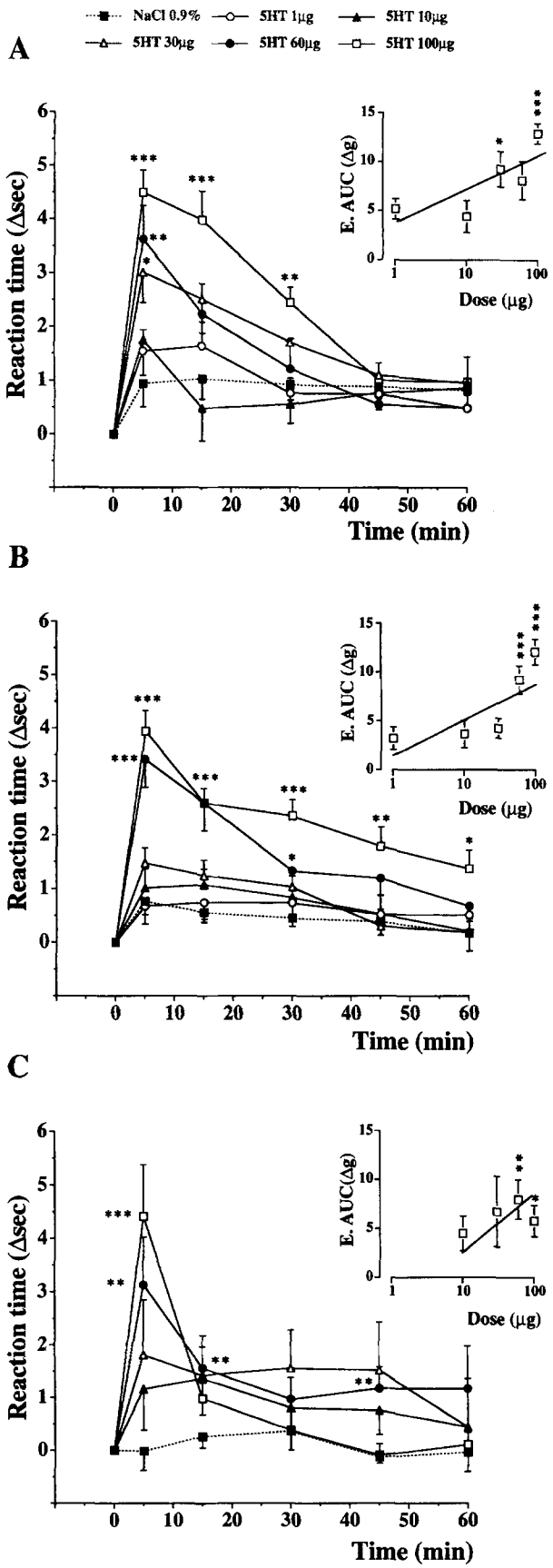

Fig. 2A–C Time-course of the antinociceptive effect of i.t. administered 5-HT on tail-flick test (**A**), on tail-immersion test in warm (46°C) (**B**) and in cold (5°C) (**C**) water. Tail withdrawal latency was used as a pain parameter. The values shown are the means±SEM of variations between post and predrug values (*Δg*; n=12 for each dose). Estimated area under the curves (*E. AUC*) were calculated by summing the variation scores obtained for each individual time. The dose-effect relationship was linear in the 1–100 µg range (r^2=0.612, P=0.002; r^2=0.628, P<0.001; r^2=0.337, P=0.003; in the tail-flick, tail immersion in warmth and cold, respectively). * P<0.05; ** P<0.01; *** P<0.001 as compared to corresponding saline-treated rats

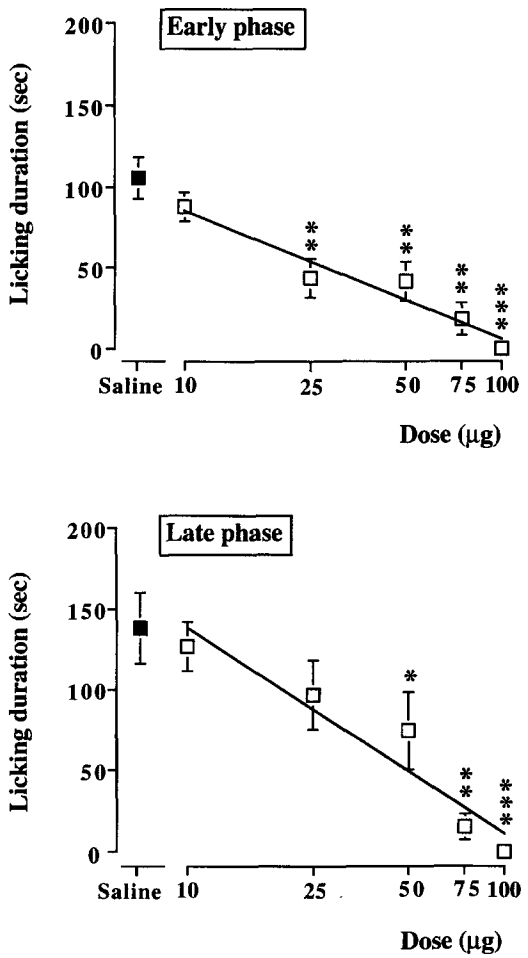

Fig. 3 Dose-response effect of 5-HT on the formalin test. The duration of licking of the right injected hindpaw, in the early (0–5 min) and the late (20–30 min) phase, was used as a pain parameter (n=8 for each dose). The dose-effect relationship was linear in the 10–100 μg range (r^2=0.934, P<0.001; r^2=0.905, P<0.001 for the early and the late phase, respectively). * P<0.05; ** P<0.01; *** P<0.001 as compared to saline-treated rats

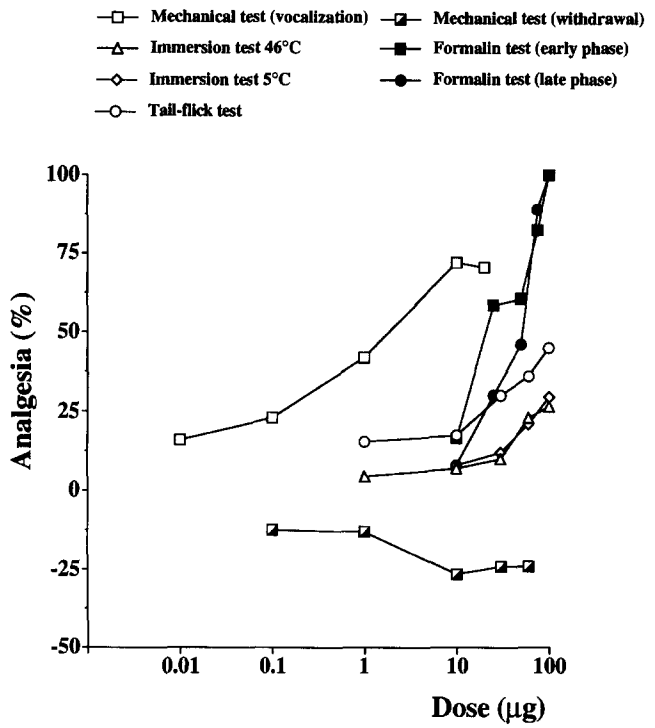

Fig. 4 Compared antinociceptive effect of i.t. injected 5-HT in the mechanical, thermal and chemical pain tests. The percentage of analgesia was calculated by the following formula: [(maximum postdrug value)–(predrug value)]/[(cut off value)–(predrug value)]×100. Values for each dose are the means of scores of 8–12 animals according to the test

sessment of vocalization threshold) was applied (ED_{50} value was 1.4±0.4 μg). The sensitivity of other tests decreased as follows: formalin test (ED_{50} values were 26±3 and 35±4 μg for early and late phase, respectively); tail-flick test (ED_{20} value was 13±2 μg); tail-immersion tests (ED_{20} values were 55±8 and 48±6 μg in warmth and cold, respectively); mechanical test (with assessment of paw withdrawal threshold) with evidence of an hyperalgesic effect of 5-HT. When the relative antinociceptive efficacy of 5-HT on the different pain tests was compared, the obtained scale from the best to the least efficacy was: chemical (100±0%), mechanical (vocalization; 72±7%) and thermal tests (27±3%, 30±6% and 45±4% in warm or cold immersion and in tail-flick tests, respectively).

Discussion

Present data confirm the spinal antinociceptive effect of 5-HT as previously described by several authors (Yaksh and Wilson 1979; Solomon and Gebhart 1988; Crisp et al. 1991). As generally found, 5-HT is rapidly effective (maximum effect appearing 5 min after i.t. injection), which can account for a specific spinal action. Indeed, Schmauss et al. (1983), performing both a pharmacodynamic and a pharmacokinetic study using i.t. injections of [^{14}C] 5-HT, showed a correlation between the peak of the antinociceptive effect and the spinal peak concentration (5–15 min), while radioactivity only appeared in the brain 45 min after the injection, when analgesia was significantly diminished. Interestingly, the duration of antinociception was similar to that obtained in the present work. However, if the present results confirm the spinal antinociceptive action of 5-HT, they show differences in both potency and efficacy of 5-HT, according to the behavioural pain test used.

The high potency and the relatively good efficacy of 5-HT observed in the mechanical test (based on the assessment of vocalization thresholds) suggest that this test may be a useful tool for further investigating 5-HT-mediated antinociception. The low effective doses (0.1 or 1 μg/rat), compatible with the amount of 5-HT present in the spinal cord (0.4 ng/mg tissue; Basbaum et al. 1987), might allow studies close to physiological conditions to be performed and provide a better understanding of serotonergic mechanisms involved in pain control. Even though 5-HT receptors may not directly mediate all these effects [interactions with dopaminergic (Blandina et al. 1989; Schmidt and Black 1989) or adrenergic (Post and

Archer 1990; Sawynok and Reid 1992) systems might be evoked], the doses used (e.g. 0.1 μg/10 μl per rat, corresponding to a 6.10^{-5} M injected solution), leads to a tissue concentration of 5-HT high enough to activate $5HT_1$ receptors (the affinity of 5HT is in the nanomolar range) as well as $5HT_2$ and $5HT_3$ receptors (the affinity of 5HT is in the micromolar range; Humphrey et al. 1993; Hoyer et al. 1994). On the other hand, it is low enough to avoid unspecific effects induced by high doses. Intrathecal injection of 200 μg 5-HT in clorgyline-treated rats induces a behavioural motor syndrome consisting of hyper-reactivity, resting tremor and rigidity (Schmauss et al. 1983). 5-HT (100 μg i.t.) has also been shown to induce scratching behaviour in mice (Hylden and Wilcox 1983). 8-OH-DPAT (50 μg i.t.), a $5\text{-}HT_{1A}$ receptor agonist, induces spontaneous tail-flicks in rats (Bervoets et al. 1993). All these effects are in line with the facilitatory activity on spinal motoneurons, as described for 5-HT (Le Bars 1988). Besides, this property might explain the lack of antinociceptive effect and the hyperalgesia observed with 10 μg and 60 μg of 5-HT, respectively, on the mechanical test when the paw withdrawal was assessed. The potent antinociceptive effect of 5-HT obtained with similar doses (10 μg) when vocalization was assessed is likely neutralized by the facilitating influence of the monoamine on the paw withdrawal, a spinal motor reflex. To conclude with the mechanical test, we must also underline that with doses up to 20 μg/rat the maximal analgesia was not reached (maximum increase 71±6%), and higher doses (100 μg) might have increased the antinociceptive effect but perhaps at the expense of the specificity.

The data obtained with the chemical test show a lower sensitivity to 5-HT than that observed in the mechanical test, but the analgesia is complete after 100 μg/rat. The noxious stimulus used would account for this apparent lower sensitivity. A 5% formol solution is the highest concentration generally used and a dose-dependent pronociceptive effect of formol has been demonstrated (Rosland et al. 1990; Coderre et al. 1993). It may be suggested that lower doses of 5-HT would have been active by using lower formalin concentrations, as Rosland et al. (1990) found that indomethacin was active against formalin 1%, but ineffective when a 5% solution was used. Furthermore, increasing doses of morphine and aspirin had to be used to induce antinociception with the highest formalin concentration (5%). Whatever the real sensitivity of this test to 5-HT, it is also interesting to observe that 5-HT is as effective in the early phase as in the late one (dose-effect relationship and ED_{50} are very similar). Such a result is unusual, since morphine and acetylsalicylic acid generally induce a higher effect in the late phase than in the early one (Hunskaar and Hole 1987; Rosland et al. 1990; Wheeler-Aceto and Cowan 1991). In addition, nonsteroidal anti-inflammatory drugs are known to be ineffective in the first phase (Hunskaar et al. 1986; Malmberg and Yaksh 1992). Although this may be due to the differential properties of analgesics taking into account the different nature of the two phases (Hunskaar and Hole 1987; Shibata et al. 1989), variations in the techniques used to assess formalin-induced pain behaviours might participate in the observed differences and explain the particular profile of action of 5-HT in this test. However, licking has been shown as one of the more consistent behaviours (Abbott et al. 1995) and the potential motor excitatory effect of 5-HT would increase the paw-licking duration, which would be opposite to the behavioural changes due to its antinociceptive action. Thus the effect of 5-HT on the formalin test is clearly due to its antinociceptive activity.

5-HT is less potent and efficient in the thermal tests, whatever the nature of the heat source, the temperature and the experimental conditions used. The minimal effective dose was 60 μg in the tail immersion tests (warm and cold) and 30 μg in the tail-flick test. These results agree with those of several other authors who found the lowest active doses of 5-HT between 50 and 100 μg, using a similar noxious stimulus (Yaksh and Wilson 1979; Schmauss et al. 1983; Xu et al. 1994). This low potency of 5-HT is associated with a low efficacy, as the maximal percentage of analgesia obtained with 100 μg/rat is 27±3%, 30±6% and 45±4% in warmth and cold immersion and tail-flick tests, respectively. This agrees with observations that 5-HT produces only weak antinociceptive effects in thermal tests (Kuraishi et al. 1985). These characteristics might suggest that the stimulation of 5-HT receptors would be poorly able to reduce the transmission of a nociceptive impulse induced by a thermal stimulation. However, methodological problems might also account for these results: excitatory motor effects of 5-HT inducing a decrease in reaction time might again antagonize its antinociceptive action (increase in reaction time).

The presently demonstrated, test-dependent antinociceptive effect of 5-HT recalls that obtained with other neuromediators involved in pain modulation. Intrathecal noradrenaline also produces a more potent antinociceptive effect on mechanical than on thermal pain tests (Kuraishi et al. 1985). This is of interest taking into account the demonstrated interaction between serotonergic and noradrenergic systems in analgesia (Post and Archer 1990) and the fact that mixed serotonin and noradrenaline reuptake inhibitors are more effective in relieving neuropathic pain than specific serotonin reuptake inhibitors (Eschalier 1990; Onghena and Van Houdenhove 1992). NMDA antagonists have different efficacy depending upon the type of noxious stimulus involved (Coderre and Empel 1994). Pain tests based on chemical and mechanical noxious stimuli have been found to be much more sensitive to the antinociceptive activity of opioid ligands than were tests based on noxious heat (Millan 1986; Hill 1994). Finally, concerning 5-HT, the efficacy of very low doses (much lower than those usually used) shown in this work allows working with concentrations closier to the endogenous amount of this neuromediator in the central nervous system, which may give a new insight into its mechanism of action as pain modulator.

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