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Effect of acute and chronic tramadol on [^{3}H]-5-HT uptake in rat cortical synaptosomes

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1 Tramadol hydrochloride is a centrally acting opioid analgesic, the efficacy and potency of which is only five to ten times lower than that of morphine. Opioid, as well as non-opioid mechanisms, may participate in the analgesic activity of tramadol.

2 [^{3}H]-5-hydroxytryptamine (5-HT) uptake in rat isolated cortical synaptosomes was studied in the presence of tramadol, desipramine, fluoxetine, methadone and morphine. Methadone and tramadol inhibited synaptosomal [^{3}H]-5-HT uptake with apparent K_is of 0.27 ± 0.04 and 0.76 ± 0.04 μM, respectively. Morphine essentially failed to inhibit [^{3}H]-5-HT uptake (K_i 0.50 ± 0.30 M).

3 Methadone, morphine and tramadol were active in the hot plate test with ED_{50}s of 3.5, 4.3 and 31 mg kg^{-1}, respectively. At the highest tested dose (80 mg kg^{-1}) tramadol produced only 77 ± 5.3% of the maximal possible effect.

4 When [^{3}H]-5-HT uptake was examined in synaptosomes prepared from rats 30 min after a single dose of morphine, methadone or tramadol, only tramadol (31 mg kg^{-1}, s.c., equal to the ED_{50} in the hot plate test) and methadone (35 mg kg^{-1}, s.c., equal to the ED_{90} in the hot plate test) decreased uptake.

5 Animals were chronically treated for 15 days with increasing doses of tramadol or methadone (5 to 40 mg kg^{-1} and 15 to 120 mg kg^{-1}, s.c., respectively). Twenty-four hours after the last drug injection, a challenge dose of methadone (35 mg kg^{-1}, s.c.) or tramadol (31 mg kg^{-1}, s.c.) was administered. [^{3}H]-5-HT uptake was not affected in synaptosomes prepared from rats chronically-treated with methadone, whereas chronic tramadol was still able to reduce this parameter by 42%.

6 Rats chronically-treated with methadone showed a significant increase in [^{3}H]-5-HT uptake (190%) 72 h after drug withdrawal. In contrast, [^{3}H]-5-HT uptake in rats chronically-treated with tramadol (110%) did not differ significantly from control animals.

7 These results further support the hypothesis that [^{3}H]-5-HT uptake inhibition may contribute to the antinociceptive effects of tramadol. The lack of tolerance development of [^{3}H]-5-HT uptake, together with the absence of behavioural alterations after chronic tramadol treatment, suggest that tramadol has an advantage over classical opioids in the treatment of pain disorders.

Keywords: Tramadol; methadone; morphine; [^{3}H]-5-HT uptake; antinociception; pain

Introduction

Tramadol hydrochloride (1**RS**, 2**RS**)-2-[(dimethylamino) methyl]-1-(3-methoxyphenyl)-cyclohexanol HCl) is a centrally acting analgesic with documented antinociceptive effects in animal models (Raffa *et al*., 1992) and in man (Collart *et al*., 1993). The analgesic efficacy and potency of acutely administered tramadol is comparable to that of codeine, pentazocine or dextropopoxyphene (Hennies *et al*., 1988), while its analgesic and antinociceptive potency is only five to ten fold lower than that of morphine (Lehmann *et al*., 1990). Tramadol has a unique profile among opioid analgesics. Importantly, its acute therapeutic use is not associated with clinically significant side effects, such as respiratory depression, constipation or sedation (Lee *et al*., 1993). Long-term clinical trials indicate absence of abuse potential (Preston *et al*., 1991) or euphoric effects (Huber, 1978) of tramadol.

Tramadol-induced antinociception appears to be mediated via both opioid and non-opioid mechanisms (Raffa *et al*., 1992). Subcutaneous naloxone only partially inhibits tramadol analgesic activity in the hot plate test in rats (Kayser *et al*., 1991; Raffa *et al*., 1992). In displacement studies, the K_i values of tramadol for μ, δ and κ opioid receptors are 2.1, 57.5 and 42 μM, respectively (Hennies *et al*., 1988). In spite of its low affinity for opioid receptors, a high correlation between tramadol analgesic potency and selectivity for μ receptors has been shown (Raffa *et al*., 1992). Interestingly, in the tail-flick test the antinociceptive effect of tramadol is antagonized by both yohimbine, an α_2-adrenoceptor antagonist, and ritanserin, a 5-hydroxytryptamine (5-HT) antagonist (Raffa *et al*., 1992). Tramadol has also been shown to inhibit *in vitro* [^{3}H]-noradrenaline and [^{3}H]-5-HT uptake with a K_i of 0.79 μM and 0.99 μM (IC_{50} 3.1 μM), respectively (Raffa *et al*., 1992; Driessen & Reimann, 1992).

The present study was designed to probe further the contribution of non-opioid mechanisms in the antinociceptive effects of tramadol, as assessed by [^{3}H]-5-HT uptake after acute and chronic drug administration in rats. The effects of acute or chronic tramadol treatment, as well as termination of chronic treatment, on [^{3}H]-5-HT accumulation were also investigated. For comparative purposes, the classical opioids methadone and morphine were included.

Methods

Animals

Adult male Sprague-Dawley rats (200–250 g) were obtained from Harlan-Nossan (S. Pietro Natisone, UD, Italy) and housed under controlled temperature (23 ± 2°C) and illumination (12 h light–12 h dark) (18 h 00 min–06 h 00 min). Experiments were performed between 10 h 00 min and 17 h 00 min.

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Synaptosomal preparation

Brain synaptosomes were isolated according to Whittaker & Barker (1972). Rats were decapitated, the brains rapidly excised, and the frontal cortex dissected out on ice. The tissue was homogenized in 10 volumes (w/v) of ice-cold 0.32 M sucrose and then centrifuged at 1800 *g* for 10 min (4°C). The pellet was discarded and the supernatant centrifuged at 17000 *g* for 60 min (4°C). The pellet was resuspended in a small volume of 0.32 M sucrose, layered on a 1.2–0.8 M sucrose gradient and centrifuged at 37000 *g* for 60 min (4°C). The synaptosomal fraction at the interface between 0.8 and 1.2 M sucrose was collected and further centrifuged at 17000 *g* for 30 min. The pellet was resuspended in buffer (composition in mM: NaCl 115, KCl 4.97, $CaCl_2^{-1}$, $MgSO_4$ 1.22, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 11.1, pH 7.4, plus 0.01 mM pargyline) and immediately used. Protein content was determined according to Lowry *et al.* (1951) with bovine serum albumin as standard.

[3H]5-HT uptake in synaptosomes

Synaptosomes (200–250 μg protein) were preincubated for 5 min at 37°C in the absence or presence of different concentrations of drug. Uptake was started by addition of 5 nM [3H]-5-HT and incubation continued for 6 min. Control samples were incubated at 0°C to evaluate membrane diffusion. The reaction was stopped by cooling the tubes in ice. The samples were then filtered through Whatman GF/C glass fibre filters (Whatman Inc., Clifton, NJ, U.S.A.) and washed twice with 150 mM Tris HCl, pH 7.4. Filter-bound radioactivity was counted by liquid scintillation spectrometry with Filter Count (Packard). The difference in [3H]-5-HT accumulation at 37°C and 0°C was taken as a measure of active uptake. K_i ($\pm$ s.e. mean) values were calculated by a computer-assisted curve fitting programme (EBDA) (McPherson, 1987).

Hot plate test

The hot-plate test of Eddy & Leimbach (1953) was utilized. Rats (5 animals per group) were placed individually on a plate maintained at 55 ± 0.5°C by feedback from a surface-mounted thermocouple. The response latency was evaluated on the basis of either hind paw lick or jump reaction, following contact with the plate. After three readings at 30 min intervals, response changes were assessed 30 min after s.c. drug administration.

The percentage of antinociceptive maximal possible effect (MPE) was calculated from the formula:

%MPE = 100 (test latency-predrug latency)/(cut-off time-predrug latency) by use of the predrug latency of each animal and a cut-off time of 45 s.

Systemic drug treatment

Four animals were used for each acute treatment group, with a single s.c. injection of methadone, morphine or tramadol dissolved in saline. Drug doses chosen corresponded to the respective hot plate ED_{50}. Methadone and morphine were also administered at their ED_{50} (i.e., $\sim$35 and $\sim$40 mg kg^{-1}, respectively). Control animals were treated with saline only. Thirty minutes later animals were killed and synaptosomes prepared. Chronic treatment consisted of single daily injections (s.c.) for 15 days. The initial dose of methadone or tramadol was 5 or 15 mg kg^{-1}, respectively. The methadone and tramadol doses were then increased daily by 2.5 and 7.5 mg kg^{-1} until the animals received 40 and 120 mg kg^{-1}, respectively. Eight animals per group were used. Control rats ($n = 12$) received daily injections of saline. Seventy-two hours after the last narcotic or saline injection, 4 rats from each experimental group were killed and synaptosomes prepared. The remaining rats chronically treated with methadone or tramadol ($n = 4$ per group) received, 24 h after the last injection, a challenge dose of methadone (35 mg kg^{-1}) or tramadol (31 mg kg^{-1}), respectively. Methadone (35 mg kg^{-1}) or tramadol (31 mg kg^{-1}) was likewise administered to chronic saline-treated rats. Animals were killed 30 min later and synaptosomal [3H]-5-HT uptake assessed.

Drugs and solutions

($\pm$)-Tramadol hydrochloride was a generous gift of Formenti S.p.A. (Milan, Italy). Fluoxetine hydrochloride was obtained from Eli Lilly & Co. (Indianapolis, IN, U.S.A.). ($\pm$)-Methadone hydrochloride and ($\pm$)-morphine hydrochloride were purchased from S.A.L.A.R.S., S.p.A. (Como, Italy) and desipramine and pargyline were obtained from Sigma (St. Louis, MO, U.S.A.). [3H]-5-HT (specific activity 27.3 Ci mmol^{-1}) was obtained from NEN (Boston, MA, U.S.A.). All drug concentrations and doses refer to the free base. Compounds were dissolved in isotonic saline and administered s.c. in a volume of 1 ml kg^{-1}. All other reagents were of analytical grade.

Statistics

The ED_{50} and ED_{90} (doses of drug that give 50% and 90% of MPE, respectively) in the hot plate test were derived from the percentage protection data by Probit analysis according to Finney (1971). The confidence limits were referred to $P = 0.05$. In uptake studies results are expressed as means $\pm$ s.e.mean; significant differences between means were determined by one way ANOVA followed by REGWF test. The probability level chosen was $P < 0.05$.

Results

[3H]-5-HT uptake by isolated synaptosomes

Incubation of cortical synaptosomes in the presence of methadone and tramadol, as well as other known 5-HT uptake inhibitors (desimipramine, fluoxetine) dose-dependently inhibited [3H]-5-HT uptake. The K_i values, estimated from the concentration of drug required to inhibit 50% of [3H]-5-HT uptake, are shown in Table 1. Synaptosomal uptake of [3H]-5-HT was most strongly reduced by fluoxetine. Methadone, tramadol and desimipramine, although less potent, were still clearly effective. Morphine produced only nominal inhibition of [3H]-5-HT uptake ($K_i = 0.5 \pm 0.3$ M).

Hot plate test

The antinociceptive efficacy against thermal nociception of acutely administered methadone, morphine and tramadol is illustrated in Figure 1. Methadone and morphine antinociception was dose-dependent and achieved MPE, whereas tramadol at the highest tested dose (80 mg kg^{-1}) reached 77 ± 5.3% of the MPE. In all cases drug performance peaked between 20–45 min, with the antinociceptive effect exceeding 120 min (data not shown). The ED_{50} for methadone, morphine and tramadol are given in Table 2. The results indicate the

Table 1 Drug-induced inhibition of [3H]-5-HT uptake in rat cortical synaptosomes

Drug	K_i (μM)
Desimipramine	0.43 ± 0.037
Fluoxetine	0.049 ± 0.0046
Methadone	0.27 ± 0.038
Morphine	$>300{,}000$
Tramadol	0.76 ± 0.045

Data are means $\pm$ s.e.mean from three different experiments each performed in triplicate. For experimental details, see Methods.

antinociceptive potency of tramadol to be 8.7 (confidence limits: 2.5–31) and 7.2 (2.1–25) times lower than that of methadone and morphine, respectively ($P<0.01$).

Effects of acute drug administration on [3H]-5-HT uptake

The influence of acutely given methadone, morphine and tramadol on [3H]-5-HT uptake by cortical synaptosomes was next investigated. Lower doses of methadone (3.5 mg kg^{-1}) and morphine (4.3 mg kg^{-1}) (ED_{50} in the hot-plate test) did not affect uptake (Figure 2). In contrast, the hot plate equieffective dose of tramadol (31 mg kg^{-1}) significantly inhibited [3H]-5-HT uptake (63±3.4%) ($P<0.01$ vs control). 5-HT uptake could be inhibited at higher methadone doses (35 mg kg^{-1}, ED_{90} in hot plate test) (52±3.8%) ($P<0.01$), while morphine was still ineffective even at 40 mg kg^{-1} (ED_{90} in the hot plate test). An equieffective dose of tramadol (302 mg kg^{-1}) was not used, due to its toxicity (LD_{50} 286 mg kg^{-1}, s.c.) (Lagler *et al.*, 1978).

Effects of chronic drug administration on [3H]-5-HT uptake

Rats were treated for 15 days with increasing doses of methadone and killed 72 h after the last drug injection. Synaptosomes prepared from these animals showed a significantly increased (+90±15%) [3H]-5-HT uptake ($P<0.01$) (Table 3; 'drug deprivation' column). Surprisingly, rats chronically treated with tramadol did not differ significantly from the chronic-saline group (Table 3). A challenge dose of methadone (35 mg kg^{-1}), given 24 h after conclusion of chronic methadone treatment did not affect synaptosome [3H]-5-HT uptake with respect to the chronic saline group (Table 3). This contrasts with the results obtained for acute methadone administration (Figure 2) and with methadone challenge in the chronic saline group (Table 3), where [3H]-5-HT uptake was decreased. Repeated administration of methadone thus resulted in a loss of inhibitory effects on the uptake process.

Conversely, when rats chronically-treated with saline or increasing doses of tramadol for 15 days were challenged with tramadol (31 mg kg^{-1}) a significant reduction in [3H]-5-HT uptake was observed (Table 3). This reduction was similar to that caused by acute tramadol administration (Figure 2).

Behaviour

Relevant behavioural changes were observed in rats acutely treated with high doses of methadone (35 mg kg^{-1}) or morphine (40 mg kg^{-1}). The former appeared cataleptic and showed body rigidity, absence of spontaneous movements and a preserved righting reflex. Morphine produced sedation with a classical Straub tail reaction. Tramadol (80 mg kg^{-1}), given acutely produced only a slight sedation.

Behavioural changes were also observed in rats that received chronic methadone treatment. In these animals, the day 4 dose (12.5 mg kg^{-1}) induced a 4 h cataleptic state. The same

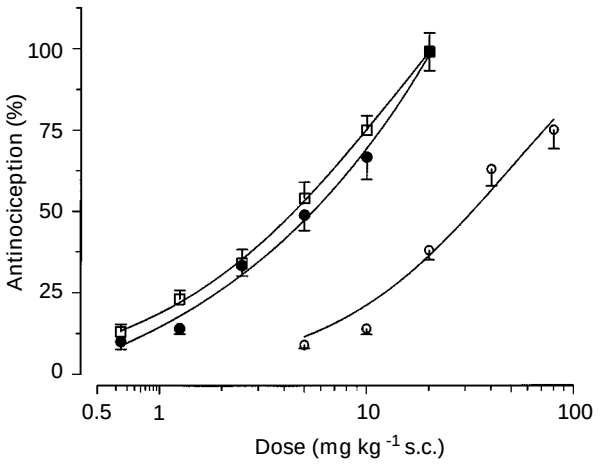

Figure 1 Antinociceptive efficacy of methadone (□), morphine (●) and tramadol (○) in the hot plate test. Compounds were administered s.c. 30 min before the test. Values are means and vertical lines show s.e.mean ($n=5$). For experimental details, see Methods.

Table 2 Antinociceptive effects of methadone, morphine and tramadol in the hot plate test

Drug	*ED_{50}* (mg kg^{-1})
Methadone	3.5 (1.8–6.7)
Morphine	4.3 (2.3–8.1)
Tramadol	31 (15–63)

Data are means±s.e.mean of five animals per group. For experimental details, see Methods.

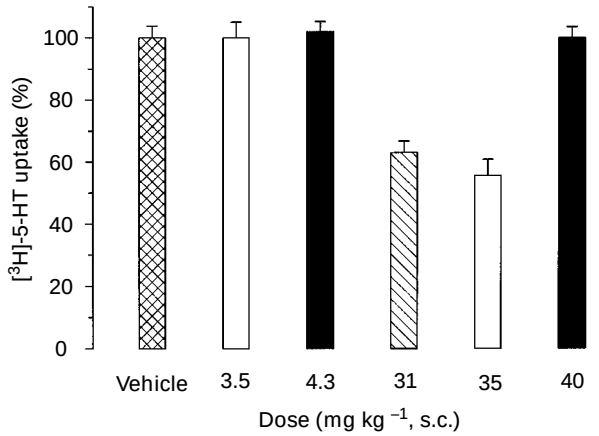

Figure 2 Effect of acute administration of methadone (open columns; 3.5 and 35 mg kg^{-1}), morphine (solid columns; 4.3 and 40 mg kg^{-1}), tramadol (hatched column; 31 mg kg^{-1}) and vehicle (cross-hatched column; 1 ml kg^{-1}) on [3H]-5-HT uptake by rat cortical synaptosomes. Compounds were administered s.c. 30 min before the test. Values are means±s.e.mean ($n=4$). For experimental details, see Methods.

Table 3 [3H]-5-HT uptake in cortical synaptosomes from chronically-treated rats

Treatment group	*Drug deprivation*	*% uptake Methadone challenge*	*Tramadol challenge*
Saline	100±6.6[a]	52±3.8[c]	62±4.0[c]
Methadone	190±15[b]	102±4.4[a]	–
Tramadol	110±4.8[a]	–	58±4.2[c]

Methadone and tramadol refer to groups of rats treated with single daily injections of methadone or tramadol for 15 days, respectively. The starting doses of methadone and tramadol were 5 and 15 mg kg^{-1}, respectively. Methadone and tramadol were then increased daily by 2.5 and 7.5 mg kg^{-1} until the animals received 40 and 120 mg kg^{-1}, respectively, Saline indicates rats receiving daily injections of saline. 'Drug deprivation' indicates animals killed 72 h following the last drug injection. Methadone challenge and tramadol challenge denote rats receiving a challenge dose of methadone (35 mg kg^{-1}) or tramadol (31 mg kg^{-1}) 24 h after the last chronic administration and killed 30 min later. Data are mean±s.e.mean of four animals per group, and are expressed relative to the respective saline-treated groups (0.35 pmol mg^{-1} protein = 100%). Mean values having different superscript letters indicate significant differences ($P<0.01$) between groups. For experimental details, see Methods.

phenomenon was observed at day 10 (30 mg kg^{-1}) but persisted for only 90 min. After the cataleptic state had subsided, rats began to exhibit long-lasting (more than 5 h) head nodding or masticatory behaviour. Rats chronically treated with tramadol were devoid of gross behavioural abnormalities.

Discussion

Tramadol has long been appreciated for its analgesic effects which are obtained at doses producing only weak classical opioid side effects, including respiratory depression and abuse. This pharmacological profile probably represents a concerted action between both the opioid and other systems involved in the control of pain transmission, e.g. the aminergic system. In the present study, tramadol produced a dose-dependent antinociception in the hot plate test that was, not unexpectedly, 8.7 and 7.2 times less potent than methadone and morphine, respectively. These results are in agreement with the tail flick response test in rats (Hennies *et al*., 1988), and in clinical studies on patient-controlled analgesia (Lehmann *et al*., 1990). Moreover, only a slight sedation was observed in acute and chronic tramadol-treated rats, unlike the typical behavioural signs of opiate agonist administration, i.e. catalepsy and Straub tail phenomena, seen in methadone- and morphine-treated rats. The data are in accord with the low incidence of tramadol side effects in man (Huber, 1978; Barth *et al*., 1987).

Tramadol has been found to have low affinity for opioid receptors with dissociation constants (K_i) in the micromolar range (Hennies *et al*., 1988), and either a lack of selectivity for μ, κ and binding δ sites (Hennies *et al*., 1988) or some selectivity for μ-receptors (Raffa *et al*., 1992). Despite a 1000 fold lower affinity for μ-opiate receptors, tramadol has been found to induce antinociception with a potency only 5 to 10 fold less than that of morphine. While the analgesic efficacy of tramadol could be due to the generation of active metabolites, *O*-demethyl tramadol (M1), the only tramadol metabolite with high affinity for μ-opioid receptors (K_i: 0.22 nM) (Frink *et al*., 1996) seemed not to contribute to the analgesic effect of tramadol (Lee *et al*., 1993).

Analgesia can also be produced by tricyclic antidepressants (amitriptyline or desimipramine) (Sharav *et al*., 1987; Magni, 1991) or adrenoceptor agonists (clonidine) (Yask, 1985), which inhibit 5-HT or noradrenaline (NA) uptake. The latter are more effective than opiate analgesics in treating neuropathic or deafferentation pain (Ventafridda *et al*., 1988; Max *et al*., 1992). Furthermore, they are devoid of abuse potential.

The antinociceptive potency of some semisynthetic (such as levorphanol and levomethorphan, i.e. phenanthrene opioids having neither an oxygen bridge between C4-C5 nor an oxygen-containing substituent at C6), as well as synthetic (such as methadone and tramadol, i.e. non-phenanthrene opioids) opioids may actually be better described by a model which includes both their μ-receptor affinity and capacity to inhibit 5-HT uptake (Codd *et al*., 1995). Here, we observed that methadone and tramadol were as potent as desimipramine in inhibiting [3H]-5-HT uptake when added to rat isolated cortical synaptosomes. However, when rats were acutely treated with equieffective doses (i.e., the ED_{50} in the hot plate test) of methadone or tramadol, only synaptosomes prepared from the latter animals had significantly reduced [3H]-5-HT uptake. This effect of methadone on [3H]-5-HT uptake was apparent at 31 mg kg^{-1}, i.e. at the ED_{90} in the hot plate test. A similar effect was described in rat hypothalamic slices after a single injection of methadone (30 mg kg^{-1}) (Moffat & Jhamandas, 1976). At such drug doses, opioid activation probably predominates over monoaminergic potentiation. It thus appears that of the opioids tested, only tramadol displays 5-HT uptake inhibition and antinociceptive activity in a correlative fashion at pharmacologically relevant doses.

To investigate the effect of chronic drug administration on pharmacological responses, a challenge dose of opiate was administered 30 min before synaptosomal preparation from chronically drug-treated rats. Interestingly, methadone challenge (35 mg kg^{-1}, ED_{90} in the hot plate) in this chronic treatment paradigm was no longer able to inhibit 5-HT uptake. Such loss of activity might be explained by the development of a tolerance mechanism caused by chronic methadone administration. In contrast, a challenge dose of tramadol, following chronic administration of the drug, inhibited 5-HT uptake as effectively as when given acutely (Figure 1; Table 3). Tramadol thus differs from methadone in its ability to affect the 5-HT uptake system, without imposing a state of tolerance.

Tramadol was further differentiated from classical opioids by its action in chronically-treated, drug-deprived rats. Seventy-two hours after termination of chronic methadone, a lag time sufficient to bring out possible withdrawal effects, [3H]-5-HT baseline uptake was significantly elevated compared to controls. Although this effect could be due to an unmasking of a compensatory increase in [3H]-5-HT uptake occurring during chronic methadone administration, it seems unlikely, given that chronic methadone has been shown not to alter 5-HT baseline uptake (Moffat & Jhamandas, 1976). Alternatively an increase in 5-HT uptake, due to opiate withdrawal stress, may have taken place (Moffat & Jhamandas, 1976; Keller *et al*., 1994). Unlike methadone, chronic treatment with increasing doses of tramadol did not alter 5-HT uptake 72 h after drug deprivation. Clearly, under the present experimental conditions neither withdrawal nor a compensatory increase in 5-HT uptake occur after chronic tramadol.

In summary, the antinociceptive action of tramadol is actually concomitant with a potentiating effect on the 5-hydroxytryptaminergic system. Together with the opioid activity, this effect may indeed participate in tramadol-induced analgesia, thus differentiating its molecular mechanism of action from that of pure opioids – as suggested by the absence of noticeable opioid behavioural signs. The lack of tolerance in 5-HT uptake and the absence of behavioural alterations after chronic tramadol treatment suggest that this drug may offer advantages in the long term treatment of pain, with respect to other opioid analgesics including methadone and morphine.

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