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Interaction of the central analgesic, tramadol, with the uptake and release of 5-hydroxytryptamine in the rat brain *in vitro*

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1 Tramadol is a centrally acting analgesic with low opioid receptor affinity and therefore presumably other mechanisms of analgesic action. Tramadol inhibits noradrenaline uptake but since 5-hydroxytryptamine (5-HT) is also involved in the modulation of pain perception, we tested the effects of tramadol on 5-HT uptake and release *in vitro*.

2 Tramadol inhibited the uptake of [^{3}H]-5-HT into purified rat frontal cortex synaptosomes with an IC_{50} of 3.1 μM. The (+)-enantiomer was about four times more potent than the (−)-enantiomer; the main metabolite of tramadol, O-desmethyltramadol, was about ten times less potent.

3 Rat frontal cortex slices were preincubated with [^{3}H]-5-HT, then superfused and stimulated electrically. Tramadol facilitated the basal outflow of [^{3}H]-5-HT, at concentrations greater than 1 μM, while the uptake inhibitor 5-nitroquipazine enhanced both basal and stimulation-evoked overflow. Effects of the (+)-enantiomer were more potent than either the racemate, the (−)-enantiomer or the principal metabolite.

4 The effects of tramadol on the basal outflow of [^{3}H]-5-HT were almost completely abolished when the superfusion medium contained a high concentration of the selective 5-HT uptake blocker, 6-nitroquipazine.

5 The results provide evidence for an interaction of tramadol with the neuronal 5-HT transporter. An intact uptake system is necessary for the enhancement of extraneuronal 5-HT concentrations by tramadol indicating an intraneuronal site of action.

Keywords: Tramadol; 6-nitroquipazine; 5-HT uptake; 5-HT release; frontal cortex slices; frontal cortex synaptosomes

Introduction

Pain perception is modulated by a variety of neurotransmitters including opioids, noradrenaline and 5-hydroxytryptamine (5-HT) (Yaksh, 1988). The centrally acting analgesic, tramadol, (1**RS**;2**RS**)-2-[(dimethylamino)methyl]-1-(3-methoxyphenyl)-cyclohexanol hydrochloride, has a low affinity for opioid receptors in the micromolar range (Hennies *et al.*, 1988) which may not adequately explain its analgesic and antinociceptive potency which is only five to ten times lower than that of morphine (Friderichs *et al.*, 1978). We have recently shown that noradrenaline uptake blockade may be a further mechanism contributing to its antinociceptive effects (Driessen *et al.*, 1990). Since 5-hydroxytryptaminergic pathways are particularly important in pain modulation (Besson & Chaouch, 1987) we investigated whether tramadol may also interact with this neurotransmitter system.

Methods

General

Male Sprague Dawley rats weighing 160–380 g were killed by decapitation and the brain was quickly removed and chilled. Frontal cortices were dissected according to Glowinski & Iversen (1966). Slices from frontal cerebral cortex (0.4 mm thick, 5 mm diameter) were prepared as described and characterized previously (Reimann *et al.*, 1981).

Accumulation of [^{3}H]-5-hydroxytryptamine in synaptosomes

Cortices were homogenized in ice-cold 0.32 M sucrose (1.5 ml per 100 mg tissue) in a glass homogenizer with a loosely fitting teflon pestle by 10 full up and down strokes at 800 revolutions per min. The homogenate was filtered through Thomapor gauze, 100 μm mesh, and centrifuged at 1000 *g* for 15 min at 4°C. Further subfractionation was performed according to Dodd *et al.* (1981) by discontinuous gradient centrifugation. The supernatant was layered on 4.5 ml 1.2 M sucrose and centrifuged at 190000 *g* for 25 min. Particles at the gradient interface were collected in a volume of about 2.5 ml and diluted with ice-cold 0.32 M sucrose to a final volume of 8.0 ml. This suspension was layered on 4.5 ml 0.8 M sucrose and centrifuged again for 25 min at 190000 *g*. The resulting synaptosomal pellet was gently resuspended in 4 ml ice-cold incubation medium per 100 mg original tissue weight by use of a teflon/glass homogenizer.

The incubation medium was as follows (mM): NaCl 119, KCl 3.9, $CaCl_2$ 0.51, $MgSO_4$ 0.65, Na_2HPO_4 15.6, NaH_2PO_4 3.4, glucose 10, ascorbic acid 0.57. Pargyline, 10 μM, was added for inhibition of monoamine oxidase. The pH was adjusted to 7.4 by addition of NaOH.

The synaptosomes were preincubated for 5 min at 37°C in the absence or presence of the drugs to be tested. The uptake was started by addition of 10 μl [^{3}H]-5-HT yielding a concentration of 0.1 μM. The final incubation volume was 1 ml, and the protein concentration, determined according to Lowry *et al.* (1951), was 90–193 μg ml^{-1}. The incubation lasted for 30 s if not stated otherwise and was stopped by addition of 6 ml ice-cold medium, immediately followed by vacuum filtration through nitrocellulose membranes (pore seize 0.65 μm; Sartorius) presoaked in incubation medium. For determination of radioactivity, the wet filters were solubilised in 2 ml ethylene glycol monoethyl ether. Radioactivity was counted after addition of 10 ml Ready Safe (Beckman). Values were corrected for the accumulation of radioactivity in synaptosomes incubated at 0°C. All assays were performed in triplicate.

Outflow of tritium from slices after preincubation with [^{3}H]-5-hydroxytryptamine

Cortical slices were preincubated with 0.1 μM [^{3}H]-5-HT at 37°C for 30 min, then transferred to glass superfusion chambers equipped with platinum electrodes and superfused with

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medium at a rate of 1 ml min^{-1} for 105 min at 37°C. Starting 50 min after the onset of superfusion 5 min samples of the perfusate were collected. The slices were stimulated electrically for two periods of 2 min each, after 60 and 90 min of superfusion (S_1; S_2). Rectangular pulses of 2 ms duration, 24 mA current strength and a frequency of 3 Hz were delivered from a stimulator constructed by the Biomedical Technics Department of Grünenthal.

At the end of the experiment, the slices were solubilised in 0.5 ml Soluene 350 (Packard). The radioactivity in superfusates and slices was measured by liquid scintillation spectrometry after addition of Ready Gel (Beckman) and Ready Safe (Beckman), respectively. The incubation and superfusion medium contained (mM): NaCl 118, KCl 4.8, $CaCl_2$ 1.3, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 11, ascorbic acid 0.57, disodium EDTA 0.03; the medium was saturated with 5% CO_2 in O_2. The pH was adjusted to 7.4 by the addition of NaOH. Pargyline, 10 μM, was added to the medium throughout the superfusion. Drugs were added 15 min before S_2 or were present throughout the superfusion as indicated.

Calculations and statistics

The kinetic parameters of synaptosomal uptake, K_m and V_{max}, were estimated by adaptation of the data to the Michaelis-Menten hyperbola, and IC_{50}s by adaptation to the IB_1 model of the Top-fit programme package (Thomae) on a Wang 2236-D type computer.

The fractional rate of tritium outflow in release experiments was calculated by dividing the tritium content in the superfusate by the calculated tritium content in the slice at the start of the respective collection period. The stimulation-evoked overflow of tritium was calculated by subtraction of the estimated basal outflow and was expressed as a percentage of the tissue tritium content at the start of stimulation. For further evaluation of basal tritium efflux, ratios were calculated between the fractional rate of outflow observed in the 5 min period before S_2 (pre-S_2) over that observed in the 5 min period before S_1 (pre-S_1).

Means ± s.e.mean are given throughout. Differences between means were tested for significance by the Mann-Whitney test.

Compounds

5-[1,2-^{3}H(N)]-hydroxytryptamine-creatinine-sulphate, specific activity 21.0, 25.4 and 29.6 Ci $mmol^{-1}$ (New England Nuclear); pargyline HCl (Serva); morphine HCl (E. Merck); tramadol HCl, (−)- and (+)-enantiomer of tramadol, O-desmethyltramadol HCl (Grünenthal). 6-Nitroquipazine maleate was a kind gift of Duphar Pharma, and zimeldine dihydrochloride monohydrate of Astra Chemicals. All drugs were dissolved in water.

Results

Effects on the accumulation of [^{3}H]-5-hydroxytryptamine in synaptosomes

Preliminary experiments indicated that the rapid initial rate of tritium accumulation in frontal cortex synaptosomes lasted only for 30 s. This time period was therefore chosen for subsequent experiments. Kinetic analysis showed an apparent K_m of 0.051 ± 0.005 μM, and V_{max} was 5.4 ± 0.2 pmol $30\,s^{-1}\,mg^{-1}$ protein ($n = 4$ with 7 concentrations per experiment).

Synaptosomes were incubated with 0.1 μM [^{3}H]-5-HT either in the presence or absence of drugs. Accumulation of tritium under control conditions (no drug present) was 4.2 ± 0.1 pmol [^{3}H]-5-HT equivalent mg^{-1} protein ($n = 36$). The accumulation of radioactivity was most potently inhibited by 6-nitroquipazine, followed by zimeldine and tramadol (Figure 1). Calculated IC_{50} values are given in Table 1. The principal metabolite of tramadol, O-desmethyltramadol, was less active by about one order of magnitude (Figure 1; Table 1). Some stereoselectivity of the uptake inhibition by tramadol was observed since the (+)-enantiomer is about four times more potent than the (−)-form (Table 1). Inhibition of uptake by the test drugs was not complete. Maximum inhibition at 100 μM amounted to 85.6 ± 1.5% with 6-nitroquipazine, 88.2 ± 1.8% with zimeldine, 80.2 ± 2.4% with tramadol, 86.9 ± 2.2% with its (+)-enantiomer, 76.9 ± 1.1% with its (−)-enantiomer and 65.4 ± 1.9% with its metabolite O-desmethyltramadol. For comparison, morphine was practically devoid of 5-HT uptake inhibition; its maximum effect was 12.8 ± 3.1% inhibition at 100 μM ($n = 3$) (data not shown).

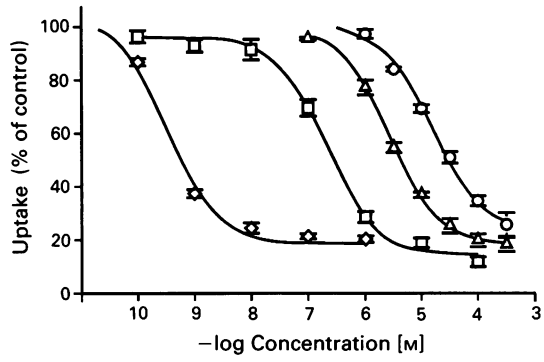

Figure 1 Effects of various drugs on the accumulation of tritium in frontal cortex synaptosomes incubated with 0.1 μM [^{3}H]-5-hydroxytryptamine. The accumulation in the presence of nitroquipazine (◇), zimeldine (□), tramadol (△) and O-desmethyltramadol (○) is expressed as a percentage of control. Each data point represents the mean of 6 experiments; s.e.mean shown by vertical bars.

Effects on tritium outflow from slices preincubated with [^{3}H]-5-hydroxytryptamine

Slices from the frontal cortex were preincubated with [^{3}H]-5-HT then superfused with medium containing 10 μM pargyline and stimulated electrically for two periods (S_1, S_2). When no drug was present, stimulation at S_1 resulted in an overflow of 1.2 ± 0.02% of the tritium in the tissue ($n = 97$) and was enhanced to 4.9 ± 0.1% in the presence of 1 μM 6-nitroquipazine ($n = 23$).

Tramadol, when added 15 min before S_2, enhanced the basal outflow without major effects on the stimulation-evoked overflow (Figure 2). The basal outflow was evaluated in the fractions preceding the stimulation periods; 10 μM tramadol enhanced the overflow by about 100% (Table 2). Basal outflow was facilitated more potently by the (+)-enantiomer

Table 1 Effect of drugs on the accumulation of tritium in frontal cortex synaptosomes incubated with 0.1 μM [^{3}H]-5-hydroxytryptamine

Drug	*IC_{50} (μM)*
6-Nitroquipazine	0.00038 ± 0.00002
Zimeldine	0.23 ± 0.03
Tramadol	3.1 ± 0.4
Tramadol (+)-enantiomer	2.1 ± 0.2
Tramadol (−)-enantiomer	8.6 ± 0.6
O-desmethyltramadol	24.2 ± 2.3

In each experiment, at least six drug concentrations were used. Means ± s.e.mean of $n = 6$ individual experiments.

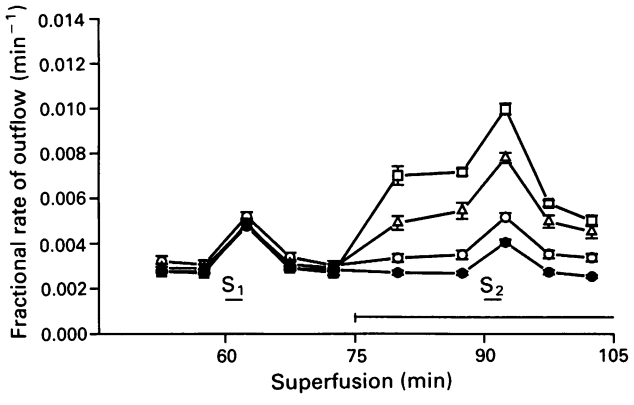

Figure 2 Effect of tramadol on the outflow of tritium from brain cortical slices preincubated with 0.1 μM [^{3}H]-5-hydroxytryptamine. After preincubation, slices were superfused with medium containing 10 μM pargyline and were stimulated electrically for two periods (S_1/S_2). Tramadol was added 15 min before S_2 as indicated by the bar. (●) Controls without tramadol; (○) tramadol 1 μM; (△) tramadol 10 μM; (□) tramadol 100 μM. Each data point represents the mean of 5–6 experiments; s.e.mean shown by vertical bars.

as compared to the (−)-enantiomer, and the metabolite was considerably less potent (Table 2). An evaluation of the stimulation-evoked overflow was not possible since the enhancement of the basal outflow precludes an exact estimation of the basal outflow for the subtraction from the total overflow.

Addition of the 5-HT uptake inhibitor 6-nitroquipazine, 1 μM, to the superfusion medium results in an attenuation of the effects of tramadol (Figure 3; Table 2). Residual effects were only observed at 100 μM. Under these conditions, a small but significant inhibition by tramadol of the stimulation-evoked overflow was observed. The ratio S_2/S_1 dropped to 0.85 ± 0.01 at 10 μM (n = 6) and 0.68 ± 0.02 at 100 μM tramadol (n = 5) as compared to 0.96 ± 0.01 (n = 6) under control conditions (absence of tramadol).

6-Nitroquipazine, added at S_2, also enhanced the basal outflow at the concentrations tested (Figure 4); the facilitation was almost 60% (Table 2). But it also enhanced the stimulation-evoked overflow, as can be seen from the graph. Effects were even more pronounced on the stimulation-evoked as compared to the basal overflow and differed therefore from the effects of tramadol (cf. Figure 2).

Discussion and conclusions

Neuronal amine uptake is a primary mechanism for the termination of neurotransmitter action and therefore drugs may interfere with the uptake mechanism to modulate neurotransmission. To determine the influence of a drug on the uptake of a specific neurotransmitter, some precautions have

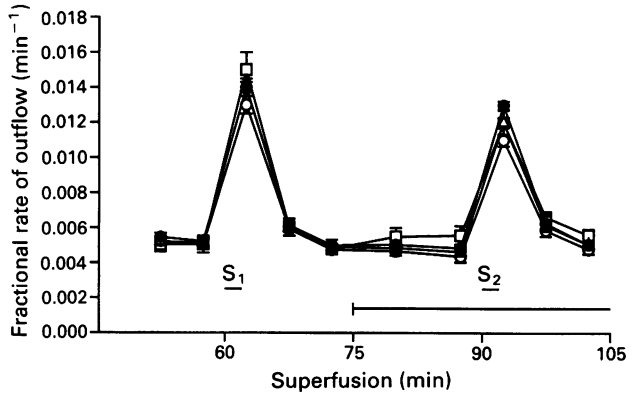

Figure 3 Effect of tramadol on the outflow of tritium from brain cortical slices preincubated with 0.1 μM [^{3}H]-5-hydroxytryptamine. After preincubation, slices were superfused with medium containing 10 μM pargyline and 1 μM nitroquipazine and were then stimulated electrically for two periods (S_1, S_2). Tramadol was added 15 min before S_2 as indicated by the bar. (●) Controls without tramadol; (○) tramadol 1 μM; (△) tramadol 10 μM; (□) tramadol 100 μM. Each data point represents the mean of 5-6 experiments; s.e.mean shown by vertical bars.

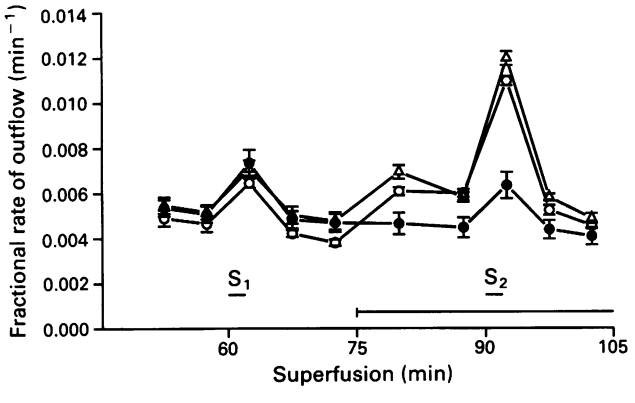

Figure 4 Effect of nitroquipazine on the overflow of tritium from brain cortical slices preincubated with 0.1 μM [^{3}H]-5-hydroxytryptamine. After preincubation, slices were superfused with medium containing 10 μM pargyline and were stimulated electrically for two periods (S_1, S_2). Nitroquipazine was added 15 min before S_2 as indicated by the bar. (●) Controls without nitroquipazine; (○) nitroquipazine 1 μM; (△) nitroquipazine 10 μM. Each data point represents the mean of 6 experiments; s.e.mean shown by vertical bars.

Table 2 Effects of the investigated drugs on the basal outflow of tritium from rat cerebral cortex slices preincubated with [^{3}H]-5-hydroxytryptamine

Drug added at S_2	*Drug added throughout superfusion*	*Basal outflow (pre-S_2/pre-S_1) at a drug concentration of* 0	1 μM	10 μM	100 μM
Tramadol	—	0.97 ± 0.02 (6)	1.14 ± 0.03 (6)*	1.88 ± 0.04 (6)*	2.67 ± 0.04 (5)*
Tramadol	6-Nitroquipazine 1 μM	0.91 ± 0.01 (6)	0.90 ± 0.03 (6)	0.95 ± 0.03 (6)	1.19 ± 0.06 (5)*
Tramadol (+)-enantiomer	—	0.89 ± 0.01 (8)	1.27 ± 0.16 (4)*	1.53 ± 0.08 (4)*	2.21 ± 0.12 (4)*
Tramadol (−)-enantiomer	—	0.89 ± 0.01 (8)	0.96 ± 0.01 (4)*	1.35 ± 0.08 (4)*	2.22 ± 0.15 (4)*
O-desmethyl-tramadol	—	0.82 ± 0.02 (4)	0.83 ± 0.01 (4)	1.05 ± 0.03 (4)*	1.71 ± 0.02 (4)*
6-Nitroquipazine	—	0.87 ± 0.03 (6)	1.37 ± 0.02 (6)*	1.14 ± 0.03 (6)*	

After preincubation, slices were superfused with medium containing pargyline and, when indicated, 6-nitroquipazine. They were stimulated electrically for two periods (S_1/S_2). Drugs given at S_2 were added to the medium 15 min before the onset of stimulation. Fractional rates of tritium outflow were determined in the fractions preceding the stimulation periods (pre-S_1, pre-S_2). Significant differences from corresponding controls (concentration of drug added at S_2 = 0): * $P < 0.05$. Means ± s.e.mean of number of experiments in parentheses.

to be taken. In order to approach true uptake, i.e. unidirectional transmitter flow, only the initial linear rate of uptake should be measured (Graefe, 1976). This precondition was met by the short incubation time of 30 s in the present study. The uptake of [^{3}H]-5-HT into the rat frontal cortex synaptosomes followed Michaelis-Menten-kinetics and the K_m-value, indicative for high affinity, agrees with that of other investigators (Richelson & Pfenning, 1984). 5-HT may also accumulate in catecholaminergic nerve terminals (Iversen, 1974) but with a much lower affinity for the carrier (K_m 8 μM). Therefore we can assume that, under the present conditions, most of the radiolabelled transmitter has been taken up by 5-hydroxytryptaminergic terminals. Purification of the synaptosomes by gradient centrifugation was performed in order to minimize uptake in or attachment to other cell organelles.

Nitroquipazine was a very potent 5-HT uptake blocker in the present study. As with zimeldine, inhibition of total uptake of exogenous 5-HT was monophasic, albeit not complete, indicative of a highly selective interaction with the uptake mechanism for 5-HT (Ross *et al.*, 1976; Vaatstra *et al.*, 1981). Inhibition by zimeldine of 5-HT uptake was comparable to previously published data (Ross & Renyi, 1977; Richelson & Pfenning, 1984) while the efficacy of nitroquipazine was about two orders of magnitude higher than that reported by Vaatstra *et al.* (1981) who, however, studied uptake in whole brain homogenates, which may explain the apparent discrepancy. Nevertheless, the potency of uptake inhibition by nitroquipazine in our experiments is in good agreement with data from binding studies with [^{3}H]-6-nitroquipazine (K_i 0.12 nM) recently published by Hashimoto & Goromaru (1990).

Tramadol blocked the uptake of 5-HT in a similar manner to nitroquipazine and zimeldine. Its potency was only about one order of magnitude less than that of zimeldine and contrasts with a previous study which reported the blockade of 5-HT uptake with considerably lower potency (Hennies *et al.*, 1982), differences which may be due to the use of whole brain synaptosomes. The investigation of the enantiomers of tramadol showed evidence of a stereoselective interaction with a four fold higher potency in favour of the (+)-enantiomer. Investigations on the interaction with the 5-HT transporter often yield small stereoselective effects although some results are in favour of a clearcut stereoselective uptake mechanism (Smith, 1986). An unexpected finding is that the loss of the O-methyl group results in a strong reduction of activity; this small alteration of the molecule did not affect the interaction with the noradrenaline transporter (unpublished observations).

Apparent neurotransmitter uptake inhibition in experiments measuring accumulation of transmitter into synaptosomes may be the result of either true blockade of uptake sites or induction of neurotransmitter release (Baumann & Maitre, 1976; Maxwell *et al.*, 1976). To avoid misinterpretation of the uptake inhibition observed, we tested tramadol and nitroquipazine in a concomitant investigation of 5-HT release from slices of the same brain region.

Overflow of tritium from cortical slices preincubated with [^{3}H]-5-HT is likely to represent authentic 5-HT when monoamine oxidase is inhibited by pargyline. The model is often used for the study of the presynaptic regulation of 5-HT release (Cerrito & Raiteri, 1979; Göthert & Weinheimer, 1979; Göthert, 1980; Baumann & Waldmeier, 1981), and using this system, facilitation of [^{3}H]-5-HT overflow can be observed with uptake inhibitors which prevent the reuptake of previously released transmitter.

6-Nitroquipazine is a potent and highly selective inhibitor of neuronal 5-HT uptake (Vaatstra *et al.*, 1981; Hashimoto & Goromori, 1990). Its facilitating effect on the stimulation-evoked overflow has been described (Göthert *et al.*, 1983; Classen *et al.*, 1984) and was also observed in the present investigation. Tramadol, which was demonstrated to be an uptake inhibitor in the synaptosomal uptake experiments, showed a concentration-dependent enhancement of the basal overflow in brain slice release experiments. This effect cannot be due to interference with autoreceptors, since they are known not to modulate the basal transmitter outflow (Göthert, 1980; Galzin *et al.*, 1985) and since affinity for 5-HT receptor sites has not been shown for tramadol (Vaught *et al.*, 1991). The effect is, however, dependent on a functional uptake site since it was almost completely abolished in the presence of 6-nitroquipazine. Summarising the results from uptake and release studies, it appears that tramadol competes with 5-HT for the 5-HT carrier to enter the nerve terminal where it subsequently induces the stimulus-independent release of 5-HT. A similar mechanism has been claimed for the actions of the indirect 5-HT-mimetic, fenfluramine (Fuxe *et al.*, 1975). The release studies confirm the stereoselectivity of the effects of tramadol found in the uptake experiments, and the loss of activity of the metabolite O-desmethyltramadol was also observed. The small inhibition of the stimulation-evoked overflow in the presence of 6-nitroquipazine may be due to enhancement by tramadol of extraneuronal noradrenaline (Driessen *et al.*, 1990) and not to an interaction with 5-HT receptors since it lacks affinity for 5-HT receptor sites (Vaught *et al.*, 1991).

5-Hydroxytryptamine pathways are part of the spinoreticulospinal system which plays an important role in the modulation of pain transmission at the spinal level (Roberts, 1984; Besson & Chaouch, 1987). Intrathecal injection of 5-HT inhibits the activity of dorsal horn neurones and causes antinociception (Yaksh & Wilson, 1979; Schmauss *et al.*, 1983; Solomon & Gebhart, 1988). It has been postulated that enhancement of 5-hydroxytryptaminergic neurotransmission by 5-HT uptake inhibitors may explain the analgesic efficacy of these drugs (Yaksh & Wilson, 1979; Keilstein *et al.*, 1988). The effects of tramadol on 5-HT uptake occur at serum concentrations which are achieved with analgesic doses in the mouse tail-flick test, 0.8 μM at the threshold and 10.6 μM at the maximum effective dose (Friderichs & Becker, 1991). In addition, four fold higher concentrations were observed in the CNS as compared to plasma levels when [^{11}C]-tramadol concentrations were measured by positron emission in men and mice (G. Stoecklin, KFA Jülich, personal communication). Further support for a participation of the 5-hydroxytryptaminergic component of tramadol is derived from the antagonism of the 5-HT$_2$ receptor blocking drug, ritanserin, to the antinociceptive effect of intrathecally injected tramadol in rats (Driessen *et al.*, 1991). These observations provide evidence that reinforcement of 5-HT neurotransmission by tramadol may be of relevance to its *in vivo* antinociceptive effects.

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