Effects of tramadol stereoisomers on norepinephrine efflux and uptake in the rat locus coeruleus measured by real time voltammetry

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Despite its structural similarity to codeine, tramadol is an unusual analgesic whose antinociceptive efficacy is not solely a result of opioid actions but also of its apparent capacity to block monoamine uptake. Tramadol is a mixture of stereoisomers. In this study, we have examined the actions of racemic, (+)- and (−)-tramadol, in addition to O-desmethyltramadol (the main human metabolite), on electrically evoked norepinephrine efflux and uptake in the locus coeruleus brain slice, measured by fast cyclic voltammetry. Racemic tramadol and its (+)- and (−)-enantiomers (all at 5 μmol litre⁻¹) significantly increased stimulated norepinephrine efflux (P<0.01) by mean 66 (SEM 10)%; 57 (7)% and 64 (13)% respectively. However, only (−)-tramadol blocked norepinephrine reuptake (P<0.01), increasing the reuptake half-time to 499 (63)% of pre-drug values. The metabolite O-desmethyl tramadol was inactive at the concentration tested (5 μmol litre⁻¹). In the case of (−)-tramadol, the effect on norepinephrine efflux was directly proportional to, but significantly smaller than, the effect on norepinephrine uptake (P<0.01). This appeared to be a result of compensatory α₂A autoreceptor tone as the selective α₂A autoreceptor antagonist BRL 44408 (1 μmol litre⁻¹) eliminated this difference when its own effects on norepinephrine reuptake were taken into account. The efficacy of (−)-tramadol on norepinephrine uptake, at clinically relevant concentrations, may contribute to its antinociceptive efficacy.

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Tramadol is a unique centrally acting analgesic, clinically effective in the treatment of moderate to moderately severe pain.¹ Compared with opioids, tramadol has low potential for respiratory depression and gastrointestinal side effects² ³ and thus has a role in the management of pain in acute and chronic settings.¹ The weak opioid receptor affinity of tramadol means that the drug has minimal abuse potential.⁴ However, its antinociceptive efficacy suggests that other mechanisms have a contributory role. For example, the analgesic effects of tramadol, unlike those of classical opioids, are not fully reversed by the opioid antagonist naloxone.⁵ Various studies have now indicated that tramadol analgesia is likely to involve monoaminergic pathways, primarily the serotonergic and noradrenergic systems,⁶ both of which have been implicated in descending pain pathways.⁷ This combination of opioid and monoaminergic activity enhances the analgesic profile of tramadol and there is evidence of pharmacological synergism.

The perception of pain is subject not only to modulation during its ascending transmission from the periphery to the cerebral cortex, but also to descending monoaminergic control from higher centres.⁸ In particular, brainstem cell groups, such as the locus coeruleus–sub-coeruleus complex, periaqueductal grey and raphe nuclei play a pivotal role in the descending control of pain.⁷

The involvement of serotonin (5-HT) in the modulation of pain has long been recognized.⁸ We have reported previously that tramadol blocks 5-HT uptake in the rat dorsal raphe nucleus⁹ and it is possible that this action may, in part, help to mediate tramadol-induced analgesia.

The evidence for a noradrenergic component to tramadol-induced analgesia is equally compelling. Certainly, intrathe-
nal norepinephrine produces an analgesic effect in the rat which is blocked by phentolamine and yohimbine, implying that antinociceptive activity is mediated by $\alpha_2$ adrenoceptors. Furthermore, intrathecal administration of desipramine potentiates morphine analgesia and drugs that block norepinephrine uptake or stimulate $\alpha_2$ adrenoceptors (e.g. clonidine) are useful adjuncts to conventional analgesic regimens in addition to being able to produce analgesia in their own right. Tramadol has been shown to block norepinephrine uptake in synaptosomes and to increase norepinephrine efflux in brain slices. This and the fact that $\alpha_2$ adrenoceptor block reduces its antinociceptive efficacy indicates that enhancement of norepinephrine function contributes significantly to its analgesic profile.

The antinociceptive pharmacology of tramadol is complicated further by the fact that the parent drug is a mixture of enantiomers and its principal metabolite O-desmethyltramadol has also been shown not only to be pharmacologically active at opioid sites but to be a more potent analgesic than its parent. Recent work from this laboratory and others has demonstrated that the efficacy of tramadol on the serotonergic systems resides principally with the (+)-enantiomer. However, the (-)-enantiomer also has some, albeit weaker, antinociceptive actions that are synergistic with the (+)-antipode, despite being devoid of effects on the serotonergic systems.

A recent study showed that tramadol and its principal metabolite O-desmethyltramadol inhibited norepinephrine cell firing in the locus coeruleus. Furthermore, inhibition by (-)-tramadol was inhibited by rauwolscine, suggesting involvement of $\alpha_2$ receptors. Therefore, we have examined the actions of tramadol, its enantiomers and principal metabolite on norepinephrine efflux and uptake in the locus coeruleus brain slice.

Materials and methods

The effects of tramadol, its two enantiomers and principal metabolite on electrically stimulated norepinephrine efflux and subsequent reuptake in rat locus coeruleus brain slices were measured by fast cyclic voltammetry at carbon fibre microelectrodes.

Preparation of rat locus coeruleus slices

Male Wistar rats (150±25 g) were stunned and killed by rapid cervical dislocation. The brain was removed quickly and washed with chilled (±1°C) artificial cerebrospinal fluid (ACSF). After initial trimming cuts, 350-μm thick brainstem slices (0.7 to 1.1 mm vs the interaural line) containing the locus coeruleus (seen as two ovoid structures, lateral to the posterodorsal tegmental nucleus and immediately medial to the mesencephalic trigeminal nucleus) were obtained with a vibratome (Campden 752M). The locus coeruleus slice was held in a superfusion-type brain slice chamber by a Nylon mesh over a stainless steel grid. The slice was superfused with oxygenated (95% oxygen–5% carbon dioxide) ACSF at 1 ml min$^{-1}$ throughout the experiment and the temperature of the chamber was maintained at 32°C.

ACSF comprised (mmol litre$^{-1}$) NaCl 124; NaHCO$_3$ 25; (±)-glucose 11; KCl 2; CaCl$_2$ 2; MgSO$_4$ 2; and KH$_2$PO$_4$ 1.25.

Measurement of norepinephrine efflux and reuptake by fast cyclic voltammetry

A standard glass-encased carbon fibre (8 μm diameter, 50 μm length) recording electrode was inserted approximately 80 μm below the surface of the slice between the poles of a bipolar tungsten stimulating electrode (A-M Systems, Seattle, USA) of tip separation 250 μm. The auxiliary (stainless steel) and reference (Ag–AgCl) electrodes were positioned at a convenient location elsewhere in the tissue bath.

An input triangular voltage waveform (1.5 cycles, −1.0 to +1.4 V vs Ag/AgCl, 480 V s$^{-1}$ scan rate) was applied via a Millar voltammetric analyser potentiotstat (PD Systems, West Molesey, UK) twice per second. The output current of the carbon fibre microelectrode was displayed on a digital storage oscilloscope (Hameg 205–3 or Nicolet 310). The output of a sample and hold circuit, set to monitor the current at the oxidation potential for norepinephrine (+600 mV vs Ag/AgCl), was displayed on a chart recorder (Lloyd PL5). The signal was also digitized via a CED 1401 Plus (Cambridge Electronic Design) analogue-to-digital converter at a sampling rate of 10 Hz and stored on an IBM microcomputer using CED ‘Signal’ software.

Electrical stimulation of norepinephrine efflux

The slice was allowed to equilibrate in the chamber for at least 1 h before any stimulation was conducted. Norepinephrine efflux was evoked by trains of 20 pulses (0.1 ms duration, 10 mA, 100 Hz) applied every 10 min. All electrical stimulations were generated with a Neurolog modular system (Digitimer Ltd) and applied via an NL800 stimulus pulse isolator.

Norepinephrine efflux and uptake were recorded on each stimulation. Norepinephrine efflux was taken as the peak extracellular norepinephrine concentration attained on stimulation. On termination of stimulation, norepinephrine was removed from the extracellular space by reuptake. The ‘half-time’ ($T_{1/2}$) of norepinephrine uptake (the time for the extracellular norepinephrine concentration to decrease to 50% of the peak minus initial norepinephrine concentration) was taken as a simple (reciprocal) measure of the rate of norepinephrine uptake.

Experimental procedure

After three consecutive stable norepinephrine efflux events were obtained, the drug (5 μmol litre$^{-1}$) was added to the superfusate for 1 h. Initially, five groups were compared: (±)-tramadol, (+)-tramadol, (-)-tramadol, O-desmethyltramadol and control. Controls received ACSF.
In a second experiment, the effect of addition of the selective α2A antagonist BRL 44408 1 μmol litre\(^{-1}\) on the response to (−)-tramadol 5 μmol litre\(^{-1}\) was investigated. Unless otherwise stated, group sizes were 4–8.

**Drugs**
(±)-Tramadol, (+)-tramadol, (−)-tramadol and O-desmethyl tramadol were gifts from Grünenthal Gmbh (Germany). BRL 44408 (2-[2H-(1-methyl-1,3-dihydroisoindole) methyl]-4,5-dihydroimidazole) was a gift from Smith Kline Beecham (UK). Stock solutions of each drug were prepared in distilled water and all subsequent dilutions were made directly into ACSF.

**Statistical analysis**
All drug effects on norepinephrine efflux and reuptake were plotted against time. Data for (±)-tramadol, (+)-tramadol, (−)-tramadol and O-desmethyl tramadol were tested for statistical significance vs the control group using one-way analysis of variance (ANOVA) with *post hoc* application of Dunnett’s test. Where the effect of addition of BRL 44408 to (−)-tramadol was examined, the two groups were compared using the unpaired *t* test. When the effects of a given drug on norepinephrine uptake were compared with those on norepinephrine efflux, such comparisons were made by paired *t* test.

**Results**
Application of brief trains of electrical stimulation (20 pulses, 0.1 ms duration, 100 Hz) in the locus coeruleus evoked rapid episodes of norepinephrine efflux that were detected at the implanted carbon fibre microelectrode. Norepinephrine efflux was followed by removal of norepinephrine from the extracellular space by norepinephrine uptake. Typical rapid norepinephrine efflux and reuptake profiles after local stimulation in the locus coeruleus are shown in Figure 1. On control stimulations, in the absence of drug treatments (Fig. 1, bottom), peak norepinephrine efflux was mean 50.9 (SEM 4.9) nmol litre\(^{-1}\) (rc=15) and the half-life of reuptake was 2.84 (0.26) s (n=13).

Norepinephrine efflux was constant on successive stimulations in control slices for periods of at least 1.5 h (duration of the present experiments). Mean norepinephrine efflux on the ninth stimulation (t=90 min) was 102.4 (4.5)% (n=7) of the values on the first stimulation. Norepinephrine uptake was similarly constant with half-life values on the ninth stimulation 115.4 (7.9)% of those on the first.

Racemic tramadol and both its (+)- and (−)-enantiomers significantly increased norepinephrine efflux in the locus coeruleus relative to ACSF controls (P<0.01 in all cases, Dunnett’s test). Figure 1 (top) shows a typical response after administration of (−)-tramadol 5 μmol litre\(^{-1}\). The group data in Figure 2 show the effects of tramadol, its two enantiomers and the main metabolite on stimulated norepinephrine efflux. O-desmethyl tramadol had no significant effect compared with control values.

The profile of drug effects on norepinephrine reuptake was simpler. Only (−)-tramadol significantly slowed norepinephrine reuptake, manifested by an increase in the uptake \(T_{1/2}\) (P<0.01 vs control, Dunnett’s test). Figure 1 (bottom) shows a representative trace while Figure 3 shows the group data. O-desmethyl tramadol and the (+)-enantiomer were inactive. Although racemic tramadol appeared to have a modest effect, this was not statistically significant.
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**Fig 3** Effects of tramadol, its enantiomers and metabolite O-desmethyl tramadol on norepinephrine (NE) uptake in the rat locus coeruleus slice. Stimuli were applied 10 min apart. Data are calculated as the half-time of norepinephrine uptake in the period after stimulation and expressed as a percentage of the half-time of norepinephrine uptake in the pre-drug period. All values are mean (SEM) (n=5–7). **P<0.01 vs control (Dunnett’s test). The arrow indicates addition of drug.

**Fig 4** Relationship between the effects of the tramadol stereoisomers on norepinephrine (NE) efflux and uptake. ‘Scattergram’ of the individual recordings showing peak norepinephrine efflux vs half-time of norepinephrine reuptake in slices treated with (+)- or (-)-tramadol 5 μmol litre⁻¹. The effects of (-)-tramadol on norepinephrine efflux and reuptake were significantly correlated (P<0.01) (unbroken) while those of (+)-tramadol were not (broken line).

To distinguish between the effects of the (+)- and (-)-isomers on norepinephrine efflux and uptake, the two variables were plotted against each other (Fig. 4). While both isomers had similar effects on norepinephrine efflux, only the (-)-enantiomer blocked uptake. The effect of (-)-tramadol was significantly greater on norepinephrine uptake than on efflux (P<0.01, paired t test). There was nevertheless a significant linear relationship between the effects of (-)-tramadol on norepinephrine efflux and uptake (P<0.01).

Conversely, the effects of (+)-tramadol were not significantly greater on norepinephrine efflux than on uptake and there was no significant relationship between the measures.

To establish if the smaller effect of (-)-tramadol on norepinephrine efflux compared with uptake was caused by autoreceptor feedback, the effects of (-)-tramadol on norepinephrine efflux and uptake were examined alone and in the presence of the selective α₂A adrenoceptor antagonist BRL 44408 1 μmol litre⁻¹. BRL 44408 did not potentiate the effects of (-)-tramadol on norepinephrine efflux (Fig. 5A) but appeared to attenuate the effect of (-)-tramadol on uptake (Fig. 5B), reducing T½ from 438 (47)% in the (-)-tramadol alone group to 239 (32)% of pre-drug values in the group receiving both drugs (P<0.05). However, when the effect on norepinephrine efflux was expressed as a percentage of its effect on norepinephrine uptake (Fig. 5C), there was significantly greater norepinephrine efflux in the slices treated with both drugs (73.2 (9.9)% vs 35.3 (5.3)%; P < 0.01).

**Discussion**

The rat locus coeruleus is usually considered to be the main noradrenergic nucleus in the descending control of pain, sending major projections to the spinal cord. Noxious stimuli have been shown to increase the activity of locus coeruleus cells, as evidenced by an increase in norepinephrine metabolites in the locus coeruleus. Stimulation of the locus coeruleus–sub-coeruleus complex leads to antinociception, as measured by the hot plate test and inhibition of heat-evoked dorsal horn activity. Stimulation of the locus coeruleus has also been shown to cause an increase in norepinephrine metabolites within the spinal cord.

We have shown previously that norepinephrine efflux and uptake may be readily measured in rat locus coeruleus slices and that efflux and cell firing are subject to modulation by α₂ adrenoceptor agonists and morphine. Recent data from this laboratory have shown that α₂ adrenoceptor modulation of locus coeruleus norepinephrine efflux is mediated via the α₂A subtype.

The aim of our study was to examine the effects of tramadol, its enantiomers and primary metabolite on norepinephrine efflux in the locus coeruleus at clinically appropriate concentrations. Preliminary experiments (not shown) were performed to establish the concentration–response relationship and to determine appropriate concentrations. It was decided to give the drugs at a concentration of 5 μmol litre⁻¹, which approximates to the plasma concentration of tramadol after a therapeutically effective dose.

As with the dorsal raphe nucleus, we found that O-desmethyltramadol was inactive: the metabolite did not affect norepinephrine efflux or uptake. This apparent lack of monoaminergic activity at both the locus coeruleus and dorsal raphe is of interest, as O-desmethyltramadol is an effective analgesic and probably contributes significantly.
via μ opioid receptors to the overall analgesic profile of tramadol. Clearly, although O-desmethyltramadol is a more potent opioid than its parent drug,\textsuperscript{16} such opioid mechanisms do not affect norepinephrine release in this experimental paradigm. This is consistent with our previous report\textsuperscript{26} where we showed that the capacity of morphine to decrease locus coeruleus norepinephrine efflux was modest and mediated significantly by non-opioid mechanisms.

The effects of tramadol on electrically stimulated norepinephrine efflux (Fig. 2) clearly demonstrated that both enantiomers and racemic tramadol produced an equal effect. However, with regard to norepinephrine uptake, only the (-)-tramadol prolonged the uptake $T_{1/2}$ (Fig. 3). This is consistent with previous work using purified rat hypothalamic synaptosomes where (-)-tramadol was shown to be 10 times more potent than (+)-tramadol in blocking norepinephrine uptake.\textsuperscript{12} It also mirrors our previous report on the rat dorsal raphe where we showed that the (+)-isomer was the active form on 5-HT uptake.\textsuperscript{9}

It is worth noting that the action of racemic tramadol on norepinephrine uptake was smaller than might be expected, considering that 5 μmol litre$^{-1}$ of the racemate contains 2.5 μmol litre$^{-1}$ of (-)-tramadol. One would expect the racemate to exert an effect half that of (-)-tramadol and, this effect is puzzling. One possible explanation is that (+)-tramadol, being a serotonin uptake blocker, enhances inhibitory serotonergic tone on norepinephrine efflux. Such a possibility clearly requires further study and is the subject of ongoing investigation in our laboratory.

The effect of the (+)-isomer on norepinephrine efflux is initially difficult to explain. We can nevertheless exclude the possibility that there is ‘contamination’ of the norepinephrine signal by released 5-HT as if this were the case (+)-tramadol would also affect monoamine reuptake. Thus the monoamine signal is, as reported previously in the locus coeruleus, wholly norepinephrine.\textsuperscript{24} As (+)-tramadol does not affect norepinephrine uptake, the increase in norepinephrine efflux cannot be a result of such a mechanism. This is confirmed further by the data in Figure 4 which show that there is no relation between the effect of (+)-tramadol on norepinephrine efflux and uptake. More likely, the effect is mediated indirectly via modification of 5-HT efflux. There is a significant serotonergic projection to the locus coeruleus from the dorsal raphe\textsuperscript{21} and we have observed that drugs acting on 5-HT uptake (such as (+)-tramadol) have clear effects on locus coeruleus norepinephrine release \textit{in vitro} (Callado and Stamford, unpublished data).

From Figure 4, it is clear that the effect of (-)-tramadol on norepinephrine efflux is directly proportional to its effect on norepinephrine reuptake, suggesting that the two are related phenomena. This is consistent with previous reports where it has been shown that (-)-tramadol increases stimulated but not basal norepinephrine efflux in spinal slices\textsuperscript{13} and hypothalamic synaptosomes,\textsuperscript{12} suggesting that (-)-tramadol, unlike its (+)-antipode,\textsuperscript{9} is devoid of direct monoamine-releasing effects.

However, although proportional to its effect on norepinephrine reuptake, the effect of (-)-tramadol on norepinephrine efflux is of a much smaller magnitude. This might suggest that there is a counteracting autoinhibitory tone at $\alpha_2$ receptors. A similar phenomenon has been observed in the bed nucleus of stria terminalis where we have shown previously that block of $\alpha_2$ adrenoceptors with rauwolscine relieves this compensatory tone and increases the effect of desipramine on norepinephrine efflux.\textsuperscript{29}

We tested this possibility with the selective $\alpha_{2A}$ antagonist BRL 44408\textsuperscript{30} in combination with the (-)-isomer. With (-)-tramadol, initially there was no potentiation of efflux in our series of experiments, suggesting that compensatory $\alpha_2$ autoreceptor tone did not account for the smaller effect of (-)-tramadol on norepinephrine efflux than on reuptake.
This was surprising as we have found previously that the effect of desipramine is clearly potentiated by BRL 44408 in the locus coeruleus. However, BRL 44408 appeared to cause a significant reduction of the effect of (-)-tramadol on norepinephrine reuptake (P<0.05). When this action is taken into account and the effect on norepinephrine efflux is expressed as a percentage of the comparable reuptake values, there was twice as much norepinephrine efflux in those slices also treated with BRL 44408 (73.2 (9.9)% vs 35.3 (5.3)%; P<0.01). The effect of BRL 44408 on norepinephrine reuptake needs further investigation.

In summary, we have evidence that tramadol, at clinically relevant concentrations, acts as a norepinephrine reuptake blocker in the locus coeruleus. These effects are wholly a result of the (-)-isomer. (+)-Tramadol increases norepinephrine reuptake in the locus coeruleus. These effects are wholly relevant concentrations, acts as a norepinephrine reuptake or reuptake. These findings and those of our previous study reuptake, it is not surprising that tramadol shows antidepressant-type actions in mice and it would be interesting to see, as suggested recently, if this profile is manifested in humans.

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