μ-Opioid receptor activation and noradrenaline transport inhibition by tapentadol in rat single locus coeruleus neurons.

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<td>Complete List of Authors:</td>
<td>Sadeghi, Mahsa; University of Sydney, Pharmacology Tzschentke, Thomas; Grünenthal GmbH, Department of Pain Pharmacology Christie (Guest), MacDonald; The University of Sydney,</td>
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Dear Editor,

Thank you for the reviews of the paper "µ-Opioid receptor activation and noradrenaline transport inhibition by tapentadol in rat single locus coeruleus neurons." We have revised the manuscript according to the useful comments of the reviewers as outlined below. We hope the paper is now suitable for publication.

Reviewer: 1

1. No response needed

2. It would be helpful if the description of the method used to construct the concentration response curves illustrated in Figure 1 were expanded a little. Were these done with cumulative applications of agonists or were they done as illustrated in parts A&B as a single concentration/slice followed by application of naloxone. The single concentration/slice would surely result in more reliable measures.

For met-enkephalin, 2-3 concentrations per slice were applied because we were less concerned about the potency and maximal responses in LC which have been determined and published many times, i.e. it’s a full agonist versus UK14304 with potency similar to previous studies. For the direct comparisons of partial agonists we considered it very important to not confound maxima or potency of morphine or tapentadol by any possibility of desensitization so only applied a single concentration per slice. Similarly in the experiment without MOPr or alpha2 receptors blocked we applied single concentrations. These are now stated explicitly in the relevant parts of the results section.

3. The only potentially interesting addition would be to examine the inhibition of reuptake induced by tapentadol on electrically evoked noradrenergic IPSC. This experiment might be a more physiological assessment of an otherwise very complete pharmacological assay. Although this experiment would be of interest, it does not detract from the quality of the work and presentation of the results and should not delay the publication of this work.

This is an interesting question but it turns out that the effects of NET inhibition on alpha2 receptor-mediated slow IPSCs in LC are complicated. Originally Surprenant and Williams (JPhysiol 382, 87-103,1986) showed with sharp electrodes that NET inhibitors including cocaine and desipramine prolong the IPSP in LC slices. We have done several experiments with both cocaine and tapentadol and are finding much more complex results on the IPSC in whole cell mode. We find in some cells and increase in amplitude with little effect on duration and a large effect only on
duration in others. In yet other cells, particularly at high concentrations, the IPSC is suppressed. We think the latter occurs because of inhibition of excitability of LC neurons in the slice by higher NA tone. It will take a lot of work, probably 3-4 months to complete this study. We would much prefer to more fully understand these phenomena and publish them separately than to include preliminary data here or, alternatively, greatly delay publication of this study.

**Reviewer: 2**

“... the major issue with the paper in the current form is that insufficient justification is provided for using the LC slice model system in view of the fact that the analgesic action of tapentadol and the interaction between its two mechanisms of action appears to be predominantly mediated at the spinal level – as reviewed by the authors.... The findings thus would be appreciated by a broader audience if they were put into the context of the LC’s role in the nociceptive system.

We have emphasized that we have used LC because it is a very good model for MOPr, alpha2 receptor and NAT actions but appreciate some comment on the role of LC activity in pain. This is not clear in the literature. Electrical stimulation of the LC produces antinociception but opioids microinjected into LC are analgesic. The role of the nucleus itself in pain transmission and modulation are therefore complex and this could reflect the role of LC in multiple functions with multiple CNS targets. We have already discussed the effects in spinal cord in the discussion. We have now included a brief paragraph to discuss the role of LC activity in nociception and antinociception near the end of the Discussion.

“... unique receptor profile of the LC ...”.

We think it wrong of the reviewer to suggest that there is a “unique receptor profile of the LC compared with other systems”. The only other systems studied with tapentadol in any pharmacological detail (relative efficacies) have been heterologous expression systems that have very unnatural, excessive levels of MOPr expression. Where natural expression and efficacy has been examined in detail in other neurons, particularly in relation to morphine and tolerance, the results have been comparable with LC, suggesting that the MOPr tissue efficacy in LC is not unique. We have revised the last sentence at the top of page 15 to emphasise this point for readers not familiar with the issue.

**Minor issues:**

1. **Typos in second paragraph of Methods/Preparation of brain Slices (p6)**
   Corrected.

2. **The number of neurons tested for the interaction between MOPr and NAT mechanism is not provided (p12)**
   N = 5 per data point now included on p13.

3. **Typo in last paragraph p15**
   Corrected.
μ-Opioid receptor activation and noradrenaline transport inhibition by tapentadol in rat single locus coeruleus neurons.

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Running title: Tapentadol actions on brain neurons
SUMMARY

Background and purpose.
Tapentadol is a novel analgesic that combines moderate µ-opioid receptor (MOPr) agonism and noradrenaline reuptake inhibition (NRI) in a single molecule. Both mechanisms of action are involved in producing analgesia, however the potency and efficacy of tapentadol in individual neurons has not been characterized.

Experimental approach.
Whole-cell patch clamp recordings of G-protein-coupled inwardly rectifying K (GIRK) currents were made from rat locus coeruleus neurons in brain slices to investigate the potency and relative efficacy of tapentadol, and compare its intrinsic activity with other clinically used opioids.

Key results.
Tapentadol showed agonist activity at MOPr and was approximately 6 times less potent than morphine with respect to GIRK current modulation. The intrinsic activity of tapentadol was lower than met-enkephalin, morphine and oxycodone, but higher than buprenorphine and pentazocine. Tapentadol inhibited the noradrenaline transporter (NAT) with potency similar to that at the MOPr. The interaction between these two mechanisms of action was additive in individual LC neurons.

Conclusions and Implications.
Tapentadol displays similar potency for both MOPr and NAT inhibition in functioning neurons. The intrinsic activity of tapentadol at the MOPr lies between that of buprenorphine and oxycodone, potentially explaining the favourable MOPr-related side effect profile.
Keywords.

Tapentadol, Noradrenalin reuptake inhibitor, Opioid receptor agonist, Locus coeruleus, GIRK channels

Abbreviations:

ACSF, artificial cerebrospinal fluid; GIRK, G-protein-coupled, inwardly rectifying K channel; GTP$_{\gamma}$S, guanosine 5-3-O-(thio)triphosphate; LC, locus coeruleus; ME, [Met]5enkephalin; NA, noradrenaline; NAT, noradrenaline reuptake transporter; MOPr, µ-opioid receptor.
INTRODUCTION

Opioid analgesics are the mainstay of drug therapy for management of acute and chronic severe pain but their utility is limited by on-target side effects, as well as development of tolerance and dependence (Christie, 2008; Williams et al., 2013). One approach to limit side effects, tolerance and dependence clinically is to combine a MOPr agonist and another analgesic drugs with a different mode of action, which can enhance overall analgesic actions and produce opioid sparing effects. (Schroder et al., 2011; Tzschentke et al., 2007)

Tapentadol is a centrally acting analgesic that combines MOPr agonism with a similar potency for noradrenaline transporter (NAT) inhibition in a single molecule (Tzschentke et al., 2007). The rationale behind this particular combination effect is based on findings that both opioid and noradrenergic pathways contribute to production of analgesia in different ways, and can synergistically interact at spinal and supraspinal levels (Fairbanks and Wilcox, 1999; Ossipov et al., 1982). Although tapentadol exhibits approximately 50-fold lower binding affinity for MOPr than morphine, its analgesic potency is comparable to morphine for acute and chronic pain in animal models (Schiene et al., 2011; Tzschentke et al., 2007), and it is well documented that tapentadol has a favorable tolerability in humans, particularly with respect to gastrointestinal side effects (Afilalo et al., 2010; Hartrick et al., 2009; Lange et al., 2010; Pergolizzi et al., 2012; Tzschentke et al., 2009).

The mechanisms of action of tapentadol have been studied in in vitro models (Tzschentke et al., 2007), as well as in vivo neurochemical and behavioral studies in animals (Bee et al., 2011; Schiene et al., 2011; Schroder et al., 2011; Schroder et al.,
2010; Tzschentke et al., 2012) but little is known about the mechanism of action in individual neurons. The locus coeruleus (LC) is the major source of noradrenergic innervation of the brain and spinal cord (Foote et al., 1983). LC neurons express both MOPr and α2-adrenoceptors that converge via G-protein βγ-subunits to activate a single population of inwardly rectifying K (GIRK) channels (North and Williams, 1985). The LC also expresses a high density of NAT (Schroeter et al., 2000). As such, the LC is also a useful model to study inhibition of NAT by assaying enhancement of actions of α2-adrenoceptors on GIRK channels (Surprenant and Williams, 1987).

A previous study of electrical activity of LC neurons in vivo (Torres-Sanchez et al., 2013) reported that systemic administration of tapentadol inhibits spontaneous action potential activity. The authors suggested that the actions of tapentadol on these neurons is mediated predominantly by activation of α2-adrenoceptors but the direct membrane actions and relative potencies for MOPr and NAT could not be determined using this experimental approach. The aim of the present study was to investigate the relative potency and efficacy of tapentadol in functioning LC neurons using patch clamp recording in brain slices. The results establish that tapentadol is an agonist at MOPr with lower intrinsic activity than morphine and oxycodone, but greater than that of buprenorphine. Tapentadol has similar potency in single LC neurons for MOPr and NAT inhibition. At the cellular level, the interaction between these mechanisms on activation of GIRK channels is additive.

METHODS

Preparation of brain slices
Male Sprague–Dawley rats (3 to 5 weeks-old, n = 114) were used in this study. Animals were housed in groups of two to four under a 12 h/12h light–dark cycle at 22 ± 2°C with environmental enrichment and free access to food and water. The Animals Ethics Committee of the University of Sydney, Sydney, NSW, Australia, which complies with the National Health and Medical Research Council ‘Australian code of practice for the care and use of animals for scientific purposes’, approved all experiments.

LC slices were prepared as described previously (Dang et al., 2011; Dang et al., 2012). Briefly, rats were anesthetized with isoflurane (4% in air), decapitated then brains were rapidly removed and blocked. Horizontal brain slices (260-300 µm thick) containing LC were obtain from pons on a vibratome (Leica, VT1000) in cooled artificial cerebrospinal fluid (ACSF) containing the following (in mM): 126 NaCl, 2.5 KCl, 2.4 CaCl₂, 1.2 MgCl₂, 1.2 NaH₂PO₄, 21.4 NaHCO₃, 11.1 glucose, 95%O₂-5%CO₂. Slices were incubated in warm (37°C) oxygenated ACSF for at least 30 min before recording.

**Electrophysiological Recordings**

Whole-cell voltage-clamp recordings of visualized LC neurons (infrared Dopt optics, Multiclamp 700B amplifier from Molecular Devices, CA, USA) were acquired at holding potential of -60 mV using Axograph X (Axograph Scientific, Australia) as previously described (Dang et al., 2012). Data were sampled at 10 kHz and filtered at 20-50 Hz. Series resistance (≤ 15 MΩ) was compensated by 75% and monitored throughout experiments. Patch pipettes with resistance of 2–4 MΩ were filled with a solution containing the following (in mM): 115 K MES (2-[morpholino]- ethane-
sulfonic acid), 20 NaCl, 1.5 MgCl₂, 10 BAPTA, 5 HEPES, 4 Mg₂ ATP and 0.4 Na-GTP and pH 7.3–7.4 was adjusted using KOH. All drugs were applied by superfusion of the recording chamber (~2 ml/min, 35°C). Bestatin (10 µM) and thiorphan (1 µM) were included in all experiments using met-enkephalin to limit degradation of the peptide. In all experiments involving application of NA, prazosin (1 µM) was included for at least 5 min before superfusing NA to prevent potential effects of α₁-adrenoceptor activation on GIRK channels in these neurons (Osborne et al., 2002). Solutions of NA were freshly prepared when reapplication time exceeded 5 min to avoid oxidative decomposition. A single concentration of tapentadol was applied per slice.

**Data analyses**

Data were analyzed using Graph-Pad Prism (Graph-Pad V5), except for isobolographic analysis. All data are presented as mean ± SEM. Significant differences were analyzed by Student’s t-test (paired or unpaired two-tailed Student’s t tests) or, where appropriate, one way ANOVA followed by Dunnett’s test, or two way ANOVA followed by Bonferroni post-test. Concentration-response curves were calculated for each group and fitted to a logistic equation using Graph-Pad Prism, with minima constrained to zero. Isobolographic analysis was performed using a modification of the method of (Tallarida, 2007). By this method, additivity is based on the concept of concentration equivalence for drug A and B (or dual mechanisms) follows from individual concentration-effect curves (Grabovsky and Tallarida, 2004), i.e. a concentration α of drug A is equal to a concentration of drug B denoted b_{eq}(α). So combinations of these two concentrations (a,b) would be b_{eq}(α) + b. The theoretical additive EC₅₀ value was compared with the observed EC₅₀ for the
combination by t-test using FlashCalc 4.5.3 (Dr Michael Ossipov, University of Arizona, Tucson AZ).

**Drugs:**

Stock solutions of tapentadol HCl supplied by Grünenthal GmbH (Germany), [Met\(^5\)]-enkephalin (Sigma-Aldrich, Australia), bestatin (Sigma), morphine HCl (GSK Australia), idazoxan HCl (Tocris, UK), naloxone HCl (Sigma-Aldrich, Australia), noradrenaline (NA; Sigma-Aldrich, Australia), cocaine (GlaxoSmithKline, Australia) and oxycodone HCl (National Institute on Drug Abuse Drug Supply Program, USA), were dissolved in distilled water. Other drugs included UK 14,304 (Tocris, UK), prazosin HCl (Sigma-Aldrich, Australia), dissolved in 20% and 10% dimethyl sulfoxide (DMSO) respectively. Thiorphan (Sigma) was dissolved in 50% ethanol. (+)-Pentazocine from Research Biochemical (Natick, MA, USA) was dissolved in distilled water with 1-2 drops (almost 50 µL) of glacial acetic acid. Buprenorphine (National Institute on Drug Abuse Drug Supply Program, USA) was dissolved in distilled water using HCl (1M, 1-2 drops). The maximum concentration of DMSO used in the superfusion solution was < 0.01% and for ethanol was < 0.0001%.

**RESULTS**

Relative potency and efficacy of tapentadol at µ-opioid receptors.

To determine the relative potencies and efficacies of tapentadol and several other opioids at MOPr, whole-cell patch-clamp of GIRK currents were recorded at a \( V_H \) of -60 mV in the presence of idazoxan (1 µM) and prazosin (300 nM) to block any action of increased noradrenergic tone that might be caused by tapentadol’s NAT inhibition.
Concentration-response curves for met-enkephalin were constructed using 2-3 concentrations per cell. For morphine and tapentadol concentration-response curves, a single concentration of tapentadol or morphine was applied per slice to minimise any possibility that desensitization would confound potency or maximal responses. As shown in Figure 1A, a supramaximal concentration of tapentadol (30 µM) produced an outward current that was consistently smaller than the current produced by a supramaximal concentration of the full α2-adrenoceptor agonist UK14304 (3 µM), in the same cells (Table 1). Naloxone (1 µM) fully reversed the effects of tapentadol (n = 12).

As expected from previous studies (Bailey et al., 2009; Dang and Williams, 2005; Osborne et al., 2000; Virk and Williams, 2008), the effect of morphine was lower than the maximal response produced by met-enkephalin in LC neurons (Figure 1B, Table 1), ie. morphine acted as a partial agonist for activation of GIRK in rat LC neurons using whole-cell recording in brain slices. The maximum response to tapentadol was smaller than that produced by morphine (Table 1) suggesting that tapentadol has lower intrinsic efficacy than morphine at the MOPr.

Met-enkephalin was a potent and efficacious agonist in LC (Figure 1D, Table 1), with a response amplitude similar to that produced by UK14304 (3 µM). The maximum responses relative to UK14304 (3 µM) for both morphine (30 µM) and tapentadol (30 µM) were considerably smaller than that produced by met-enkephalin (Table 1). As summarized in Table 1, tapentadol is approximately 6-fold less potent than morphine. The EC50 for morphine was similar to that previously reported in LC neurons using patch-clamp recordings (Osborne et al., 2000).
Intrinsic activity of tapentadol relative to other MOPr agonists using partial antagonism

If tapentadol does indeed have lower intrinsic agonist activity than met-enkephalin and morphine, it should partially antagonise both agonists. As shown in Figure 2, both morphine (Figure 2A) and tapentadol (Figure 2B) partially reduced the actions of a supramaximal concentration of met-enkephalin. Moreover, tapentadol partially reduced the effect of a supramaximal concentration of morphine (Figure 2C).

The relative intrinsic activity of tapentadol was further compared with several other clinically used MOPr-agonists including oxycodone, buprenorphine and pentazocine. Tapentadol partially reduced the effect of a supramaximal concentration of oxycodone (Figure 2D). Because buprenorphine and pentazocine are weak partial agonists at MOPr in LC neurons (Virk et al., 2009), producing very small outward currents, they were both tested as partial antagonists, with tapentadol applied first. Buprenorphine (Figure 2E) and pentazocine (Figure 2F) both antagonised the actions of a supramaximal concentration of tapentadol, suggesting that tapentadol has greater intrinsic activity than these two partial agonists.

To test the possibility that tapentadol might inhibit GIRK in LC neurons independently of its action on MOPr (as does methadone; (Rodriguez-Martin et al., 2008)), slices were superfused with naloxone (1 µM), prior to and during application of tapentadol in the presence of a supramaximal concentration of UK14304 (Figure 2G) to fully activate GIRK currents via α2-adrenoceptors. Prazosin (300 nM) was included to avoid potential confounds from activation of α1-adrenoceptors (Osborne et
Application of a saturating concentration of UK14304 (3µM) induced a slowly desensitizing outward current, as previously reported (Dang et al., 2012) but tapentadol had no apparent effect on the current (Figure 2G). To account for the decline in the UK14304-induced current due to desensitisation, the slope of the declining current was fitted with a linear function prior to application of tapentadol and the increased slope produced by tapentadol was calculated. Superfusion of tapentadol in the presence of UK14304 did not have any effect on the steady decay of the outward current (t = 0.054, P > 0.96 paired t-test, df = 6). These results establish that partial antagonism of met-enkephalin, morphine and oxycodone by tapentadol is due to its lower intrinsic activity rather than direct block of GIRK channels.

The actions of tapentadol at the MOPr are summarised in Table 2. The results suggest a relative order of intrinsic activity of Met-enkephalin > morphine ≈ oxycodone > tapentadol > buprenorphine ≈ pentazocine.

**Tapentadol enhances the action of exogenously applied noradrenaline in LC neurons.**

The potency of tapentadol to inhibit NAT and thereby potentiate the actions of NA on LC neurons was studied by blocking MOPr, then superfusing a low concentration of NA with tapentadol as previously established for the actions of cocaine in LC neurons (Surprenant and Williams, 1987). In the presence of naloxone (1 µM) and prazosin (300 nM), without addition of NA, tapentadol (100 µM) produced little or no outward current (< 5 pA, n = 3, data not shown), suggesting little basal noradrenergic tone in slices under the present recording conditions. However, as shown in Figure 3, tapentadol potentiated α2-adrenoceptor responses when NA was exogenously applied.
Responses to NA (3 or 10 µM) were variable from cell to cell and slice to slice, presumably due to variation in the diffusion path through slices to relevant α2-adrenoceptors, which produced a large variation in subsequent NAT inhibition. Indeed, there appeared to be an inverse correlation between the amplitude of response to NA and extent of potentiation produced by tapentadol (not shown). To control for these differences, the extent of potentiation produced by different concentrations of tapentadol was determined only if the initial response to 3 or 10 µM NA was between 5% (less than 5% was considered too small to calculate a reliable percentage of enhancement) and 20% of the maximum response produced by met-enkephalin. Tapentadol produced a concentration-dependent enhancement of NA-induced GIRK currents in the concentration range of 0.1-30 µM. In the presence of high concentrations of tapentadol (30 - 100 µM), no further potentiation of the response to NA could be achieved by further addition of a supramaximal concentration of cocaine (10 µM; (Surprenant and Williams, 1987) (101 ± 1% of tapentadol response, n = 3)), indicating that NAT inhibition was maximal at these concentrations. All subsequent data were normalized to the enhancement of outward current produced by a supramaximal concentration of cocaine (10 µM). The concentration-response curve for tapentadol is presented in Figure 4. The IC$_{50}$ for tapentadol at NAT in LC slices was 2.3 µM (pIC$_{50}$ = 5.6 ± 0.1).

**Interaction between the two mechanisms of action of tapentadol in single LC neurons**

To test how the combined actions of tapentadol on MOPr and α2-adrenoceptors, via NAT inhibition, interact with respect to GIRK currents, tapentadol was applied to LC neurons in the presence of a low concentration of NA (3 µM) without addition of
antagonists of either receptor. To construct the concentration-response curve, a single concentration of tapentadol was applied per slice. Figure 4A shows the enhancement of the effect of NA (3 µM) under these conditions. Data were normalized to the maximum GIRK current induced by saturation concentration of UK 14303 (3 µM) after subtraction of the small current induced by NA (3 µM). As shown in Figure 4B, the potency of tapentadol acting on both MOPr and NAT (EC$_{50}$ = 2.6 µM, pEC$_{50}$ = 5.6 ± 0.1, n = 5 for each concentration of tapentadol) is not significantly different from the potency of tapentadol as a NAT inhibitor (EC$_{50}$ = 2.3 µM, P> 0.05, two-way ANOVA, Bonferroni post-test). As confirmed by isobolographic analysis (Grabovsky and Tallarida, 2004; Tallarida, 2006), the interaction between these two mechanisms of action on a single neuron level was additive (P> 0.15), since the theoretical additive EC$_{50}$ for independent actions on MOPr and NET (3.8 ± 1.2 µM) was not significantly different from EC$_{50}$ for tapentadol with its combined mechanism (2.53 ± 1.4 µM).

**DISCUSSION**

The present study demonstrates that tapentadol is a MOPr agonist and NAT inhibitor in single LC neurons in brain slices. Tapentadol exhibited 6-fold lower potency than morphine for activation of GIRK currents in LC neurons. A previous study (Tzschtentke et al., 2007), reported that the binding affinity of tapentadol for rat MOPr was more than 40-fold lower than that of morphine ($K_i$ = 96 nM for tapentadol versus $K_i$ = 2.2 nM for morphine). Similarly, in a human recombinant MOPr [$^{35}$S]GTP$_\gamma$S assay tapentadol (EC$_{50}$ = 670 nM) exhibited approximately 30-fold lower potency than morphine (EC$_{50}$ = 22 nM; (Tzschtentke et al., 2007)). From this, tapentadol would have been expected to exhibit substantially lower potency than morphine in LC
neurons. A likely explanation for the smaller differences between the agonist potencies of tapentadol and morphine observed here compared with the previous study is that MOP receptor reserve is substantially lower in LC neurons than the heterologous expression system used previously (Tzschentke et al., 2007). This interpretation is consistent with the overall potency difference for morphine (14-fold) between the two assay systems. The differences in relative intrinsic activities are also consistent with a large difference in receptor reserve. The maximal effect of tapentadol was 88% of that of morphine in the previous study (Tzschentke et al., 2007) but less than 50% in the LC. Agonists with relatively high intrinsic efficacy (morphine) are predicted to exhibit greater reduction in EC$_{50}$ than lower intrinsic efficacy agonists when receptor reserve increases (Strange, 2008). It is therefore likely that the relative potency and intrinsic activity differences between morphine and tapentadol reported here more closely reflect differences in MOPr interaction in brain neurons than in previous studies in heterologous expression systems, as discussed elsewhere (Law et al., 2000; Morgan and Christie, 2011).

Tapentadol exhibited a relative intrinsic activity at MOPr with respect to activation of GIRK currents that was less than met-enkephalin, morphine and oxycodone but greater than the weak partial agonists buprenorphine and pentazocine. This was confirmed, where possible, both by examining the relative maximal response to each agonist and partial antagonism. Furthermore, the present study confirmed that measurement of relative intrinsic activity of tapentadol was not confounded by direct block of GIRK channels, as was observed for other opioids such as methadone (Rodriguez-Martín et al., 2008).
Many studies have sought to separate the analgesic effect of MOPr activation from their side effects. It is well established that the MOPr mediates both analgesic activity and adverse effects of MOPr agonists (Matthes et al., 1996). Agonists with a relatively low intrinsic activity at MOPr could overcome some of the limitations of very high intrinsic efficacy MOPr agonists in terms of therapeutic window and side effects. Indeed buprenorphine is a very low intrinsic efficacy MOPr agonist, but produces strong analgesia, and shows an improved tolerability profile compared with morphine (Cowan et al., 1977; Jasinski et al., 1978). In opioid tolerant individuals, buprenorphine also shows a greatly reduced overdose liability (Fatseas and Auriacombe, 2007). It is tempting to speculate that favorable tolerability of tapentadol in humans, particularly with respect to gastrointestinal side effects (Afilalo et al., 2010; Hartrick et al., 2009; Lange et al., 2010; Pergolizzi et al., 2012; Tzschtke et al., 2009), could in part be due to its relative intrinsic activity at MOPr which is intermediate between morphine and buprenorphine.

The present study has also established that tapentadol enhances the outward current induced by application of NA in LC neurons by effectively increasing the concentration of NA reaching postsynaptic α2-adrenoceptors, as do other NAT inhibitors (Surprenant and Williams, 1987). Tapentadol exhibited potency for NAT inhibition similar to that for activation of the MOPr.

The finding that the interaction between the two mechanisms of action of tapentadol is additive with respect to activation of GIRK currents in LC neurons is not surprising because both mechanisms are known to converge via a common transduction system through G-protein βγ-subunits to activate a single population of GIRK channels.
(North and Williams, 1985; Stone and Wilcox, 2004). However, previous studies have demonstrated the interaction between the two mechanism of action of tapentadol in vivo, which is predominantly mediated at the spinal level, is synergistic rather than additive (Schroder et al., 2011). Possible explanations for the synergistic interaction of tapentadol acting on both MOPr and NAT in vivo could be due to receptor activation at multiple sites along the physiological pain-modulatory pathways rather than being mediated at the single cell level (Stone and Wilcox, 2004). The synergistic interaction observed in vivo could include a network effect activation of the descending inhibitory pathways at supraspinal level via MOPr activation together with potentiation of this effect via NAT inhibition at the spinal level, as well as inhibition of nociceptive transmission between primary afferent and spinal second order neurons via pre- and postsynaptic MOPr- and α2-adrenoceptors.

Although the LC is useful to study action of MOPr pharmacology and NAT inhibition in neurons, the influence of neuronal activity in the nucleus in pain modulation is not fully understood. Electrical or chemical stimulation of LC produces analgesia in acute and inflammatory pain states (Jones, 1991; Jones and Gebhart, 1986; West et al., 1993) as would be expected from enhancement of spinal NA release. However, direct inhibition of LC activity by microinjection of MOPr agonists in vivo is also antinociceptive (Jongeling et al., 2009). The predicted overall effect of suppression of LC neuronal activity by tapentadol on pain modulation in vivo is therefore uncertain.

In conclusion, the present study has established that tapentadol has almost the same potency for NAT inhibition and MOPr activation in single functioning neurons. Its
reduced intrinsic activity at MOPr compared with morphine, together with its inhibition of NET may both contribute to improved tolerability profile.

**Acknowledgements:**

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REFERENCES


Table 1. Membrane actions, MOPr agonism and potency of tapentadol and other opioids in LC neurons

<table>
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<tr>
<th></th>
<th>$I_{\text{max}}$ (pA)</th>
<th>$I_{\text{max}}/I_{\text{UK14304}}$ %</th>
<th>pEC$_{50}$ (-log[µM])$^a$</th>
<th>EC$_{50}$ (µM)</th>
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<tr>
<td>tapentadol</td>
<td>91 ± 15</td>
<td>29 ± 4 $^\dagger$</td>
<td>5.8 ± 0.2</td>
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<td>(n = 12)</td>
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<tr>
<td>morphine</td>
<td>151 ± 25</td>
<td>59 ± 4 $^*$</td>
<td>6.5 ± 0.3</td>
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<td>(n = 8)</td>
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<tr>
<td>met-enkephalin</td>
<td>222 ± 13</td>
<td>126 ± 7</td>
<td>6.9 ± 0.1</td>
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<tr>
<td>(n = 5)</td>
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Data are expressed as mean mean ± s.e.m.

$^a$ – s.e.m. of fitted curve

* - significantly different from met-enkephalin (unpaired t-tests, all P < 0.001)

$^\dagger$ - significantly different from morphine (unpaired t-test, all P < 0.001)

- Data are expressed as mean mean ± s.e.m, and the number of experiments is shown in parentheses.
Table 2. Relative intrinsic activity of tapentadol and other opioids in LC neurons

<table>
<thead>
<tr>
<th>Opioid (n)</th>
<th>I_{opioid} (pA)</th>
<th>I_{opioid+tapent} (pA)</th>
<th>I_{tapent/opioid} (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>met-enkephalin (4)</td>
<td>177 ± 6</td>
<td>26 ± 9</td>
<td>14 ± 5</td>
</tr>
<tr>
<td>Morphine (4)</td>
<td>153 ± 18</td>
<td>73 ± 9*</td>
<td>48 ± 3</td>
</tr>
<tr>
<td>Oxycodone (7)</td>
<td>209 ± 37</td>
<td>93 ± 27*</td>
<td>41 ± 5</td>
</tr>
<tr>
<td>Buprenorphine (5)</td>
<td>69 ± 6a</td>
<td>3.4 ± 1.7*</td>
<td>n.d.</td>
</tr>
<tr>
<td>Pentazocine (4)</td>
<td>117 ± 17a</td>
<td>1.3 ± 2.0*</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Data are expressed as mean mean ± s.e.m.

a – I_{tapentadol} applied first then combined with listed opioid

* - significantly different from met-enkephalin (unpaired t-tests, all P < 0.01)

n.d. – not determined
Figure 1

GIRK current induced by activation of MOPr in LC neurons. Superfusion (shown by bars) of tapentadol (A), morphine (B) and met-enkephalin (ME, C) activate GIRK currents at holding potential (-60 mV) in the presence of the α₂-adrenoptor antagonist, idazoxan. All actions are reversed by the MOPr antagonist, naloxone. D. Concentration-response curves for met-enkephalin (black), morphine (red) and tapentadol (green). The amplitude of the hyperpolarization plotted as a percentage of the amplitude of a supramaximal concentration of UK14304.

Figure 2

Intrinsic activity of tapentadol at MOPr (adrenergic receptors were blocked from A-F with idazoxan, 1 µM and prazosin 300 nM). A. Morphine (30 µM) reversibly antagonized the current induced by a supramaximal (and desensitized) concentration of met-enkephalin confirming the lower intrinsic activity of morphine than met-enkephalin. B. Tapentadol had lower intrinsic activity than met-enkephalin and reversibly antagonized the current induced by a supramaximal (desensitized) concentration of met-enkephalin. C. Tapentadol partially and reversibly reduced the current induced by a supramaximal concentration of morphine. D. Tapentadol partially reduced the current induced by a supramaximal concentration of oxycodone. E. Buprenorphine and F. Pentazocine almost completely antagonized currents induced by a supramaximal concentration of tapentadol. G. Tapentadol did not inhibit the current induced by supramaximal concentration of UK14304 in the presence of naloxone.
Figure 3

Tapentadol potentiates responses of LC neurons to NA A. Example showing that tapentadol in the continuous presence of NA enhances the outward current induced by NA via inhibiting NA reuptake transporter (MOPr blocked by naloxone 1 µM) B. Concentration-response curve for potentiation of exogenous NA by tapentadol (IC$_{50}$ = 2.3 µM).

Figure 4

A. Tapentadol with combined mode of action enhanced the NA effect in single LC neurons. All data were normalized by the maximum effect induced by UK 14,304 (3 µM). B. Comparison between the concentration response curve of tapentadol as a MOPr agonist alone (with adrenergic receptors blocked; blue from Figure 1), tapentadol as a NAT inhibitor alone (with MOPr blocked; orange from Figure 3) and tapentadol acting on both mechanisms in the absence both MOPr and α2-adrenoceptor antagonists (solid curve in green).

Conflict of interest:
The current work was financially supported in part by a grant from Grünenthal GmbH to MJC. TT is an employee of Grünenthal GmbH.
GIRK current induced by activation of MOPr in LC neurons. Superfusion (shown by bars) of tapentadol (A), morphine (B) and met- enkephalin (ME, C) activate GIRK currents at holding potential (~60 mV) in the presence of the α2-adrenoceptor antagonist, idazoxan. All actions are reversed by the MOPr antagonist, naloxone. D. Concentration-response curves for met-enkephalin (black), morphine (red) and tapentadol (green). The amplitude of the hyperpolarization plotted as a percentage of the amplitude of a supramaximal concentration of UK14304.

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