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Effect of tapentadol on neurons in the locus coeruleus

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ABSTRACT

Tapentadol is a novel centrally acting drug that combines mu-opioid receptor (MOR) agonism and noradrenaline reuptake inhibition (NRI), producing analgesic effects in various painful conditions. We investigated the acute effects of tapentadol in the locus coeruleus (LC), a central nucleus regulated by the noradrenergic and opioid systems that is critical in pain modulation. In single-unit extracellular recordings of LC neurons from anaesthetized male Sprague–Dawley rats, tapentadol clearly inhibited the spontaneous electrophysiological activity of LC neurons in a dose-dependent manner (ED_{50} = 0.8 mg/kg). This inhibitory effect was reversed by RX821002 (an alpha2-adrenoceptor antagonist) and naloxone (a mu-opioid receptor antagonist) by 96.7% and 28.2%, respectively. Pretreatment with RX821002, N-ethoxycarbonyl-2-ethoxy-1-2-dihydroquinoline (EEDQ, an irreversible alpha2-adrenoceptor antagonist) or naloxone shifted the tapentadol dose–effect curve to the right (ED_{50} = 2.2 mg/kg, 2.0 mg/kg and 2.1 mg/kg, respectively). Furthermore, tapentadol inhibited the LC response to mechanical stimulation of the hindpaw in a dose-dependent manner. In summary, we demonstrate that acute administration of tapentadol inhibits LC neurons *in vivo*, mainly due to the activation of alpha2-adrenoceptors. These data suggest that both the noradrenergic and opioid systems participate in the inhibitory effect of tapentadol on LC neurons, albeit to different extents, which may account for its potent analgesic effect and mild opioidergic side-effects.

1. Introduction

Tapentadol, a new drug for pain treatment, was developed based on the known synergistic interaction between opioid and noradrenergic mechanisms at the spinal and supraspinal levels (Jordan et al., 2003; Ossipov et al., 1997, 1990). Tapentadol HCl, (−)-(1R, 2R)-3-(3-dimethylamino-1-ethyl-2-methyl-propyl)-phenol hydrochloride, is an analgesic that acts centrally and that combines two mechanisms of action in a single non-racemic molecule: that of a mu-opioid receptors (MOR) agonist and as a noradrenaline reuptake inhibitor (NRI). Tapentadol is currently prescribed as an alternative to classic opioids for the treatment of moderate to severe acute and chronic pain (Daniels et al., 2009; Kavanagh et al., 2012; Xu et al., 2012). Indeed, despite its 50-fold lower affinity for MOR, tapentadol is only 2- to 3-fold less potent than morphine in inducing analgesia in preclinical models of pain, yet with a better tolerance and physical dependence profile (Tzschentke et al., 2006, 2007).

While several recent studies have investigated the mechanism of action of tapentadol at the behavioural and spinal level, it remains to be determined how tapentadol modulates the descending noradrenergic pathway. The locus coeruleus (LC) is the main site of noradrenergic cell bodies in the brain and an essential modulator of pain and opiate action. Noradrenaline is an important endogenous analgesic in the spinal cord and it is released by bulbospinal noradrenergic axons that project from LC neurons (see review in: Pertovaara, 2006). LC activity is tonically regulated by somato-dendritic and terminal alpha2-adrenoceptors (alpha2-AR), which inhibit noradrenaline release (Mateo and Meana, 1999; Starke, 2001). Activation of these G_i-protein-coupled receptors leads to a progressive increase in potassium conductance and cell hyperpolarization (Aghajanian and Wang, 1986). Accordingly, their direct activation with agonists or indirect activation with NRI (*e.g.*, antidepressants such as desipramine and venlafaxine) dampens the electrical activity of these neurons in a dose-dependent manner

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(Berrocoso and Mico, 2007; Mateo et al., 1998; Szabo and Blier, 2001; Valentino et al., 1990). The LC also contains a high density of MOR that act as heteroreceptors to inhibit LC electrical activity, as well as modulating opiate tolerance and dependence (review in: Nestler et al., 1999).

The aim of the present study was to characterize the effects of systemic tapentadol administration on the electrical activity of LC neurons, and to determine the relative contributions of the noradrenergic and opioid systems to this effect. In addition, we compared the effects of tapentadol on LC neurons with those of the NRI desipramine and of the MOR agonist morphine. Finally, we studied the effect of tapentadol on the LC response to the mechanical stimulation of the hindpaw (sensory-evoked LC activity).

2. Materials and methods

2.1. Animals

Experiments were performed on adult male Sprague–Dawley rats (body weight, 200–300 g), which were housed in polycarbonate cages in groups of four under standard laboratory conditions (22 °C, 12 h light/dark cycle, lights on at 08:00 AM, food and water *ad libitum*). The animals were acclimatised for at least 7 days prior to the initiation of the study, and every effort was made to minimize animal suffering and to use the smallest possible number of animals. All procedures and animal handling were carried out in accordance with the European Commission's directive 2010/63/EU and Spanish Law (RD 1201/2005) regulating animal research.

2.2. Drugs

All drug solutions were prepared immediately before each trial and all the drugs were dissolved in physiological saline (0.9% NaCl), with the exception of EEDQ (N-ethoxycarbonyl-2-ethoxy-1-2-dihydroquinoline) which was dissolved in ethanol and then diluted sequentially in propylene glycol and water (0.25:0.25:0.50, v/v/v). All the drugs were administered intravenously (i.v.), except for EEDQ which was injected intraperitoneally (i.p.). The following drugs were used: tapentadol HCl (Grünenthal GmbH, Germany), idazoxan (IDX), naloxone hydrochloride (NLX), EEDQ, desipramine hydrochloride (Sigma Chemicals, USA), morphine (Spanish Ministry of Health, Spain) and RX821002 hydrochloride (Tocris Bioscience, UK). The doses used were selected on the basis of previous successful experiments carried out in our laboratory and data available in the literature. Naloxone was injected intravenously at 5 mg/kg, a dose that blocks the effect of opioid compounds on LC neurons (Ruiz-Durantez et al., 2003). Idazoxan and RX821002 were injected at 100 μg/kg i.v., a dose previously shown to reverse the inhibitory effects of alpha2-AR agonists on LC neurons (Alba-Delgado et al., 2012a; Biyah and Advenier, 1995). EEDQ (6 mg/kg, i.p.) was administered 6 h before commencing electrophysiological recordings, as this duration is required to achieve complete blockade of alpha2-AR (Pineda et al., 1997).

2.3. Electrophysiological recordings in anesthetized rats

Single-unit extracellular recordings of LC neurons were performed as described previously (Berrocoso et al., 2006). Rats were first anesthetized with an i.p. injection of chloral hydrate (400 mg/kg) and subsequently, anaesthesia was maintained using a perfusion pump (60–70 mg/kg/h). The animal's body temperature was maintained at 37 °C with a heated pad. Next, the rat was placed in a stereotaxic frame (David Kopf Instrument, USA) with its head at an angle of 15° to the horizontal plane (nose down) and the recording electrode was lowered into the LC (3.7 mm caudal to lambda, −1.1 mm lateral to the midline suture and 5.0–6.0 mm ventral to the dural surface; Paxinos and Watson, 2009). The recording electrode was a single-barrel pulled glass micropipette filled with a 2% solution of Pontamine Sky Blue in 0.5% sodium acetate. The extracellular signals from the electrode were amplified with a high-input impedance amplifier and monitored. Discriminated spikes were fed into a PC and processed using CED Micro1401 and Spike2 computer software (UK). LC neurons were identified based on the following well-established criteria: long duration action potential (>2 ms), spontaneous firing at a regular rhythm, a slow firing rate between 0.5 and 5 Hz and characteristic spikes with a long-lasting positive-negative waveform. At the end of the experiments, the recording site was marked by passing a 5 μA cathodic current through the electrode to leave a blue spot. In this way brain sections could ultimately be stained with neutral red and examined microscopically (Fig. 1A). Only measurements from cells within the LC were included in the analysis.

2.4. Experimental design

2.4.1. Spontaneous firing activity

When a single LC unit was isolated, the spontaneous basal discharge was recorded at least 2 min prior to drug administration and it was assessed in terms of: (i) firing rate (Hz); (ii) coefficient of variation (expressed as the percentage ratio of the standard deviation to the mean interval value of an interspike time-interval histogram); and (iii) burst firing rate (burst/s). A LC cell was considered to exhibit burst firing when it displayed at least two spikes with an initial interspike interval <80 ms and with subsequent interspike intervals ≥160 ms (Grace and Bunney, 1984: see Table 1). Dose–response curves were then generated for tapentadol by injecting the drug at 2 min intervals, in doubling doses, until all spontaneous activity ceased (maximal effect). Subsequent injections of NLX (MOR antagonist, 5 mg/kg), IDX (alpha2-AR antagonist, 100 μg/kg) or RX821002 (selective alpha2-AR antagonist, 100 μg/kg) were administered to determine if they reversed the inhibitory effect of tapentadol. To characterize the relative noradrenergic and opioid components of tapentadol-mediated inhibition of spontaneous LC neuronal activity, RX821002 (100 μg/kg) or NLX (5 mg/kg) were administered 2 min before performing tapentadol dose–response experiments. In both assays, the inhibitory effect of tapentadol was reversed by i.v. administration of NLX (5 mg/kg) or RX821002 (100 μg/kg). In addition, the irreversible alpha2-AR antagonist EEDQ (6 mg/kg) was administered 6 h before performing tapentadol dose–response experiments. The complete inhibition of spontaneous activity induced by tapentadol was reversed by NLX administration (5 mg/kg). Finally, to compare the effect of tapentadol on LC neurons with that of desipramine and morphine, dose–response experiments were performed for both drugs. Desipramine or morphine was administered in increasing doubling doses at 3 and 2 min intervals, respectively, until electrical activity ceased. The capacity of IDX (100 μg/kg) or NLX (1 mg/kg) to reverse this inhibitory effect was then quantified.

Changes in firing rate were expressed as a percentage of the basal firing rate and in reversion studies, they were expressed as the percentage recovery with respect to the basal firing rate. Dose–response curves were analysed for the best non-linear fit to the following logistic three-parameter equation (Parker and Waud, 1971): $E = E_{max}[C]^n/(ED_{50}^n + [C]^n)$, where $[C]$ is the i.v. dose of drug; E is the effect of the drug on the firing rate; E_{max} is the maximal percentage change at "infinite" dose (100%); ED_{50} is the effective dose that elicits 50% of E_{max}, and n is the slope factor of the function. Only one dose–response curve was obtained per rat.

2.4.2. Sensory-evoked firing activity

The LC evoked response was recorded in another set of animals. Accordingly, three consecutive mechanical compressions of the hindpaw were applied before

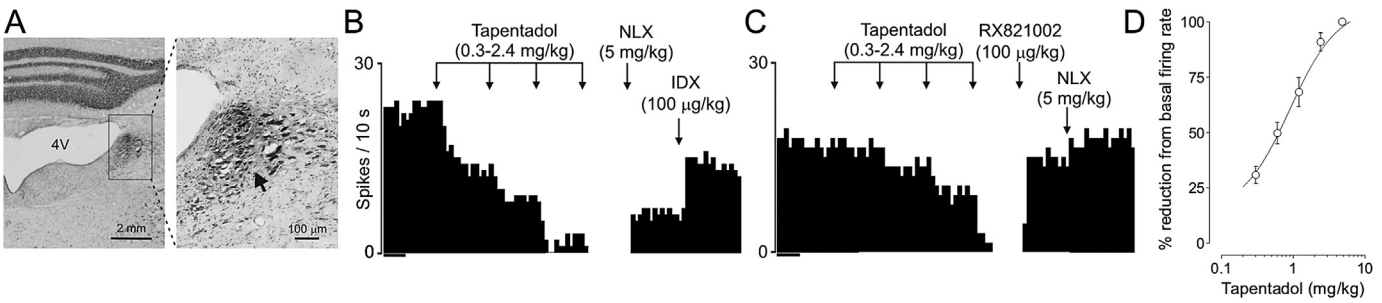

Fig. 1. Effect of tapentadol on the spontaneous firing activity of locus coeruleus (LC) neurons. (A) Photomicrograph of a coronal section (Neutral Red stain) of the rat brainstem showing the recording site in the LC (black arrow: 4V, 4th ventricle). (B–C) Representative histogram from firing rate recordings of LC neurons demonstrating the dose-dependent inhibitory effect of tapentadol. This effect was reversed by subsequent intravenous administration of (B) naloxone (NLX) and idazoxan (IDX), or (C) RX821002 and NLX (scale bar = 1 min). (D) Dose–response curve showing the inhibitory effect of tapentadol ($n = 8$). Symbols represent the percentage reduction with respect to the basal firing rate (mean ± SEM). The horizontal axis represents the logarithm of cumulative tapentadol doses.

(baseline) and 1.5 min after the i.v. administration of increasing doses of tapentadol (0.3–2.4 mg/kg). Each paw compression lasted for 1.0 s, delivered at 2.0 s intervals using a pair of 15.0 cm surgical forceps (Ref. 501742-G, World Precision Instruments, UK) and applying pressure midway along the forceps such that the opposite leaves of the forceps came into contact. Such stimuli evoked a burst discharge followed by post-activation inhibition or suppression (Fig. 4A: Grant and Weiss, 2001). A LC neuron was considered to exhibit a sensory-evoked response when it displayed at least two spikes, with an interspike interval <160 ms (defined as a burst event). This response was assessed in terms of: (i) incidence, i.e., the proportion of cells exhibiting an evoked response; (ii) latency (ms), the time to the onset of the response to the stimulus; (iii) number of sensory-evoked responses per train; (iv) number of spikes per train; and (v) period of suppression (s), i.e., the time after each evoked response in which there was no neuronal activity.

2.5. Data analysis

All the data are presented as the mean ± S.E.M. and they were analysed with Prism 5.0 GraphPad (GraphPad, USA). As no differences were found between the spontaneous electrical parameters of LC neurons before pharmacological treatment, these data were pooled for analysis ("baseline", Table 1). Student's *t*-test was used to compare values between two groups. Comparisons of more than two groups were performed with a one-way analysis of variance (ANOVA) followed by the Tukey's multiple comparison test (Table S1). The Fisher's exact test was used to analyse two categorical variables. A $p < 0.05$ was considered as significant.

3. Results

3.1. Acute effect of tapentadol on the electrical activity of LC neurons

Tapentadol administration (0.3–4.8 mg/kg) inhibited spontaneous activity in the LC in a dose-dependent manner. Complete inhibition was achieved in all cells tested and the mean ED_{50} value estimated from dose–response curves was 0.8 ± 0.1 mg/kg (Fig. 1D). This complete inhibition of firing activity was reversed (28.2 ± 0.9%) by NLX (5 mg/kg) administration, while subsequent IDX (100 μg/kg) administration produced a further reversal of the residual activity of 26.4 ± 7.3% (total reversion of 54.5 ± 6.7% respect to basal activity: Fig. 1B and Table 2). As IDX also displays significant affinity for other receptors expressed in the LC (Hoyer, 1988; Miralles et al., 1993; Newman-Tancredi et al., 1998), additional studies were performed using this selective alpha2-AR antagonist, RX821002. Accordingly, the inhibitory effect of tapentadol was reversed by RX821002 (96.7 ± 4.6%), while subsequent NLX administration resulted in a further reversal of 18.9 ± 0.8% of the residual activity (total reversion of 115.5 ± 3.9% respect to basal activity: Fig. 1C and Table 2).

3.2. Effect of alpha2-AR blockade on the tapentadol-induced inhibition of LC activity

The contribution of alpha2-AR to the inhibitory effect of tapentadol in LC neurons was studied using two approaches. Firstly, rats were pretreated with RX821002 (100 μg/kg) 2 min before tapentadol administration, this exposure to RX821002 producing no effect on the basal firing properties of LC neurons ($p > 0.05$; Fig. 2A, Table 1). However, the dose–response curve of tapentadol was shifted to the right when administered after RX821002, and the ED_{50} increased by 175.0% (ED_{50} tapentadol 0.8 ± 0.1 mg/kg; ED_{50} RX821002 + tapentadol 2.2 ± 0.4 mg/kg: $p < 0.05$; Fig. 2B, Tables 2 and S1). The complete inhibition of LC neurons following RX821002 + tapentadol administration was reversed by 59.5 ± 10.2% following the subsequent administration of NLX (5 mg/kg; Fig. 2A and Table 2).

Pretreatment with the irreversible alpha2-AR antagonist, EEDQ (6 mg/kg), 6 h before tapentadol administration significantly increased spontaneous firing: the mean basal firing rate ($p < 0.001$, Table S1), the regular firing pattern (variation coefficient; $p < 0.001$, Table S1) and the burst firing ($p < 0.001$, Table S1) of LC neurons compared to the basal parameters (Table 1: Pineda et al., 1997). In these conditions, the dose–response curve of tapentadol shifted to the right and the ED_{50} increased by 150% (ED_{50} tapentadol 0.8 ± 0.1 mg/kg; ED_{50} EEDQ + tapentadol 2.0 ± 0.4 mg/kg: $p < 0.05$; Fig. 2D, Tables 2 and S1). Total inhibition was observed in all neurons tested, an effect that was rapidly reversed by subsequent administration of NLX (5 mg/kg, 75.7 ± 7.6%; Fig. 2C and Table 2).

3.3. Effect of opioid receptor blockade on tapentadol-induced inhibition of LC activity

NLX administration had no effect on the basal firing properties of LC neurons ($p > 0.05$; Fig. 2E, Tables 1 and S1: Berrocoso and Mico, 2007; Berrocoso et al., 2006; Illes and Norenberg, 1990) and thus, we also examined the effects of administering NLX (5 mg/kg) 2 min before generating tapentadol dose–response curves. This opioid receptor blockade shifted the tapentadol dose–response curve to the right and increased the ED_{50} by 162.5% when compared with the control group (ED_{50} tapentadol 0.8 ± 0.1 mg/kg; ED_{50} NLX + tapentadol 2.1 ± 0.2 mg/kg: $p < 0.05$; Fig. 2F, Tables 2 and S1). This shift was similar to that induced by pretreatment with RX821002 and EEDQ ($p > 0.05$; NLX vs RX821002; NLX vs EEDQ; Table S1). Complete inhibition of firing was observed in all neurons

Table 1
Spontaneous firing activity of locus coeruleus neurons.

	Firing rate (Hz)	Variation coefficient (%)	Burst firing (burst/s)	*n*
Baseline	1.5 ± 0.1	36.3 ± 2.5	0.010 ± 0.004	29
RX821002 (100 μg/kg)	2.0 ± 0.3	47.4 ± 5.9	0.017 ± 0.007	7
EEDQ (6 mg/kg)	3.5 ± 0.2***	59.4 ± 2.3***	0.169 ± 0.050***	17
Naloxone (5 mg/kg)	1.4 ± 0.2	34.1 ± 5.7	0.002 ± 0.002	6

Data are expressed as the mean ± S.E.M of *n* cells per group. The distinct parameters were obtained from the locus coeruleus neurons in basal conditions (before any pharmacological treatment), and after a single i.v. dose of RX821002 (selective alpha2-adrenoceptor antagonist) or naloxone (opioid receptor antagonist), and after i.p. pretreatment with N-ethoxycarbonyl-2-ethoxy-1-2-dihydroquinoline (EEDQ, irreversible alpha2-adrenoceptor antagonist) administered 6 h before recording: ***$p < 0.001$ vs baseline (one-way analysis of variance test followed by Tukey's multiple comparison test).

Table 2
Summary of the ED_{50} and the reversal of tapentadol-induced inhibition.

Group	ED_{50} (mg/kg)	Reversion studies (%)	
Tapentadol	0.8 ± 0.1 (8)	NLX	IDX[#]
		28.2 ± 0.9	26.4 ± 7.3
		RX821002	NLX[#]
		96.7 ± 4.6	18.9 ± 0.8
RX821002 + Tapentadol	2.2 ± 0.4* (7)	NLX	
		59.5 ± 10.2	
EEDQ + Tapentadol	2.0 ± 0.4* (6)	NLX	
		75.7 ± 7.6	
NLX + Tapentadol	2.1 ± 0.2* (6)	RX821002	
		79.8 ± 2.8	
Desipramine	0.2 ± 0.1[$$] (5)	IDX	
		29.8 ± 7.3	
Morphine	1.0 ± 0.2 (5)	NLX[&]	
		94.3 ± 6.6	

Data are expressed as the mean ± S.E.M. The effective dose 50 (ED_{50}) was estimated from the dose–response curves for tapentadol, desipramine or morphine. The values within parentheses represent the number of pharmacologically tested neurons. The reversal of tapentadol-induced inhibition is expressed as the percentage of recovery of the firing rate by a single i.v. dose of naloxone (NLX, 5 mg/kg or 1 mg/kg[&]), idazoxan (IDX, 100 μg/kg) or RX821002 (100 μg/kg). [#]The effect of the second drug (IDX or NLX) administered in the reversion studies is expressed as the percentage of recovery of the residual activity. *$p < 0.05$ vs tapentadol group (one-way analysis of variance test followed by Tukey's multiple comparison test), [$$]$p < 0.01$ vs tapentadol group (Student's *t*-test).

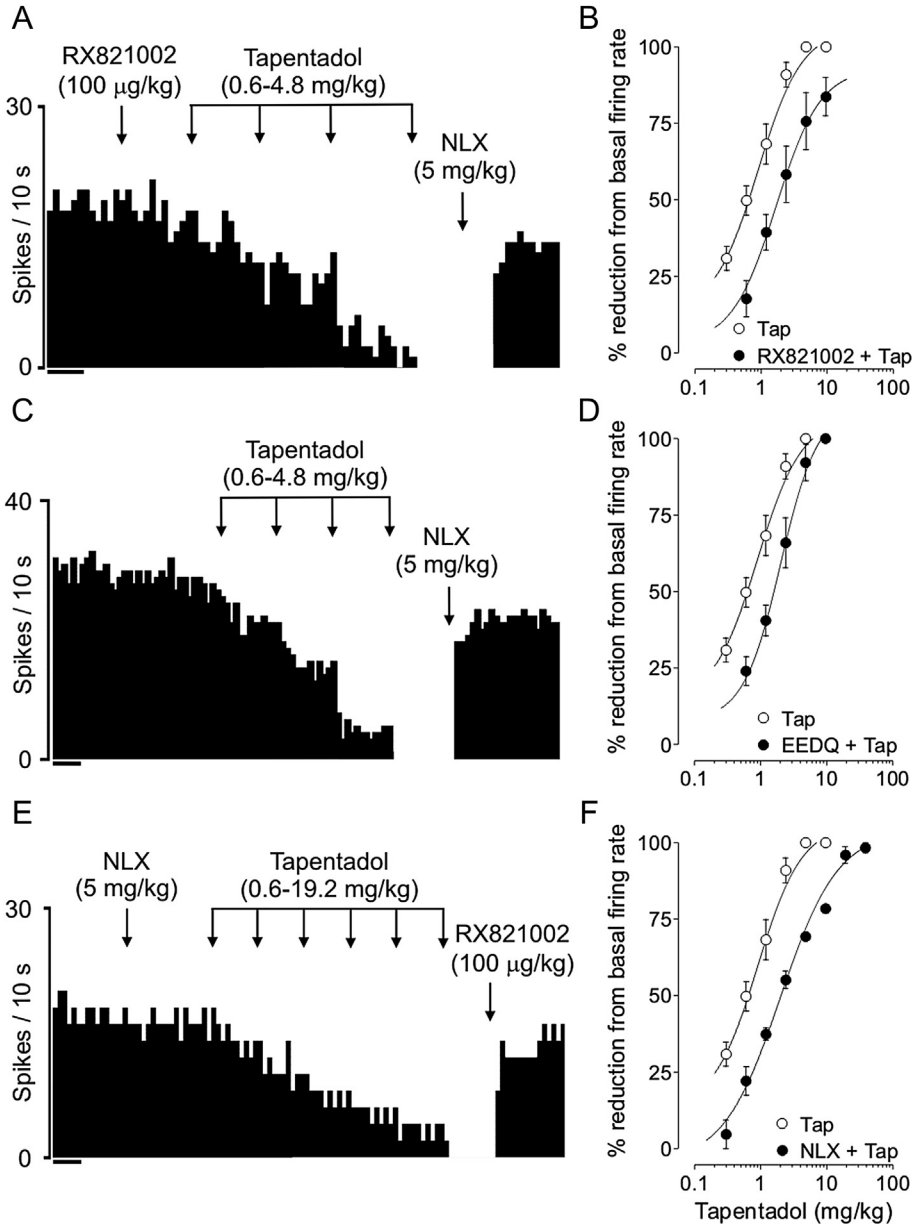

Fig. 2. Inhibitory effect of RX821002, EEDQ or naloxone (NLX) on the spontaneous firing activity of locus coeruleus (LC) neurons induced by tapentadol. (A, C, E) Representative histograms of the firing rate recordings to show the effect of tapentadol after pretreatment with RX821002, EEDQ (6 h prior) and NLX, respectively. The inhibitory effect of tapentadol was reversed by subsequent administration of (A, C) NLX or (E) RX821002 (scale bar = 1 min). (B, D, F) Dose–response curves showing the inhibitory effect of tapentadol in the control group (Tap, $n = 8$) and after pretreatment with (B) RX821002 (RX821002 + Tap, $n = 7$), (D) EEDQ (EEDQ + Tap, $n = 6$) or (F) NLX (NLX + Tap, $n = 6$). Symbols represent the percentage reduction with respect to the basal firing rate (mean ± SEM). The horizontal axis represents the logarithm of the cumulative tapentadol doses.

following tapentadol administration, an effect that was reversed by 79.8 ± 2.8% by subsequent RX821002 (100 μg/kg) administration (Fig. 2E and Table 2).

3.4. Comparison of the effects of tapentadol with those of desipramine and morphine

The administration of desipramine (0.05–1.60 mg/kg) or morphine (0.15–4.80 mg/kg) inhibited spontaneous LC activity in a dose-dependent manner (Fig. 3A and C) and complete inhibition was observed in all the cells tested. The mean ED_{50} value estimated from the dose–response curve for desipramine was 0.2 ± 0.0 mg/kg (Fig. 3B). Subsequent IDX administration (100 μg/kg) reversed the total inhibition of spontaneous LC activity by desipramine by 29.8 ± 7.3% with respect to basal activity (Fig. 3A and Table 2), an effect similar to that observed in the tapentadol dose–response experiments (Fig. 1B and Table 2). The mean ED_{50} value for morphine estimated from the dose–response curves was 1.0 ± 0.2 mg/kg (Fig. 3D) and subsequent NLX administration (1 mg/kg) reversed the total inhibition of spontaneous LC activity induced by morphine by 94.3 ± 6.6% (Fig. 3C and Table 2).

3.5. Acute effect of tapentadol on the sensory-evoked activity of LC neurons

Mechanical hindpaw stimulation evoked the well-documented response of LC neurons in all the cells tested, characterized by a marked increase in the spikes after paw compression followed by a

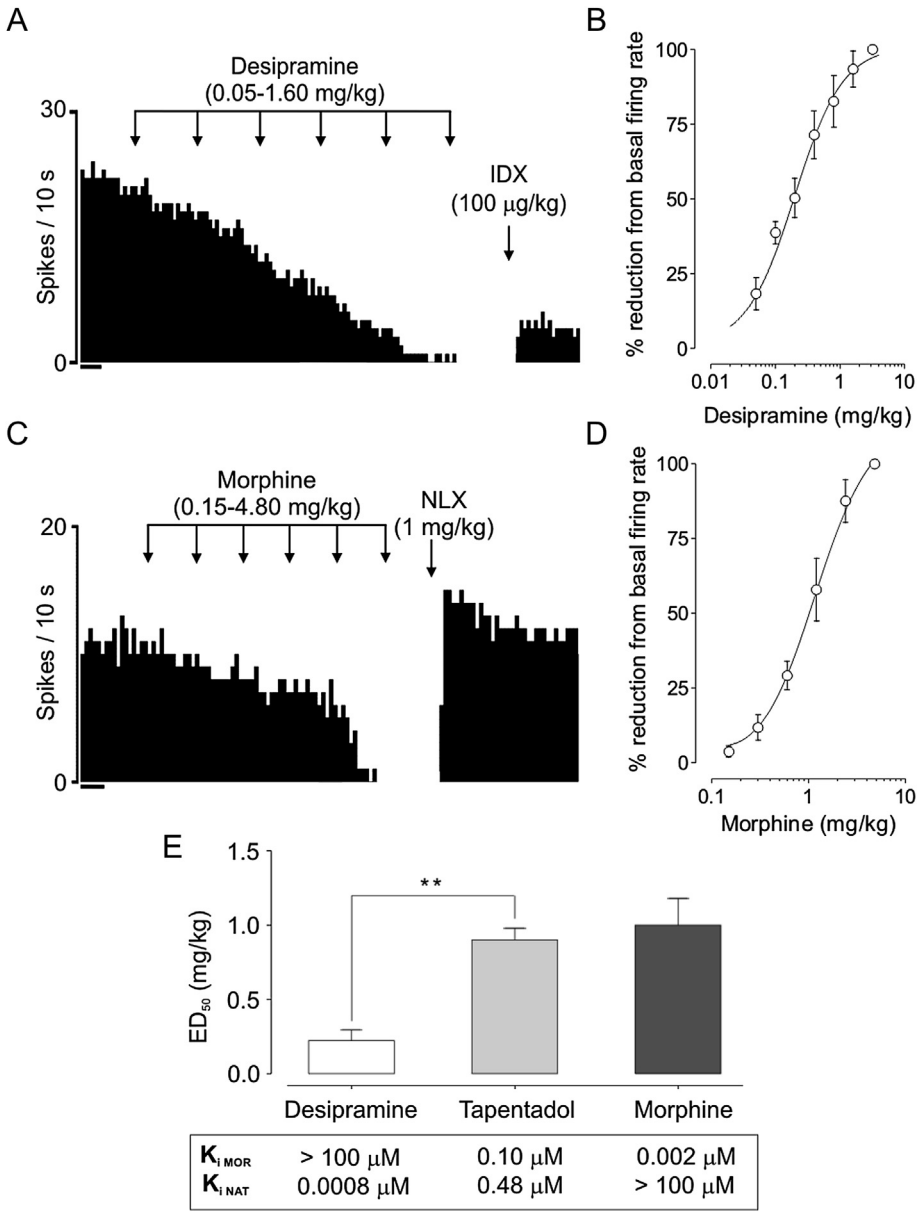

Fig. 3. Effects of desipramine and morphine on the spontaneous firing activity of locus coeruleus (LC) neurons. (A, C) Representative histograms of firing rate recordings from LC neurons showing the dose-dependent inhibition by desipramine and morphine. These effects were reversed by subsequent administration of (A) idazoxan (IDX) or (C) naloxone (NLX: scale bar = 1 min). The dose–response curves demonstrate the inhibitory effect of (B) desipramine and (D) morphine (both $n = 5$), and the symbols represent the percentage reduction with respect to the basal firing rate (mean ± SEM). The logarithm of the cumulative drug doses is reflected in the horizontal axis. (E) Estimated 50% effective dose (ED_{50}) taken from the dose–response curves for desipramine ($n = 5$), tapentadol ($n = 8$) and morphine ($n = 5$). The affinity constants (K_i) for the mu-opioid receptors (MOR) and noradrenaline transporter (NAT) reflect the ligand concentrations (µM) determined in MOR-binding and synaptosomal monoamine reuptake assays in the rat brain (Sanchez and Hyttel, 1999; Tzschentke et al., 2007): $^{**}p < 0.01$ vs desipramine (Student's t-test).

period of quiescence (suppression period, Fig. 4B: Grant and Weiss, 2001). After tapentadol administration, the number of cells that responded to the mechanical stimulus was reduced to 33% and 0% at doses of 1.2 and 2.4 mg/kg, respectively. Accordingly, this drug reduced both the number of responses, as well as the number of spikes in each response, in dose-dependent manner (Fig. 4C and D, Tables 3 and S1).

4. Discussion

The present study demonstrates that systemic tapentadol administration inhibits the spontaneous and mechanically-evoked firing of LC neurons *in vivo* in a dose-dependent manner. The effect on spontaneous activity is associated with the activation of both alpha2-AR and MOR. These results confirm the dual mechanism of action of acute tapentadol administration at the level of the LC, in agreement with previous behavioural findings in models of acute and chronic pain, and electrophysiological data from spinal neurons (Bee et al., 2011; Christoph et al., 2010; Kogel et al., 2011; Tzschentke et al., 2007).

The inhibitory effect of tapentadol on LC neurons appears to be mediated by both the noradrenergic and opioid systems, albeit to different extents. This effect was almost entirely reversed by RX821002, but only partially by NLX treatment, suggesting that alpha2-AR primarily mediate the acute inhibitory effect of tapentadol on LC neurons. The activation of alpha2-AR in the LC

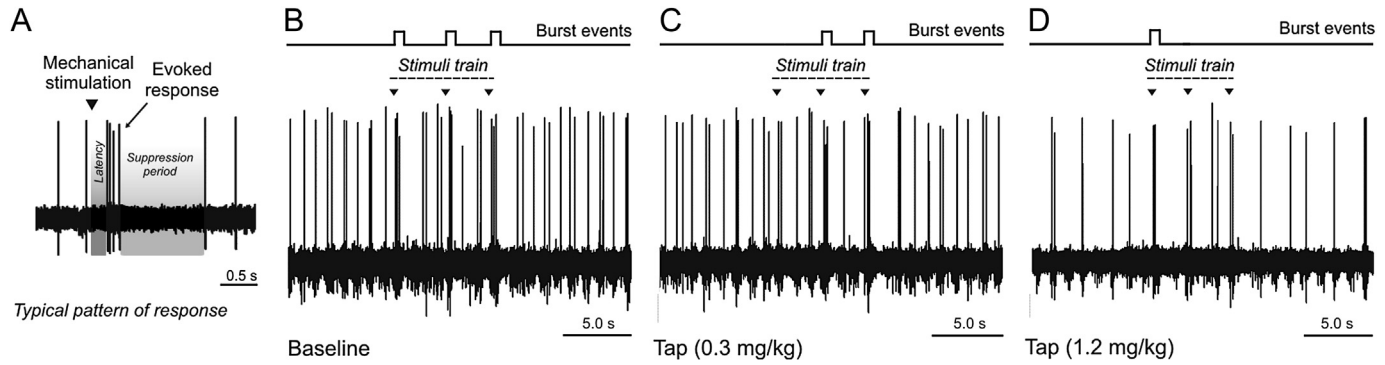

Fig. 4. Effect of tapentadol (Tap) on the sensory-evoked firing activity of locus coeruleus (LC) neurons. (A) Schematic representation of a typical LC neuron response to mechanical stimulation (black triangle). (B–D) Representative oscillography traces illustrating the sensory-evoked response of LC neurons to a train of mechanical stimuli. The notches in upper traces of each panel represent the burst events at the (B) baseline (before tapentadol), and after administration of (C) 0.3 mg/kg and (D) 1.2 mg/kg tapentadol. Note the progressive inhibition of LC response produced by tapentadol.

decreases noradrenaline release (Mateo and Meana, 1999) and the firing rate via a negative feedback loop (Svensson et al., 1975); whereas the blockade of alpha2-AR enhances the LC firing rate, as evident following exposure to the irreversible antagonist EEDQ. Administration of an NRI (*e.g.*, desipramine) produces an increase in the availability of noradrenaline in the synaptic cleft at the LC and terminal level. This results in alpha2-AR activation in the LC and ultimately, inhibition of the neuronal firing that in part counteracts the effects of desipramine on the terminal (Mateo et al., 1998). Accordingly, the inhibition of spontaneous LC firing by NRI provides a measure of alpha2-AR agonist activity in the LC, reflecting the increase in noradrenaline bioavailability in terminal areas. Microdialysis studies have demonstrated that tapentadol produces a dose-dependent increase in extracellular levels of noradrenaline in LC projection areas, such as the spinal cord and hippocampus (Tzschentke et al., 2007, 2012). Thus, like NRI, tapentadol is likely to produce a similar increase in extracellular noradrenaline at the LC level, which would activate inhibitory alpha2-AR, inhibiting LC activity and partially counteracting the net amount of noradrenaline released at both the LC and terminal levels (spinal cord and hippocampus). In agreement with this rationale, it was previously shown that local microinjection of alpha2-AR antagonists into the LC produces analgesia that is reduced by intrathecal alpha2-AR antagonists (Wei and Pertovaara, 2006), suggesting that spinal and LC alpha2-AR have opposite effects on pain-related behaviour. However, it is important to note that tapentadol does not bind to any adrenergic receptors (neither alpha2-AR nor other subtypes). Since tapentadol blocks noradrenaline reuptake, it will (indirectly) activate all types of adrenergic receptors that are present in the system. Until now the analgesic effect of tapentadol was blocked by the systemic administration of alpha2-AR antagonists (Schroder et al., 2010), presumably acting on primary afferent neurons (Fig. 5) given that tapentadol's inhibitory effects on evoked responses of spinal dorsal horn neurons were reversed by the spinal blockade of alpha2-AR in anaesthetized animals (Bee et al., 2011). We now show that this effect might be partially counteracted in anaesthetized animals by alpha2-AR activity in the LC. These data suggest that the spinal activation of alpha2-AR predominates over that of the LC in the global analgesic effect of tapentadol.

Conversely, NLX reversed the inhibitory effect of tapentadol, although less so than RX821002. The role of opioid signalling in LC activity is of particular interest, as MOR in the LC play a key role in mediating opiate dependence and withdrawal. Acute morphine administration acts on MOR to enhance the opening of inwardly-rectifying potassium channels, resulting in hyperpolarization. However, chronic morphine treatment impairs the ability of MOR to open potassium channels, leading to the development of tolerance and dependence (Aghajanian, 1978; Rasmussen et al., 1990). The mild activation of MOR by tapentadol may explain the milder physical dependence associated with this compound than with traditional opioids (Tzschentke et al., 2006). Overall the decrease of LC activity through a predominant alpha2-AR dependent mechanism rather than MOR activation would contribute to a mild opioidergic side effect. Thus, further behavioural studies using local tapentadol administration into LC would be necessary to elucidate this issue.

To determine the relative contribution of alpha2-AR to the effects of tapentadol observed, we administered the selective alpha2-AR antagonist RX821002 prior tapentadol. This antagonist provoked a shift of the tapentadol dose–response curve to the right, increasing the ED_{50}, as also occurred after pretreatment with the irreversible alpha2-AR antagonist EEDQ, indicating that alpha2-AR antagonism attenuated the inhibitory effect of tapentadol. Subsequent administration of NLX reversed the inhibitory effect of

Table 3
Sensory-evoked firing activity of locus coeruleus neurons.

	Incidence (%)	Latency (ms)	Number of responses	Spikes per response	Suppression period (s)
Baseline	100.0 (6/6)	113.3 ± 43.3	2.3 ± 0.3	6.5 ± 0.6	2.0 ± 0.2
Tapentadol doses					
0.3 mg/kg	75.0 (3/4)	103.3 ± 58.1	1.0 ± 0.4	6.3 ± 0.7	1.6 ± 0.1
0.6 mg/kg	100.0 (4/4)	27.5 ± 18.9	1.3 ± 0.3	4.8 ± 0.9	1.1 ± 0.3*
1.2 mg/kg	33.3 (1/3)	~	0.3 ± 0.3*	~	~
2.4 mg/kg	0.0 (0/2)$	~	0.0 ± 0.0*	~	~

Data represent the mean ± S.E.M. LC activity evoked by a train of mechanical stimuli. The pattern of the response was measured before (baseline) and after tapentadol doses. The values in parentheses represent the LC neurons ratio exhibiting evoked response. The factor significance was represented as $^{*}p < 0.05$ vs baseline (one-way analysis of variance test followed by Tukey's multiple comparison test). The incidence was analysed by Fisher's exact test: $^{\$}p < 0.05$ vs baseline. ~, not listed as sensory-evoked activity was not observed.

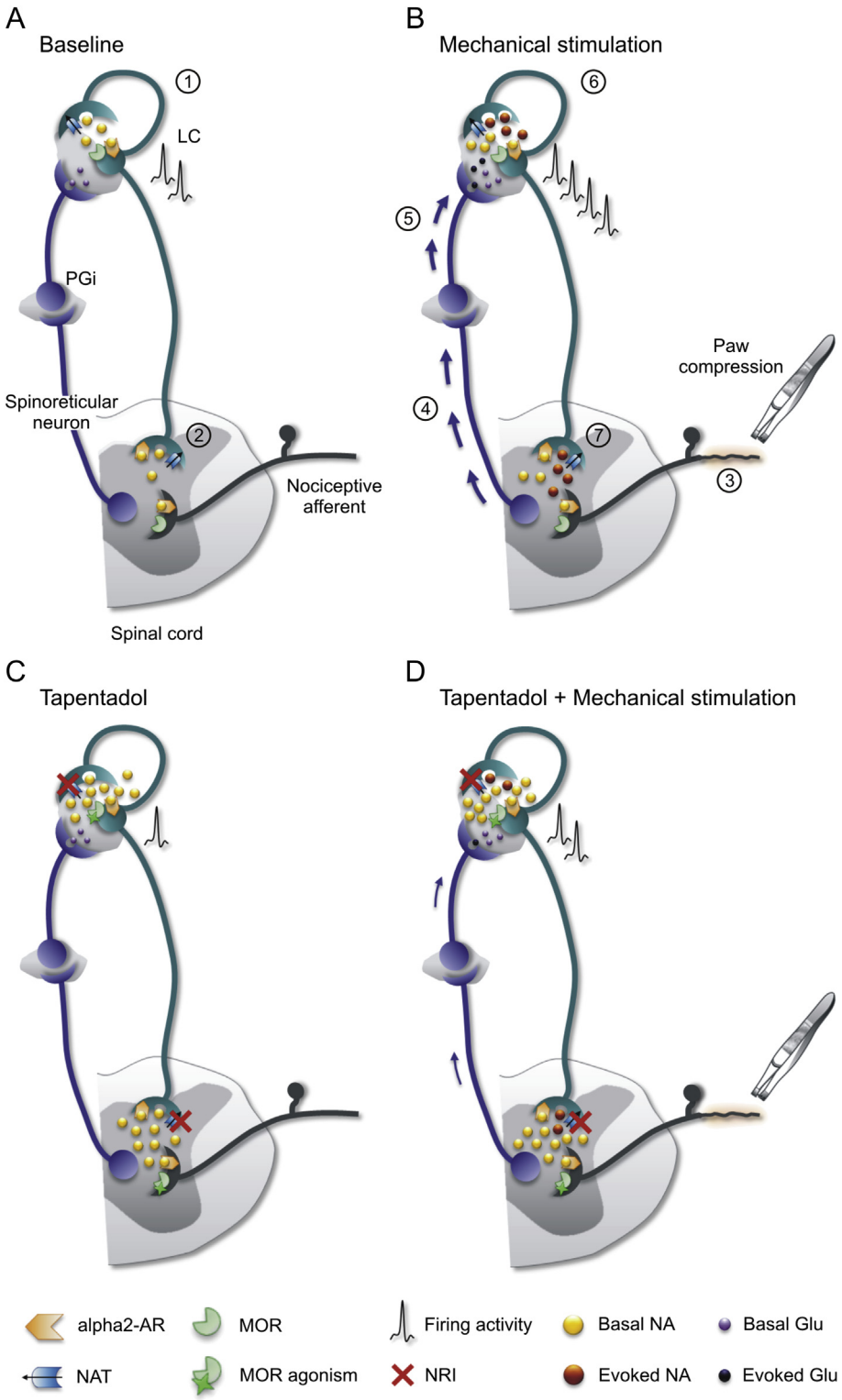

Fig. 5. Scheme representing the hypothetical effect of tapentadol on pain circuits in basal conditions and following mechanical stimulation. (A) In basal conditions (untreated), locus coeruleus (LC) neurons fire tonically and release noradrenaline (basal NA) at the level of the soma (1) and terminals (spinal cord, 2), under the negative control of presynaptic alpha2-AR and neurotransmitter reuptake. The influence of the ascending nociceptive pathway, transmitted to the LC by the paragigantocellularis nucleus (PGi: basal glutamate, Glu), is very weak. (B) Inhibition of noxious information at the level of the spinal cord is triggered by nociceptive inputs to the LC. When a mechanical stimulation (untreated) is elicited, nociceptive inputs enter the spinal cord through primary afferents (3), which strongly activate the fibres of the spinoreticular tract (4). The input along the ascending pathway rises to the PGi (5), which in turn sends an excitatory amino acid input to the LC (evoked Glu). Excitatory inputs produce a sensory-evoked response of LC neurons, resulting in an increase of firing activity and NA release (evoked NA) at the LC (6) and in the spinal cord (7). This effect is partially counteracted by the activation of alpha2-AR in the LC. (C) Acute tapentadol administration produces the blockade of noradrenaline transporters (NAT) at the LC and spinal level, as well as activation of the MOR. The increase of NA availability will mainly inhibit LC activity through the activation of LC alpha2-AR. (D) In conditions of mechanical stimulation, the ascending noxious inputs to the LC are reduced as tapentadol augments the spinal NA and opioidergic activity (mu-opioid receptor, MOR), resulting in a weaker sensory-evoked response at the LC level.

tapentadol by 60–80% and thus, blockage of alpha2-AR augments the opioid-mediated inhibition in the LC, as indicated previously (Illes and Norenberg, 1990; Schoffelmeer et al., 1986). This suggests that the concomitant activation of alpha2-AR and MOR produced by tapentadol decreases the global opioid effects at LC level. This reduced activation of MOR in LC neurons may explain the mild opioidergic side effects of tapentadol. Administration of NLX prior to tapentadol treatment increased the ED_{50} of tapentadol and interestingly, similar ED_{50} values were obtained by inhibiting alpha2-AR or opioid receptors. Moreover, subsequent RX821002 administration reversed the effect of tapentadol to levels comparable to those of untreated animals (79.8% vs 96.7%). Taken together, these findings confirm that the inhibitory effects of tapentadol are mediated by the activation of both alpha2-AR and MOR.

The potency of tapentadol was higher than that expected based on its K_i values for noradrenaline transporter (NAT) and MOR (Fig. 3E). Indeed, the inhibitory effect of tapentadol was comparable to that induced by morphine in the same neurons, even though the affinity of tapentadol for MOR was 50-fold lower than that of morphine ($K_i = 0.096$ and 0.002 μM, respectively: Tzschentke et al., 2007). The potent effects of tapentadol appear to be mainly due to its inhibition of noradrenaline reuptake, although the NRI activity of tapentadol was more moderate than that of desipramine ($K_i = 0.48$ and 0.0008 μM, respectively: Sanchez and Hyttel, 1999). These observations suggest that an interaction between both receptor systems underlies the overall effect of tapentadol. A number of behavioural studies have demonstrated that signalling via both neurotransmitter systems is required for the potent analgesic effects of tapentadol, both in preclinical models of pain (Tzschentke et al., 2006) and in isobolographic studies (Schroder et al., 2011). This may be because alpha2-AR and MOR activate the same intracellular signalling pathways to promote potassium channel opening, thereby dampening neuronal excitability. However, although MOR and alpha2-AR are concomitantly involved in the inhibitory effect of tapentadol on LC activity, the contribution of latter appears to be stronger than that of the former.

In addition to inhibiting the spontaneous firing rate of LC neurons, tapentadol also inhibits the acute noxious-evoked response in a dose-dependent manner. It has been shown that the inhibition of nociceptive information at the spinal cord level by the LC is triggered by nociceptive inputs to the LC, forming a feedback loop (Fig. 5). Indeed, noxious hindpaw stimulation will activate the ascending pain pathway, such as the glutamatergic nucleus paragigantocellularis (PGi: Chiang and Aston-Jones, 1993; Ennis and Aston-Jones, 1988), which will in turn activate LC neurons, thereby increasing the noradrenaline release at the spinal cord level. Thus, the ascending noxious information will allow LC neurons to engage descending feedback systems that regulate the output from the spinal cord (Fig. 5A and B). Administration of tapentadol enhances the availability of noradrenaline and MOR activation in the spinal cord, which will reduce LC sensory-evoked activity when the hindpaw is stimulated, given that the dampening of the transmission of the acute noxious information from the spinal cord (Fig. 5C and D). This is consistent with the noradrenergic impact of tapentadol in acute models of pain (Kogel et al., 2011; Schroder et al., 2010). However, additional studies in models of neuropathic pain will be necessary to confirm this hypothesis given that predominant noradrenergic mediation has been demonstrated in pathological pain states (Schroder et al., 2010; Tzschentke et al., 2007). In this sense, we recently demonstrated that alpha2-AR and NAT function are initially preserved when animals are subjected to chronic constriction injury (Alba-Delgado et al., 2012a), indicating that tapentadol probably exerts similar effects at the LC level to those described here in conditions of neuropathic pain. However, increased LC alpha2-AR and NAT function have been described after long term neuropathic pain (Alba-Delgado et al., 2013), and it remains unknown whether MOR expression and/or activity is altered in this condition. In addition, it will be important to determine if chronic tapentadol treatment desensitizes alpha2-AR and/or NAT at the LC level, as happens with other NRI (Alba-Delgado et al., 2012b), or if they sensitize MOR as occurs with morphine (Dang and Williams, 2004).

5. Conclusions

In conclusion, tapentadol induces a dose-dependent inhibition of the electrical activity of LC neurons by activating both alpha2-AR and MOR, both of which are required to achieve a maximal effect. Moreover, our data demonstrate the predominant role of alpha2-AR, which probably accounts for the milder tolerance and dependence associated with tapentadol than with other opioidergic analgesics. Further electrophysiological studies in pathological pain conditions (*e.g.*, neuropathies) will be required to define the mechanism of action of tapentadol and related compounds more precisely.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.neuropharm.2013.04.053.

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