Spinal-supraspinal and intrinsic μ-opioid receptor agonist-norepinephrine reuptake inhibitor (MOR-NRI) synergy of tapentadol in diabetic heat hyperalgesia in mice

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Running title: Tapentadol – spinal supraspinal synergy

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Abbreviations: 5-HT, 5-hydroxytryptamine-serotonin; ANOVA, analysis of variance; CI, confidence interval; CL, confidence limits; ED50, median effective dose; i.c.v., intracerebroventricular; i.p., intraperitoneal, i.t., intrathecal; MPE, maximal possible effect; MOR, µ opioid receptor; NE, norepinephrine; NRI, NE reuptake inhibition; SNL, spinal nerve ligation; STZ, streptozotocine; tapentadol HCl, (-)-(1R,2R)-3-(3-dimethylamino-1-ethyl-2-methyl-propyl)-phenol hydrochloride

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Abstract

Tapentadol is a Mu Opioid Receptor (MOR) agonist and Norepinephrine reuptake inhibitor (NRI) with established efficacy in neuropathic pain in patients and intrinsic synergistic interaction of both mechanisms as demonstrated in rodents. In diabetic mice, we analyzed the central antihyperalgesic activity, the occurrence of site-site interaction as well as the spinal contribution of opioid and noradrenergic mechanisms in a hot plate test. Tapentadol (0.1–3.16 µg/animal) showed full efficacy after i.t. as well as after i.c.v. administration (ED50 0.42 µg/animal i.t., 0.18 i.c.v.). Combined administration of equi-analgesic doses revealed spinal-supraspinal synergy (ED50 0.053 i.t.+i.c.v.). Morphine (0.001–10 µg/animal) also showed central efficacy and synergy (ED50 0.547 i.t., 0.004 i.c.v., 0.014 i.t.+i.c.v.). Supraspinal potencies of tapentadol and morphine correlated with the 50-fold difference in their MOR affinities. In contrast, spinal potencies of both drugs were similar and correlated with their relative systemic potencies (ED50 tapentadol 0.27, morphine 1.1 mg/kg i.p.). Spinal administration of the opioid antagonist naloxone or the α2 adrenoceptor antagonist yohimbine prior to systemic administration of equi-analgesic doses of tapentadol (1 mg/kg, i.p.) or morphine (3.16 mg/kg, i.p.) revealed pronounced influence on opioidergic and noradrenergic pathways for both compounds. Tapentadol was more sensitive towards both antagonists than was morphine, with ID50 values (i.t.) of 0.75 and 1.72 ng/animal naloxone and 1.56 and 2.04 ng/animal yohimbine, respectively. It is suggested that the antihyperalgesic action of systemically administered tapentadol is based on opioid spinal-supraspinal synergy as well as intrinsic spinally mediated MOR-NRI synergy.
Introduction

Neuropathic pain is defined as pain initiated or caused by a primary lesion or dysfunction in the nervous system (IASP) and occurs as a common consequence of diabetes, where 16-26% of diabetic patients suffer from diabetic polyneuropathy pain (Jensen, et al., 2006). While drugs with a number of different pharmacological mechanisms are available for treatment, many patients still suffer from neuropathic pain and there is a high medical need for the development of alternative, more efficacious and/or more tolerable treatment options (Finnerup, et al., 2010).

Streptozotocine (STZ) leads to depletion of pancreatic islet cells rendering rodents diabetic within 1-2 weeks after a single treatment. Symptoms of neuropathic pain such as hyperalgesia or allodynia can be demonstrated in these animals and clinically effective drugs such as the $\alpha_2\delta$-selective Ca$^{2+}$ channel subunit blocker pregabalin (Field, et al., 1999) and the norepinephrine (NE)/serotonin (5-HT) reuptake inhibitors duloxetine (Kuhad, et al., 2009) and venlafaxine (Marchand, et al., 2003) have been reported to show efficacy in this rodent model of diabetic polyneuropathic pain. The analgesic efficacy of opioids such as morphine in neuropathic pain has been demonstrated in clinical studies (Finnerup, et al., 2010).

Expression analysis of µ-opioid receptor (MOR) in the DRGs of rats with mononeuropathy reveals decreased mRNA levels and reduced inhibitory function of presynaptic spinal MOR (Kohno, et al., 2005a), which might explain limitations of opioid analgesia seen with mechanical stimuli in mononeuropathic rats (Bian, et al., 1999). In diabetic mice, morphine shows dose-dependent efficacy against heat hyperalgesia. However, heat nociception in non-diabetic controls is reduced in the same dose-range suggesting an anti-nociceptive rather than a selective anti-hyperalgesic effect of morphine in this model of polyneuropathic pain (Christoph, et al., 2010).
Co-administration of morphine by the intrathecal (i.t.) and intracerebroventricular (i.c.v.) routes reveals spinal-supraspinal synergy in acute nociception which might contribute to the high overall efficacy of opioids in moderate to severe pain states (Yeung and Rudy, 1980), and which was shown to involve bulbospinal MOR-induced spinal α2 adrenoceptor activation (Wigdor and Wilcox, 1987). In contrast, a similar site-site synergy could not be demonstrated in mononeuropathic pain models with mechanical stimulation (Bian, et al., 1995), where multisegmental elevation of spinal dynorphin was shown to be involved (Bian, et al., 1999; Malan, et al., 2000; Lai, et al., 2006). Intrathecal administration of dynorphin antiserum restored the site-site synergy, suggesting that the spinal cord is crucially important among the different levels of pain transmission along the neuraxis (Bian, et al., 1999). To our knowledge, neither polyneuropathic pain states nor heat hyperalgesia have been tested for the occurrence of opioid site-site synergy.

Tapentadol with its MOR agonism and NE reuptake inhibitory (NRI) activity (Tzschentke, et al., 2007) is considered to be a representative of a proposed new pharmacological class called MOR-NRI (Kress, 2010), and shows high efficacy in neuropathic and non-neuropathic chronic pain conditions (Afilalo and Morlion, 2013). The MOR affinity of tapentadol is 50-fold lower than that of morphine (Tzschentke, et al., 2007), while the potency in the spinal nerve ligation model in rats and in diabetic hyperalgesia in mice was shown to be in the same range as that of morphine (Christoph, et al., 2007; Christoph, et al., 2010; Schröder, et al., 2010). This shift in potency in vivo as compared to MOR affinity in vitro is thought to be due to the NRI component of tapentadol, which was shown to contribute more to the anti-hypersensitive effect in spinal nerve ligated rats than to the anti-nociceptive effect in naïve rats (Schröder, et al., 2010). Furthermore, isobolographic analysis suggests an intrinsic synergism of the two mechanisms of action of tapentadol in nociceptive and
neuropathic pain conditions in rats (Schröder, et al., 2011). This intrinsic synergy might be mediated mainly at the level of the spinal cord where both ascending and descending pain modulatory pathways sensitive to MOR activation and NRI are integrated, and where both mechanisms contribute to the systemic effect of tapentadol as demonstrated by spinal administration of opioid and noradrenergic antagonists in the spinal nerve ligation model (Bee, et al., 2011).

Thus, the spinal cord is involved in the site-site synergy of opioids. The aim of this study was to test, in a model of diabetic polyneuropathic pain, whether spinal-supraspinal synergy can be demonstrated for tapentadol, and to characterize the spinal contribution of MOR-NRI, which might lead to the intrinsic synergy of tapentadol.
Methods

Animals

Male C57BL/6 mice (18-20 g) (Charles River, Germany) were housed under standard conditions (room temperature 20°-24°C, 12-h light/dark cycle, relative air humidity 45%-70%, 15 air changes/h, air movement <0.2 m/s) with food and water available ad libitum, except for the time of the experiment. There were at least 5 days between delivery of the animals and the start of the experiments. Animal testing was performed in accordance with the recommendations and policies of the International Association for the Study of Pain (Zimmermann, 1983) and the German Animal Welfare Law. All study protocols were approved by the local government committee for animal research, which is also an ethics committee.

Streptozotocin model (diabetic polyneuropathy)

Diabetic heat hyperalgesia was tested as described previously (Christoph, et al., 2010). Mice, randomly assigned to treatment groups (STZ, citrate), were treated i.v. with 200 mg/kg STZ or vehicle (sodium citrate, pH 5). Induction of diabetes was confirmed by blood glucose levels >25 mM 1 week after STZ treatment. One and 2 weeks after treatment, diabetic and non-diabetic control animals were randomly allocated to the different treatment groups (n = 10) as outlined below. Animals were used for a maximum of 2 tests, with a wash-out period of at least 7 days. For nociceptive and hyperalgesic testing, animals were placed on a 50°C metal plate under a transparent Plexiglas box (13 x 13 x 10 cm, l x w x h) for 2 minute periods, and the number of nocifensive reactions (licking/shaking of the hind paws, licking of the genitals, jumping) was counted 30 minutes (baseline 1) and 15 minutes (baseline 2) before and 15, 30, 45 and 60 minutes after drug or vehicle treatment.
Experimental design

Methods of local administrations were adapted from Hylden and Wilcox for the i.t. (Hylden and Wilcox, 1980) and from Haley and McCormick for the i.c.v. route (Haley and McCormick, 1957) and were performed under brief isoflurane anesthesia.

Systemic administration: Dose-response curves for tapentadol and morphine were generated by assigning diabetic animals randomly to treatment groups of different doses of compound, and vehicle. Non-diabetic controls were treated with vehicle only. Combinations of spinal antagonists or vehicle with systemic tapentadol, morphine or vehicle were done by assigning diabetic animals randomly to one of the following treatment groups: vehicle, naloxone or yohimbine i.t. + tapentadol or vehicle i.p.; vehicle, naloxone or yohimbine i.t. + morphine or vehicle i.p. starting with i.t. administration of vehicle or antagonist followed 15 min later by i.p. administration of vehicle, tapentadol or morphine. Non-diabetic control animals were treated at the same time course with vehicle (i.t. + i.p.) only. Individual dose levels are indicated in the figures.

Spinal supraspinal interaction: Diabetic animals were randomly assigned to treatment groups. Each animal received two simultaneous administrations (i.t. + i.c.v.). Dose response curves for i.c.v. tapentadol and i.c.v. morphine were generated in animals that received an administration of i.t. vehicle. Dose response curves for i.t. tapentadol and i.t. morphine were generated in animals that received an administration of i.c.v. vehicle. The interaction studies were done by combining increasing dose combinations (i.c.v + i.t.) based on equi-analgesic ratios (tapentadol i.t. : i.c.v. = 1:2; morphine i.t. : i.c.v. = 1:150). Each dose-response curve was accompanied by a group of non-diabetic controls treated with i.c.v. + i.t. vehicle only. Individual dose levels are indicated in the figures.
Although the operators performing the behavioral tests were not formally "blinded" with respect to the treatment, they were not aware of the study hypothesis or the nature of differences between drugs.

**Data Analysis**

Primary data were the number of nocifensive reactions occurring within 2 minutes. Using baseline 2 of diabetic and of non-diabetic controls as 0% and 100% MPE, respectively, % maximal possible effects (MPE) were calculated. Antihyperalgesic ED50 as well as antagonistic ID50 values (with 95% confidence intervals [CIs]) were calculated by linear regression based on % MPE. Data were analyzed by means of a repeated measures analysis of variance (ANOVA) with post hoc Bonferroni test (level of significance P <0.05).

The isobolographic analysis was done as described by Tallarida et al., 1989 (Tallarida, et al., 1989). The isobologram, introduced by Loewe and Muschinek 1926 (Loewe and Muschinek, 1926), provides a graphical display of the respective dose by using rectangular coordinates (x,y) to represent equi-effective pairs of doses of drugs. In the calculation according to Tallarida (Tallarida, et al., 1989), the ED50 values of both components and their combination are used for comparison of equi-effective doses. The isobologram was constructed by connecting the ED50 of i.t. tapentadol plotted on the ordinate with the ED50 of i.c.v. tapentadol plotted on the abscissa, to obtain the line of additivity. The ED50 value of the combination of both routes of administration can be represented as point on the X-Y isobole. The ED50 of the combination was calculated based on the experimental data and statistically compared with the theoretically additive ED50 that would be expected if the combination was additive. Points on the isobole that are significantly below the line of additivity indicate synergistic (or super-additive) interaction. Combinations that are
significantly above this line indicate sub-additivity and those that do not differ significantly from the line of additivity represent additive interaction. Synergism is indicated by an interaction index $ED_{50, \text{experimental}}/ED_{50, \text{theoretical}}$ below 1.

Note that our definition of ‘$ED_{50}$ value’ differs from the original strict mathematical definition in that our $ED_{50}$ values refer to the calculated dose that would yield 50% of the MPE in the test population, rather than to the dose that would yield a given effect in 50% of the test population. Although this modified definition deviates from the formal mathematical definition, it is commonly used for the analysis of behavioral data.

**Drugs and Chemicals**

The following drugs were used: tapentadol HCl (M.W. 257.8; Grünenthal GmbH, Germany), morphine HCl (M.W. 321.8; Merck AG, Germany), naloxone HCl (Sigma, Germany), yohimbine HCl (Sigma, Germany) and streptozotocin (Sigma, Germany).

Central administration of drugs or vehicle was performed i.t. and i.c.v. under brief ether anesthesia. All drugs were dissolved in saline and injected at 5 µl/animal i.t. or i.c.v. and at 10 ml/kg i.p..

For all drugs, the salt form has been omitted from the text. All doses indicated refer to the respective salt form as indicated in this section.
Results

ED$_{50}$ values, Emax values and slopes of dose response curves (with 95% confidence limits) from all experiments are summarized in Table 1. The mean baseline 2 values (number of nocifensive reactions within 2 minutes) were in the range of 15.4 to 16.9 for non-diabetic controls and 35.3 to 37.5 for diabetic animals (Table 2).

Supraspinal Administration

When given by the i.c.v. route, tapentadol [F(4,45) = 198.46, p < 0.001] and morphine [F(4,45) = 147.75, p < 0.001] induced dose-dependent anti-hyperalgesic effects and reached efficacies of 95% and 77% MPE, respectively. Potencies differed by a factor of 45 as seen by comparison of the ED50 values (95% CI) of 0.180 (0.138-0.233) and 0.004 (0.001-0.009) µg/animal, respectively (Fig. 1 A).

Spinal Administration

After i.t. administration, tapentadol [F(4,45) = 218.22, p < 0.001] and morphine [F(4,45) = 83.61, p < 0.001] induced dose-dependent anti-hyperalgesia with efficacies of 92% and 80% MPE, respectively, and comparable potency, with ED50 values (95% CI) of 0.420 (0.258-0.580) and 0.547 (0.239-1.057) µg/animal, respectively (Fig. 1 B).

Spinal Supraspinal Interaction

Comparing the ED50 values of tapentadol resulted in a ratio i.t. : i.c.v. of 2:1 that was used for the analysis of combined equi-analgesic i.t. + i.c.v. administration. Simultaneous i.t. + i.c.v. administration of tapentadol induced dose-dependent anti-hyperalgesia [F(4,45) = 168.98, p < 0.001] with an efficacy of 83% MPE and an ED50 value (95% CI) of 0.053 (0.032-0.074) µg/animal. Isobolographic analysis of i.t., i.c.v., and combined i.t. + i.c.v. dose-response curves suggested site-site synergistic interaction of tapentadol with an interaction index of 0.181 (Tab. 1, Fig. 2). Morphine
was used in an equi-analgesic ratio i.t. : i.c.v. of 1:150 for simultaneous i.t. + i.c.v. administration and resulted in dose-dependent anti-hyperalgesia \[F(4,45) = 166.04, p < 0.001\] with an efficacy of 84% MPE and an ED50 value (95% CI) of 0.014 (0.003-0.026) µg/animal. Isobolographic analysis revealed site-site synergistic interaction of morphine with an interaction index of 0.0486 (Tab. 1).

**Systemic Administration**

When given by the i.p. route, tapentadol \[F(3,45) = 137.454, p < 0.001\] and morphine \[F(3,56) = 40.07, p < 0.001\] induced dose-dependent anti-hyperalgesia with efficacies of 82% and 84% MPE, respectively. Potencies differed by a factor of 0.25 as seen by comparison of the ED50 values (95% CI) of 0.274 (0.215-0.343) and 1.1 (0.83-1.51) mg/kg, respectively (Fig. 1 C).

**Spinal Mechanisms of Tapentadol**

While the potency ratio of i.c.v. administered tapentadol and morphine (1 : 45) correlates well with the MOR affinity ratio of both compounds in vitro (1 : 50), the potency ratio of tapentadol and morphine after i.t. administration (1 : 1) suggested the spinal cord to be crucial for the analgesic potency of tapentadol. Hence, we combined systemic i.p. administration of tapentadol with i.t. administration of naloxone and yohimbine.

Equi-analgesic doses of tapentadol (1 mg/kg i.p.) and morphine (3.16 mg/kg i.p.) were combined with i.t. pre-treatment of vehicle or antagonist and analyzed at the time of maximal efficacy (15 min after i.p. administration). ID50 values were calculated taking the mean (± SEM) of the control groups (tapentadol 87.1 ± 1.8 %MPE; morphine 77.0 ± 2.4 %MPE) as maximal effect. Dose-dependent antagonism was seen with naloxone (0.3-10 ng/animal, i.t.) for tapentadol (Fig. 3 A) and morphine (Fig. 3 B) resulting in an ID50 (95% confidence interval) of 0.75 (0.61 – 0.90) and 1.72 (1.22 – 2.22) ng/animal, respectively. The minimal effective dose (MED) was 1
ng/animal for tapentadol and 3.16 ng/animal for morphine. Likewise, yohimbine (0.3-10 ng/animal, i.t.) dose-dependently reduced the anti-hyperalgesic effects of tapentadol (Fig. 3 C) and morphine (Fig. 3 D) with ID50 values (95% confidence intervals) of 1.56 (1.33 – 1.82) and 2.04 (1.47 – 2.64) ng/animal, respectively. MEDs were identified at 1 ng/animal for tapentadol and 3.16 ng/animal for morphine.
Discussion

The present data characterize tapentadol as centrally acting analgesic in a mouse model of diabetic polyneuropathic pain. Dose-dependent anti-hyperalgesia was shown after i.c.v. administration of tapentadol and morphine (Fig. 1 A), with a potency difference of a factor 45, which is close to the difference in affinity (factor 50) of both drugs for MOR in vitro (Tzschentke, et al., 2007). Thus, the supraspinal analgesia of tapentadol seems to be mainly driven by MOR, and supraspinal tapentadol is clearly less potent as compared to supraspinal morphine. Similar data on both drugs reported for rodent models of acute somatic and visceral nociception after systemic and i.c.v. administration (Tzschentke, et al., 2006) are in line with this finding. As with the supraspinal route, spinal administration resulted in similar efficacy for tapentadol and morphine. However, the difference in potency seen on the supraspinal level was completely absent at the spinal level (Fig. 1 B). When given systemically via the i.p. route, tapentadol even showed a higher potency than morphine (Fig. 1 C). This corroborates data reported in the same model after i.v. administration, demonstrating similar potency and efficacy for tapentadol and morphine (Christoph, et al., 2010). Morphine as well as tapentadol have good central availability and to the best of our knowledge there is no reported evidence suggesting region-specific distribution within the CNS. Therefore, a comparison of potencies based on administered local and systemic dose rather than on measured central and peripheral drug concentrations seems to be justified. The latter would not be feasible in the current experimental setting with combined spinal/supraspinal or systemic/spinal administrations in a pathological model with a complex behavioral read-out. Loss of peripheral analgesic potency using heat stimulation has been demonstrated for morphine in a mouse model of mono-neuropathic pain, and it was hypothesized that loss of peripheral MOR expression is the main reason for this opioid insensitivity (Rashid, et al., 2004).
A clear rightward shift in morphine potency was reported (Rashid, et al., 2004) when comparing the i.c.v. and the i.t. route, which correlates well with our morphine data. In contrast, the spinal potency of tapentadol was shifted to a much smaller extent suggesting that the non-opioid mechanism of tapentadol contributes significantly to its analgesic potency at the spinal level. Furthermore, since similar potency ratios were found for tapentadol and morphine after systemic and spinal but not after supraspinal administration, it is hypothesized that the synergistic interaction of MOR activation and NRI as demonstrated with tapentadol in rat models of acute nociceptive and chronic neuropathic pain (Schröder, et al., 2011) is predominantly mediated at the spinal level.

Combined i.c.v. and i.t. administration of tapentadol resulted in an experimentally determined ED50 value that was significantly below the theoretical additive ED50 value (Fig. 2; Tab. 1), suggesting spinal-supraspinal synergy. This kind of site-site synergy is well known for opioids such as morphine in acute heat nociception (Yeung and Rudy, 1980; Wigdor and Wilcox, 1987; Roerig and Fujimoto, 1989) although it could not be demonstrated in mononeuropathic rats using tactile allodynia, due to complete loss of spinal efficacy after nerve ligation (Bian, et al., 1999). In light of these data it may seem surprising that site-site synergism could be demonstrated for morphine in this study (Tab. 1). However, thermal hyperalgesia and tactile allodynia are mechanistically distinct. Noxious thermal stimuli are believed to be transmitted primarily through high-threshold, thin, unmyelinated primary afferent C-fibers, while non-noxious tactile stimuli are thought to be transmitted mainly through low-threshold, large diameter, myelinated Aβ fibers (Ossipov, et al., 2000). Whereas morphine does not show site-site synergy in Aβ fiber mediated tactile allodynia, it is feasible that C fiber mediated heat hyperalgesia supports this kind of interaction. In line with this argument, MOR were shown to be localized mainly on presynaptic C-
fibers (Besse, et al., 1990; Scherrer, et al., 2009). Furthermore, mono- and polyneuropathic pain states might differ in terms of receptor regulation. Mononeuropathic lesions lead to decreased MOR expression on both A and C fibers (Kohno, et al., 2005b). In diabetic polyneuropathic rats the number and affinity of spinal MOR as measured by $^3$H]DAMGO binding was not altered, their function as measured by DAMGO-stimulated $^{35}$S[GTP$_\gamma$S binding was reduced as compared to control rats (Chen and Pan, 2003; Chen, et al., 2002). In addition, comparison of the present data measuring heat hyperalgesia in diabetic polyneuropathic mice, where morphine was much more potent after i.c.v. as compared to i.t. administration (Fig. 1, Tab. 1), with published data in rodent models of acute heat nociception demonstrating stronger potencies of spinal vs supraspinal morphine (Yeung and Rudy, 1980; Wigdor and Wilcox, 1987; Roerig and Fujimoto, 1989), suggest loss of spinal MOR activity also in diabetic mice. Indeed, the antihyperalgesic effect of i.t. morphine as measured by the paw pressure test was not completely lost but its potency was reduced two-fold in diabetic polyneuropathic rats as compared to control rats (Chen and Pan, 2003). On the other hand, the potency of supraspinal morphine was markedly increased in this study as compared to acute nociception (Wigdor and Wilcox, 1987). This difference might be explained both by changes in MOR sensitivity in diabetic as compared to non-diabetic mice and by differences in the nociceptive test reflecting a spinal reflex in the tail flick test and contribution of supraspinal input in the hot plate test. Nevertheless, as demonstrated in the current study, spinal MOR appears to be sufficiently available to maintain the opioidergic spinal-supraspinal synergy. In contrast to morphine, tapentadol contributes not only MOR agonism but also NRI activity, which is able to amplify spinal $\alpha$2-adrenoceptor mediated inhibition of pain transmission (Tzschentke, et al., 2007), and thus amplifies the opioid-induced norepinephrine-based site-site synergy at the spinal level. Taken together, the
findings from local administration of tapentadol and morphine suggest that the spinal cord has a key role in the analgesic activity of tapentadol. Therefore, spinal administration of the MOR antagonist naloxone and the α2-adrenergic antagonist yohimbine was used to further analyze the spinal contribution to the anti-hyperalgesic effects of systemic tapentadol in diabetic mice. Both antagonists are able to completely block the anti-hyperalgesic effect of tapentadol and morphine in a dose-dependent manner. While ID50 values tend to be smaller for naloxone as compared to yohimbine, MEDs are identical (1 ng/animal tapentadol; 3.16 ng/animal morphine) suggesting comparable contribution of opioid and noradrenergic components for both drugs at the spinal level. Interestingly, there is a higher sensitivity of tapentadol for both antagonists. This finding supports the concept of intrinsic synergism of tapentadol on the spinal level, as reported earlier (Schröder, et al., 2011). Synergistic interaction depends on two independent mechanisms that are able to potentiate each other. If one of the contributing mechanisms is blocked, the remaining mechanism can still work by itself, although only with a clearly weaker potency as compared to the synergistic effect. Regarding the effect of systemic morphine, the strong spinal noradrenergic component as shown by the potent ID50 and full antagonistic efficacy of yohimbine was unexpected. Several aspects can be considered. First, systemic morphine very likely will have a strong supraspinal action as suggested by the comparison of local efficacies (Table 1). MORs are located in the brainstem and are able to activate descending inhibitory pathways, resulting in antinociception mediated by spinal release of norepinephrine (Millan, 2002). Since the potency of i.c.v. morphine is more than 100-fold higher as compared to i.t. morphine, a potent noradrenergic effect of systemic morphine can be expected. Second, animals with neuropathic pain show a tendency towards increased noradrenergic sensitivity. For example, i.t. injection of norepinephrine or clonidine produced stronger anti-
nociception in diabetic mice as compared to non-diabetic controls (Omiya, et al., 2008; Omiya, et al., 2005). Furthermore, an increased density of α2-adrenoceptors was found in diabetic mice (Omiya, et al., 2008), and increased noradrenergic innervation was demonstrated in animals with mononeuropathic lesions (Ma and Eisenach, 2003).

Opioids are not the only class of analgesics known to activate descending inhibitory mechanisms. For example, gabapentin and ketamine have also been shown to increase spinal release of norepinephrine (Kawamata et al., 2000; Hayashida et al., 2008). However, unlike pure opioids, gabapentin or ketamine, tapentadol not only activates descending inhibitory projections by a (presumably) supraspinal mechanism, but its NRI activity also blocks the reuptake of NE at the spinal level thus greatly amplifying the effect of the supraspinally-mediated increase in NE release. As outlined above, this may be of particular relevance under neuropathic conditions.

In summary, the present study demonstrates, in diabetic heat hyperalgesia, site-site synergy of MOR agonists, with a strong supraspinal, MOR-dependent descending inhibitory drive projecting to the spinal cord. Furthermore, the intrinsic synergism of the combined MOR-NRI mechanism of tapentadol, that was reported in mono-neuropathic rats (Schröder, et al., 2011), appears to arise mainly from the spinal cord level, as suggested by comparison of systemic, spinal and supraspinal anti-hyperalgesic potencies. Both, spinal-suprasinal site-site synergy and local intrinsic MOR-NRI synergy in the spinal cord can contribute to the high analgesic potency and efficacy of systemic tapentadol in patients with neuropathic pain.
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Authorship Contributions

Participated in research design: Christoph, Schröder, De Vry, Tzschentke

Conducted experiments: Christoph

Performed data analysis: Christoph, Tallarida

Wrote or contributed to the writing of the manuscript: Christoph, Schröder, Tallarida, Tzschentke
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Footnotes

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Legends for Figures

Fig. 1: Effect of tapentadol and morphine on heat hyperalgesia in diabetic mice (A) after i.c.v. administration; (B) after i.t. administration; (C) after i.p. administration.

Fig. 2: Isobolographic analysis of the effect of tapentadol on heat hyperalgesia in diabetic mice after i.t., i.c.v and combined administration.

Fig.3: Effect of i.t. naloxone (0.316-10 ng/animal) on the effect of (A) tapentadol (1 mg/kg i.p.) or (B) morphine (3.16 mg/kg i.p.) on heat hyperalgesia in diabetic mice.
Effect of i.t. yohimbine (0.316-10 ng/animal) on the effect of (C) tapentadol (1 mg/kg i.p.) or (D) morphine (3.16 mg/kg i.p.) on heat hyperalgesia in diabetic mice.
Table 1. ED$_{50}$-values, slopes and Emax values with 95% confidence limits of tapentadol and morphine in diabetic mice.

<table>
<thead>
<tr>
<th>Drug route</th>
<th>ED$_{50}$ value (µg/animal) (95% confidence limits)</th>
<th>Slope (95% confidence limits)</th>
<th>Emax (mean % MPE) (95% confidence limits)</th>
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</tr>
<tr>
<td>Drug</td>
<td>Vehicle -</td>
<td>-</td>
<td></td>
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</tr>
<tr>
<td>-</td>
<td>0.420 (0.258-0.580)</td>
<td>0.547 (0.239-1.057)</td>
<td>1</td>
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<tr>
<td>-</td>
<td>48.69 (34.25-61.13)</td>
<td>22.75 (14.75-30.69)</td>
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</tr>
<tr>
<td>-</td>
<td>91.72 (86.50-96.94)</td>
<td>79.58 (72.36-86.80)</td>
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<tr>
<td>Vehicle</td>
<td>Drug -</td>
<td>-</td>
<td></td>
<td>45</td>
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<tr>
<td>-</td>
<td>0.180 (0.138-0.233)</td>
<td>0.004 (0.001-0.009)</td>
<td></td>
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<tr>
<td>-</td>
<td>59.91 (47.12-72.69)</td>
<td>19.86 (11.51-28.21)</td>
<td></td>
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<tr>
<td>-</td>
<td>95.17 (89.46-100.87)</td>
<td>76.75 (64.31-89.18)</td>
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<tr>
<td>Drug</td>
<td>Drug -</td>
<td>-</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>-</td>
<td>0.053 (0.032-0.074) a</td>
<td>0.014 (0.003-0.026) a</td>
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<tr>
<td>-</td>
<td>43.46 (28.16-58.75)</td>
<td>28.51 (13.61-43.87)</td>
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</tr>
<tr>
<td>-</td>
<td>82.92 (71.61-94.23)</td>
<td>80.94 (74.04-87.84)</td>
<td></td>
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<tr>
<td>Drug</td>
<td>Drug -</td>
<td>-</td>
<td></td>
<td>n.a.</td>
</tr>
<tr>
<td>-</td>
<td>0.291 (0.229-0.353) b</td>
<td>0.288 (0.101-0.556) b</td>
<td></td>
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</tr>
<tr>
<td>-</td>
<td>n.a.</td>
<td>n.a.</td>
<td></td>
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<tr>
<td>-</td>
<td>n.a.</td>
<td>n.a.</td>
<td></td>
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</tr>
<tr>
<td>-</td>
<td>0.274 (0.215-0.343) c</td>
<td>1.107 (0.830-1.506) c</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>54.81 (41.78-67.84)</td>
<td>65.19 (45.62-84.77)</td>
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<tr>
<td>-</td>
<td>82.44 (75.63-89.26)</td>
<td>83.61 (70.30-96.92)</td>
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</tr>
</tbody>
</table>

i.t.: intrathecal, i.c.v.: intracerebroventricular, i.p.: intraperitoneal, a experimental data; b theoretical additive data; c ED$_{50}$ value (mg/kg); n.a.: not applicable, Tap: tapentadol; Mor: morphine
Table 2. Baseline 2 number of nocifensive reactions within 2 minutes of tapentadol and morphine in diabetic and non-diabetic mice.

<table>
<thead>
<tr>
<th>Drug route</th>
<th>Baseline 2 (mean ± S.E.M.)</th>
<th>Non-diabetic control group</th>
<th>Diabetic Tapentadol group</th>
<th>Diabetic Morphine group</th>
</tr>
</thead>
<tbody>
<tr>
<td>i.t.</td>
<td>i.c.v.</td>
<td>i.p.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drug Vehicle - -</td>
<td>16.2 ± 0.3</td>
<td>36.8 ± 0.4</td>
<td>36.6 ± 0.5</td>
<td></td>
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<tr>
<td>Vehicle Drug - -</td>
<td>35.5 ± 0.6</td>
<td>36.6 ± 0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drug Drug - -</td>
<td>16.4 ± 0.4 (Tap) 16.7 ± 0.4 (Mor)</td>
<td>36.3 ± 0.5</td>
<td>37.5 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>- - Drug</td>
<td>16.0 ± 0.1 (Tap) 15.4 ± 0.3 (Mor)</td>
<td>36.1 ± 0.3</td>
<td>35.5 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>Nx - Drug</td>
<td>16.3 ± 0.5 (Tap + 1, Mor + 1) 16.9 ± 0.3 (Tap + 0.3, 3) 16.9 ± 0.4 (Mor + 3) 16.2 ± 0.3 (Mor + 10)</td>
<td>36.9 ± 0.3 (Tap + 0.3) 36.9 ± 0.3 (Tap + 1) 36.7 ± 0.3 (Tap + 3) 36.4 ± 0.4 (Mor + 1)</td>
<td>36.4 ± 0.4 (Mor + 3) 37.2 ± 0.4 (Mor + 10)</td>
<td></td>
</tr>
<tr>
<td>Yoh - Drug</td>
<td>16.3 ± 0.5 (Tap + 1, Mor + 1) 16.9 ± 0.3 (Tap + 0.3, 3, 10)</td>
<td>37.0 ± 0.3 (Tap + 0.3) 36.7 ± 0.4 (Tap + 1)</td>
<td>36.1 ± 0.5 (Mor + 1) 37.4 ± 0.3 (Mor + 3)</td>
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<tr>
<td></td>
<td></td>
<td>16.9 ± 0.4 (Mor + 3)</td>
<td>37.2 ± 0.2 (Tap + 3)</td>
<td>37.5 ± 0.3 (Mor + 3)</td>
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<tr>
<td></td>
<td></td>
<td>16.2 ± 0.2 (Mor + 10)</td>
<td>36.6 ± 0.3 (Tap + 10)</td>
<td></td>
</tr>
</tbody>
</table>

i.t.: intrathecal, i.c.v.: intracerebroventricular, i.p.: intraperitoneal, Tap: tapentadol, Mor: morphine, Nx: naloxone, Yoh: yohimbine
Figure 2

Tapentadol i.t. = 0.420 (0.258 - 0.580)
Tapentadol i.c.v. = 0.180 (0.138 - 0.223)
Experimental combination:
part of Tapentadol i.t. = 0.053 (0.032 - 0.074)
part of Tapentadol i.c.v. = 0.035 (0.022 - 0.049)
Theoretical additive value:
part of Tapentadol i.t. = 0.018 (0.013 - 0.022)
part of Tapentadol i.c.v. = 0.291 (0.229 - 0.353)

ED_{50} (95% CI) mg/kg i.t. / i.c.v