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RESEARCH PAPER

Increased morphine analgesia and reduced side effects in mice lacking the *tac1* gene

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Background and purpose: Although morphine is a very effective analgesic, its narrow therapeutic index and severe side effects limit its therapeutic use. Previous studies indicated that the pharmacological responses of opioids are modulated by genetic and pharmacological invalidation of tachykinin receptors. Here we address the role of substance P and neurokinin A, which are both encoded by the tachykinin 1 (*tac1*) gene, as modulators of opioid effects.
Experimental approach: The analgesic and side effect potential of morphine was compared between wild-type and *tac1* null mutant mice.
Key results: Morphine was a more potent analgesic in *tac1* null mutant mice, that is, in the absence of substance P/neurokinin A signalling. Interestingly, the most serious side effect of acute morphine, that is respiratory depression, was reduced in $tac1^{-/-}$ animals. Comparing the addictive potential of morphine in wild-type and knockout animals we found that morphine preference was similar between the genotypes. However, the aversive effect of withdrawal precipitated by naloxone in morphine-dependent animals was significantly reduced in *tac1* knockout mice. Behavioural sensitization, the underlying mechanism of addiction, was also significantly lower in $tac1^{-/-}$ mice.
Conclusion and implications: The analgesic potential of morphine was increased in *tac1* knockout mice. In contrast, both the ventilatory suppressing effect and the addictive potential of morphine were reduced. These results suggest that reducing activity of the tachykinin system may be a possible strategy to improve the pharmacological potential of morphine.
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Keywords: substance P; knockout; morphine; analgesia; respiration; conditioned place preference; naloxone; withdrawal; sensitization

Abbreviations: CPA, conditioned place aversion; CPP, conditioned place preference; *f*, respiratory frequency; HVR, hypoxic ventilatory response; NK_1 receptor, neurokinin 1 receptor; NKA, neurokinin A; NKB, neurokinin B; SP, substance P; *tac1* gene, tachykinin 1 gene; *T*e, expiration time; *T*i, inspiration time; VE, minute ventilation; VT, tidal volume

Introduction

Morphine, the main active constituent of opium, has been used for centuries to relieve pain. Activation of the μ-opioid receptor is responsible for the majority of the physiological effects of morphine, as demonstrated in animals with a genetic deletion of μ-opioid receptors (Matthes *et al.*, 1996). Today, morphine is a widely used drug for acute and chronic pain relief, for preoperative sedation and as a supplement to anaesthesia. However, the therapeutic potential of morphine is limited by its serious side effects and narrow therapeutic index (Breivik *et al.*, 2006). The most prominent side effects are respiratory depression, decreased gastrointestinal motility and the development of dependence after chronic administration. Reduction of these side effects would enhance the therapeutic potential of morphine. Such methods could include the targeting of transmitter systems that modulate μ-receptor signalling.

A number of studies focused on the connection between the tachykinin and opioid systems. Opioids and tachykinins have opposite effect on pain sensation, because opioid agonists reduce pain (Trescot *et al.*, 2008), while injection of substance P (SP) – an agonist of the neurokinin 1 (NK_1) receptor (nomenclature follows Alexander *et al.*, 2009) – elicits pain in humans (Pedersen-Bjergaard *et al.*, 1989; Tegeder *et al.*, 2002) and in animals (Andoh *et al.*, 1998). Their interaction on pain sensitivity is antagonistic, whereby SP blocks the

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anti-nociceptive effect of morphine (Sawynok *et al*., 1984), and, *vice versa*, morphine reduces both SP-induced pain (Hylden and Wilcox, 1983; Cridland and Henry, 1988) and hyperalgesia (Hylden and Wilcox, 1983; Cridland and Henry, 1988). Experimental data suggest that a balance between the pro-algesic SP/NK_1 and the analgesic opioid signalling systems could be of importance for normal pain sensation. Unfortunately, NK_1 receptor antagonists failed to show significant analgesic effect in humans, although in preclinical studies they effectively alleviated the symptoms of post-operative, inflammatory and neuropathic pain. A number of pharmaceutical companies started clinical trials with their newly developed NK_1 antagonists, but these compounds remained inactive against post-operative and osteoarthritis pain, diabetic neuropathy and migraine. These data suggest that SP/NK_1 neurotransmission in pain signalling is less important in humans than in rodents.

Substance P and the related peptide neurokinin A (NKA) are derived from the same precursor protein encoded by the tachykinin 1 (*tac1*) gene. SP shows a higher binding affinity to the NK_1 receptor among the tachykinin receptors, whereas the preferred receptor of NKA is the neurokinin 2 receptor (NK_2). NK_1 receptors are widely expressed in the brain, and areas involved in reward, motor activity, pain sensation and regulation of vegetative functions are particularly rich in NK_1 receptors (Otsuka and Yoshioka, 1993). The concentration of NK_2 receptors is low in the brain except in the hippocampus, thalamus and septum (Saffroy *et al*., 2001). Thus, the majority of the effects on the CNS subsequent to the genetic deletion of *tac1* are a result of decreased NK_1 signalling.

The *tac1* knockout animals are viable, fertile and have a rather moderate behavioural phenotype: they show reduced anxiety and diminished susceptibility to depression-like behaviour (Bilkei-Gorzo *et al*., 2002). The pain sensitivity of this strain is either normal or reduced – depending on the intensity (Zimmer *et al*., 1998; Basbaum, 1999) and probably on the modality (Mazario and Basbaum, 2007) of pain exposure. It is thought that SP is involved in the intensity coding of pain signals (Bilkei-Gorzo *et al*., 2005).

An antagonistic role of the opioid and SP/NK_1 systems is also observed in the regulation of respiration, as μ-opioid receptor agonists suppress ventilation, while NK_1 receptor agonists have a stimulatory effect. Furthermore, μ-opioid and NK_1 receptors are found on the same neurons in the pre-Bötzinger complex, a brainstem area known to be involved in the regulation of respiration (Smith *et al*., 1991). Endogenous opioids (endorphins, enkephalins and endomorphins), as well as endogenous SP are suggested to play an important role in the regulation of ventilation during hypoxia (see Moss and Laferriere, 2002; Wickstrom *et al*., 2004).

NK_1 receptor function is also important for the addictive properties of morphine. Morphine is not rewarding for animals lacking NK_1 receptors (Murtra *et al*., 2000) and these mice do not self-administer morphine (Ripley *et al*., 2002). In wild-type animals the release and concentration of SP is elevated during morphine withdrawal (Zhou *et al*., 1998; Gu *et al*., 2005). This elevation could contribute to the severity of withdrawal symptoms (Gu *et al*., 2005), because SP mimics some symptoms of withdrawal (Johnston and Chahl, 1991), and withdrawal symptoms can be reduced with NK_1 receptor antagonists (Maldonado *et al*., 1993; Buccafusco and Shuster, 1997; Trang *et al*., 2002; Michaud and Couture, 2003).

These data suggest that the tachykinin system influences both the main therapeutic effect of morphine and also its side effects. The primary aim of the present study was to test the hypothesis about the importance of SP/NK_1 receptor signalling on the physiological effects of morphine. Thus, we compared the effect of morphine on pain sensation, intestinal motility, ventilation, rewarding effects and the severity of physical and affective withdrawal symptoms in wild-type and *tac1* null mutant animals. Our secondary aim was to compare the reactivity to morphine between *tac1* and *NK_1* deficient mice. Therefore we used the morphine-induced analgesia (De Felipe *et al*., 1998), sensitization (Ripley *et al*., 2002), place preference and naloxone-induced place aversion (Murtra *et al*., 2000) paradigms as described using the *NK_1* knockout mouse strain.

Our results showed that the analgesic potential of morphine was increased in animals lacking SP, whereas the severity of the side effects was decreased. A similarly broader therapeutic window of morphine could be observed in NK_1 receptor null mutants: in these mice, morphine analgesia was unaltered – but the reduction of the addictive effects of morphine was more pronounced.

Methods

Animals

All animal care and the experimental protocols were approved by the local ethical committees, that is, Stockholms Norra Djurförsöksetiska nämnd (Dnr N69/07) and Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen (50.203. 2 BN 34 19/05).

The generation of mice with a targeted mutation of the *tac1* gene has been described previously (Zimmer *et al*., 1998). Adult (8–12 weeks of age) male *tac1* knockout (*tac1*$^{-/-}$) and wild-type mice were used. *tac1*$^{-/-}$ animals have been crossed more than 10 generations to C57BL/6J mice and were therefore congenic for this genetic background. To avoid genetic drift we restart the homozygous breeding colonies after each fourth generation using heterozygous breeding pairs that were received from a backcross of the knockout line with mice from the original background strain. Animals were kept in a room with a reversed light/dark cycle (lights on: 9:00 PM, lights off: 9:00 AM) for behavioural studies, whereas mice for the ventilatory test were kept under normal light conditions to facilitate comparison with previous studies. Food and water were available *ad libitum*.

Analgesic activity

Animals were treated subcutaneously (s.c.) with vehicle or morphine. Pain sensitivity was measured 30 min after the treatment in hot-plate test (De Felipe *et al*., 1998). The animals were placed individually on a hot-plate apparatus (TSE Systems GmbH, Bad Homburg, Germany), which consisted of a 25 × 25 cm metal plate maintained at 52 ± 0.1°C and surrounded by a 40 cm high Plexiglas wall. The latency of the first sign of pain – for example licking, shaking of the

hind-paw or jumping, was determined to the nearest 0.1 s. The cut-off time was 60 s (Bilkei-Gorzo *et al.*, 2004; Enard *et al.*, 2009). The ED_{50} value of morphine with confidence intervals was calculated from the fitted dose–response curves using the Prism (Graphpad Inc., San Diego, CA, USA) software. The minimal effective dose is the lowest dose producing a significant effect according to one-way ANOVA followed by Bonferroni *t*-test.

Motility of the upper intestinal tract

Upper intestinal tract motility was measured as the distance which that the non-absorbable marker carmine travels along the small intestine when administered intragastrically. The animals were treated first s.c. with vehicle or morphine and 30 min later received a 0.3 mL carmine suspension p.o. (6% in 0.1% methylcellulose solution). Twenty minutes after the oral treatment, animals were killed and the intestines were removed from the pylorus to the caecum. The length of the carmine stained gut segment and total length of the small intestine was measured. Upper intestinal motility was expressed as the distance the carmine head travelled as percentage of the total length of the small intestine. The effect of morphine was assessed using one-way ANOVA separately in both genotypes. The ED_{50} with confidence intervals was also calculated.

Respiration: protocols and ventilatory measurements

Ventilatory measurements were made using dual chamber plethysmograhy (Frappell and Mortola, 2000). A mask covering the mouth and nostrils was affixed with dental impression material (Impregum F, 3M ESPE, St. Paul, MN, USA). The animals then breathe from one chamber (the headbox) while pressure changes are measured in a second, volume calibrated, chamber (100 mL, MTA, Karolinska University Hospital, Stockholm, Sweden) using a pneumotach (Hans Rudolph Inc., Shawnee, KS, USA). Humidified air or a hypoxic mixture (8% O_2 in N_2) was supplied to the headbox by manually switching between gas reservoirs. The gas flow through the headbox was maintained at 50 mL·min^{-1} resulting in a complete change of gases within 15 s. The signal was digitally converted with a sampling rate of 200 Hz and analysed using Power Lab software (Chart v5.5.4, PowerLab systems, ADInstruments, Colorado Springs, CO, USA). The obtained values were then used to calculate respiratory frequency (*f*; breaths·min^{-1}), inspiration time (*T*i), expiration time (*T*e), tidal volume (VT) and minute ventilation (VE). VT and VE were divided by body weight (g) and expressed as μL·g^{-1} and mL·g^{-1}·min^{-1} respectively. All studies were performed between 09:00 h and 15:00 h to control for possible daily fluctuations in respiratory patterns. The ambient temperature within the chamber was measured continuously using a digital thermometer (Model BAT-12, Physitemp Instruments Inc., Clifton, NJ, USA) and recorded in parallel with the flow signal. It was maintained at 28 ± 0.5°C in accordance with the thermoneutral range for mice of similar age by immersing the chamber in a thermostat-controlled water bath. The temperature in the mouth was controlled when the animal was placed into the box, before and after respiratory recordings were performed.

Animals received i.p. injection of either morphine hydrochloride (10 mg·kg^{-1}) or 0.9% saline in a volume of 10 mL·kg^{-1} and were kept 25 min in the chamber in room air for acclimatization before the recording started. The time span is corresponding to the peak effect of morphine (Kalvass *et al.*, 2007). After injection and initial acclimatization all animals were exposed to normoxic air for 3 min while collecting baseline values, followed by three episodes of intermittent hypoxia (8% O_2 balanced with N_2). Each hypoxic period lasted 5 min followed by a normoxic recovery period of 2 min. The animals were weighed after the final recording. Analysis of the respiratory signal was made on 30 s segments without movement artefacts that were chosen subjectively within assigned intervals. The number of animals in each group was $n = 6$, coming from at least two different litters. Data was analysed using three-way ANOVA (Statistica software, StatSoft Scandinavia AB, Uppsala, Sweden) with genotype and treatment as between factor, and normoxia or hypoxia as within factor. *Post hoc* comparison was made using the Tukey HSD test.

Locomotor sensitization for morphine

Twenty-three *tac1*$^{-/-}$ (10 in the saline-treated group and 13 in the morphine-treated group) and 28 *tac1*$^{+/+}$ mice (16 in the saline-treated group and 12 in the morphine-treated group) were used in this experiment. The test procedure was carried out in a dimly illuminated (20 lx at the ground level of the arena) sound-attenuated procedure room as described previously for testing morphine effects on the motility of NK_1 receptor knockout mice (Ripley *et al.*, 2002). Animals were placed into the centre of an open-field arena (45 × 45 × 22 cm), and their motor activity was measured by an automatic system (TSE Systems GmbH) as mean speed in cm·s^{-1} during the test session.

The motor activity of the mice was first measured on three consecutive days without drug treatment to habituate them to the test environment. From day 4 during the next 15 days, mice received two i.p. injections of morphine (15 mg·kg^{-1}) per day. The morning injections were given between 9:00 h and 11:20 h, and the evening injections between 18:30 h and 19:30 h. The locomotor activity was measured after the first treatment and then on every third day (days 7, 10, 13, 16 and 19) 10 min after the morning injection. The chronic treatment ended on day 19, and the animals remained drug-free between days 20 and 25. On day 26 they received a challenge dose of morphine (15 mg·kg^{-1}) or saline and locomotor activity was measured again. Their data were analysed using three-way ANOVA with treatment and genotype as between subject factors, and day as within subject factor. Subsequent one-way ANOVAs were calculated separately to each genotype followed by Bonferroni *t*-test. A significant difference between the effects of repeated and acute morphine treatments on the motor activity indicates the development of sensitization.

Conditioned place preference

The rewarding effects of morphine were evaluated using an unbiased conditioned place preference (CPP) paradigm, as described previously (Murtra *et al.*, 2000). CPP was tested in a

three-chamber apparatus (TSE Systems GmbH), in which a small middle chamber separated the two large side chambers each differing in floor and wall conditions. The boxes were equipped with one line of photocells placed above the floor to monitor the movement and location of the mice by an automatic system (TSE Systems GmbH). Eleven animals per group (*tac1*$^{-/-}$ and the control *tac1*$^{+/+}$ mice) were treated with saline or 1.5, 3 and 6 mg·kg^{-1} morphine i.p. in a volume of 10 mL·kg^{-1}. On day 1, in the pre-conditioning phase, mice were allowed to move freely in the three chambers for 30 min. On days 2–4, in the conditioning period, mice were confined to one large chamber for 20 min immediately after they had received saline. Four hours later, they received morphine (or again saline in case of the control group) and were confined to the other side chamber for 20 min. Administrations and compartments were arranged in a counterbalanced manner. The post-conditioning phase was on day 5, when mice were placed in the middle chamber and allowed to move freely in the three chambers for 30 min like in day 1. A score was calculated for each mouse as the difference between the post-conditioning and pre-conditioning time spent in the drug-paired compartment. Development of place preference was assessed comparing the score values between the saline- and morphine-treated groups using one-way ANOVA.

Conditioned place aversion

For conditioned place aversion (CPA) analysis we used the same place preference device as described above (CPP) and the protocol as described (Murtra *et al*., 2000). To produce morphine dependence, 12–12 mice from both genotypes received two daily injections of morphine. In the pre-conditioning phase at day 1, untreated animals were allowed to move freely in the three compartments for 30 min. In the following 4 days, morphine was injected s.c. at 9:00 h and 19:00 h with increasing doses: day 2, 10 mg·kg^{-1}; day 3, 20 mg·kg^{-1}; day 4, 30 mg·kg^{-1}; day 5, 40 mg·kg^{-1}. Conditioning of naloxone-induced place aversion was conducted on day 6. One hour after morphine treatment using a 50 mg·kg^{-1} dose, saline was injected i.p and the mice were confined to one chamber for 20 min. Four hours later, they were again treated with 50 mg·kg^{-1} morphine. One hour later, they were injected i.p. with 1 mg·kg^{-1} naloxone and confined to the other chamber for 20 min. On day 7 in the post-conditioning phase, mice were placed in the middle chamber and allowed to move freely in the three chambers for 30 min. A score was calculated for each mouse as the difference between the post-conditioning and pre-conditioning time spent in the drug-paired compartment. Difference in the place aversion between the groups was assessed by unpaired Student's *t*-test.

Physical withdrawal signs

Ten wild-type and 10 knockout mice were treated with morphine thrice daily (09:00 h, 13:00 h and 17:00 h) s.c. with increasing doses: day 1: 10 mg·kg^{-1}, day 2: 20 mg·kg^{-1} days 3 and 4: 40 mg·kg^{-1}. On the test day (day 5) again 40 mg·kg^{-1} morphine was administered followed by 30 mg·kg^{-1} naloxone 3 h later (Kest *et al*., 2002). The animals were placed just after the naloxone treatment to an open-field apparatus and the number of jumps was measured by an automatic system (TSE Systems GmbH) for 15 min and finally in the 16th minute the absence or presence of paw tremor, air chewing or soft stool was additionally assessed.

Materials

Morphine hydrochloride (Meda AB, Solna, Sweden and Merck, Darmstadt, Germany) and naloxone hydrochloride (Sigma-Aldrich, Deisenhofen, Germany) were used. Both drugs were dissolved in sterile 0.9% saline and administered in a volume of 10 mL·kg^{-1}.

Results

Analgesic activity in the hot-plate test

The latencies of the first sign of pain in the hot-plate test were not different between wild-type and *tac1*$^{-/-}$ animals (Figure 1). Morphine significantly increased the latency of first sign of pain in the hot-plate test in both genotypes (*tac1*$^{+/+}$ $F_{4,39}$ = 42.05; $P < 0.001$ and by *tac1*$^{-/-}$ $F_{4,39}$ = 30.69; $P < 0.001$). Subsequent Bonferroni *post hoc* comparison revealed that the minimal effective dose of morphine was 7.5 mg·kg^{-1} in wild-type and 3.75 mg·kg^{-1} in *tac1*$^{-/-}$ animals (Figure 1). The higher sensitivity of *tac1*$^{-/-}$ mice to the analgesic effect of morphine is also shown by a lower ED_{50} value of morphine in this strain [5.73 mg·kg^{-1} with 5.17–6.35 mg·kg^{-1} confidence intervals (CI) in *tac1*$^{-/-}$ and 8.98 mg·kg^{-1} with 8.80–9.16 mg·kg^{-1} (CI) in *tac1*$^{+/+}$ animals].

Intestinal motility

The intestinal motility was the same in vehicle-treated *tac1*$^{+/+}$ and *tac1*$^{-/-}$ mice (Figure 2). Morphine potently reduced the intestinal motility in a broad dose range in both strains (*tac1*$^{+/+}$ $F_{8,72}$ = 52.79; $P < 0.001$ and by *tac1*$^{-/-}$ $F_{8,71}$ = 20.00; $P < 0.001$). The similar ED_{50} values of morphine with overlapping confidence intervals in wild-type and knockout mice [1.12 (CI, 1.00–1.26) mg·kg^{-1} in *tac1*$^{+/+}$ and 0.92 (CI, 0.82–1.03)

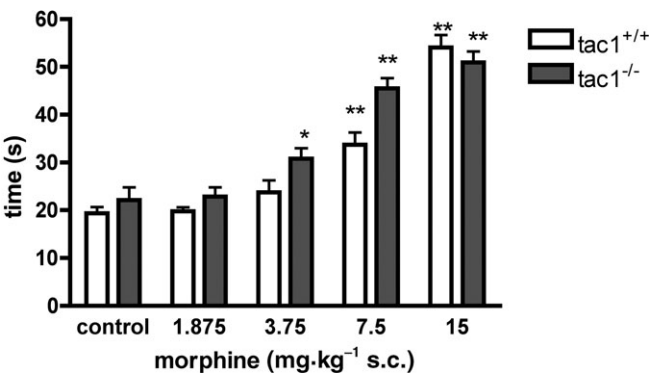

Figure 1 Effect of morphine in the hot-plate test in *tac1*$^{+/+}$ and *tac1*$^{-/-}$ mice. Knockout mice were more sensitive to the analgesic effect of morphine. *$P < 0.05$; **$P < 0.01$ difference between control and morphine-treated groups (one-way ANOVA followed by Bonferroni *t*-test, $n = 8$ in each group).

mg·kg^{-1} in *tac1*$^{-/-}$ animals] showed that genetic deletion of *tac1* did not significantly influence the intestinal motility-reducing effect of morphine.

Morphine effect on respiration under normoxic conditions

tac1 null mutant mice displayed increased basal ventilation (VE/g) as compared with *tac1*$^{+/+}$, due to non-significant increases in both *f* and VT/g. Morphine significantly lowered *f* in both *tac1*$^{+/+}$ and *tac1*$^{-/-}$ mice to the same level. However, this decrease was relatively larger in *tac1*$^{-/-}$ mice due to the higher basal *f*. A similar response was seen on VT/g, with a stronger effect on *tac1*$^{-/-}$ mice. Consequently, morphine depressed ventilation to a similar level in both groups. No significant differences were seen in *T*i or *T*e between *tac1*$^{+/+}$ and *tac1*$^{-/-}$ mice, but morphine significantly prolonged *T*i within wild-type group and both *T*i and *T*e within *tac1*$^{-/-}$ group. Basal respiratory parameters are summarized in Table 1.

Morphine effect on respiration during hypoxia

Wild-type animals displayed a normal hypoxic ventilatory response (HVR), which is a significant increase in *f*, VT/g and VE/g, to the initial hypoxic challenge. In contrast, *tac1*$^{-/-}$ mice did not significantly increase *f*, VT/g or VE/g although ventilation reached similar levels as in *tac1*$^{+/+}$ animals, due to already high baseline levels (VT/g) in *tac1*$^{-/-}$ mice. The larger increase in *f* in *tac1*$^{+/+}$ animals was due to a significant shortening of both *T*i ($P < 0.05$) and *T*e ($P < 0.05$) as compared with baseline. Morphine strongly suppressed the ventilatory response to intermittent hypoxia in *tac1*$^{+/+}$ animals ($P < 0.05$ for relative VE/g). In contrast, morphine exposure caused a significant increase in relative VE/g in *tac1*$^{-/-}$ animals as compared with morphine-treated *tac1*$^{+/+}$ animals ($P < 0.01$ in each hypoxic period), once again as a result of a significant increase in *f* ($P < 0.01$ in each hypoxic period). The increase in *f* was related to a significant shortening of both *T*i and *T*e during first and second hypoxic period ($P < 0.05$ and $P < 0.03$, respectively), whereas in the third period only to a significant shortening of *T*e ($P < 0.05$). Hence, *tac1*$^{-/-}$ mice reacted to morphine with a relatively enhanced HVR as compared with baseline during repeated hypoxia, which was not seen in saline-exposed animals. Morphine exposed *tac1*$^{+/+}$ displayed a weakened HVR to repeated hypoxia where VE/g became significantly lower in third hypoxic period as compared with an already depressed baseline ($P < 0.001$). Figure 3 shows the morphine effect on respiration parameters in absolute values.

Furthermore, the severe respiratory depression following hypoxic exposures in morphine-treated animals caused a 2/6 mortality rate in the wild-type group at the end of the third hypoxic period. In contrast, no mortality was seen in *tac1*$^{-/-}$ animals.

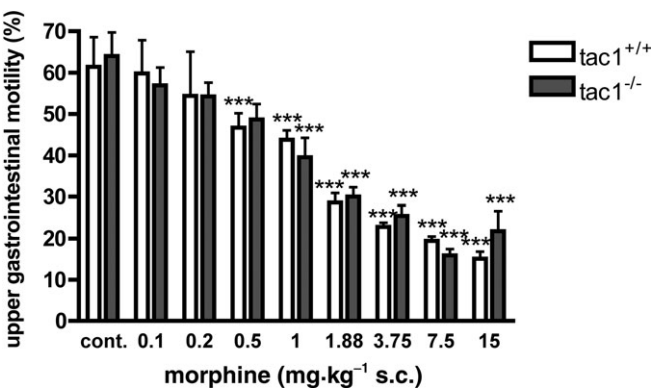

Figure 2 Effect of morphine on the motility of the upper intestinal tract was similar between *tac1*$^{+/+}$ and *tac1*$^{-/-}$ mice; $n = 8$ in each group. ***$P < 0.001$ difference between control and morphine-treated groups (one-way ANOVA followed by Bonferroni *t*-test).

Locomotor sensitization to morphine

Repeated administration of the same dose of morphine had different effects on the locomotor activity of *tac1*$^{+/+}$ and *tac1*$^{-/-}$ animals as shown by the significant interaction between treatment, time and genotype ($F_{9,42} = 2.66$; $P < 0.01$). Acute treatment with 15 mg·kg^{-1} morphine had no effect on speed of mice, regardless of the genotype (day 4 in Figure 4). However, sensitization to the locomotor effects of morphine developed in wild-type mice, because the speed of the animals after repeated morphine treatment was significantly higher compared with the acute administration, starting from day 7 onwards. In contrast, there was no locomotor sensitization in *tac1* null mutants during the chronic treatment. We only observed an enhanced response on day 26, when the animals were challenged with morphine after a drug-free period (Figure 4).

Conditioned place preference to morphine

Morphine treatment induced a place preference as shown by a significant difference between saline- and morphine-treated

Table 1 Ventilatory parameters under normoxic conditions in morphine (MO)- or vehicle (NaCl)-treated *tac1*$^{+/+}$ and *tac1*$^{-/-}$ animals

Normal values	f *(breaths·min^{-1})*	*VT/bw (µL·g^{-1})*	*VE/bw (mL·g^{-1}·min^{-1})*	T*i (s)*	T*e (s)*
tac1$^{+/+}$ NaCl	224 ± 23	1.6 ± 0.15	0.36 ± 0.027	0.12 ± 0.008	0.14 ± 0.026
tac1$^{+/+}$ MO	167 ± 10*	0.36 ± 0.051*	0.059 ± 0.010*	0.17 ± 0.009**	0.18 ± 0.008
tac1$^{-/-}$ NaCl	263 ± 11	2.23 ± 0.27	0.57 ± 0.056†	0.12 ± 0.006	0.12 ± 0.007
tac1$^{-/-}$ MO	163 ± 10***	0.34 ± 0.53***	0.055 ± 0.012***	0.19 ± 0.015***	0.18 ± 0.008*

Morphine significantly lowered frequency (*f*), tidal volume/body weight (VT/bw) and ventilation/body weight (VE/bw) within *tac1*$^{+/+}$ and *tac1*$^{-/-}$ group. VE/bw was significantly higher in *tac1*$^{-/-}$ mice as compared with wild-type. Following *f* changes, morphine significantly prolonged inspiration time (*T*i) in both genotypes whereas expiration time (*T*e) was significantly prolonged only in *tac1*$^{-/-}$ animals.
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, control (NaCl) compared with morphine (MO)-treated; †$P < 0.05$ *tac1*$^{+/+}$ compared with *tac1*$^{-/-}$; one-way ANOVA followed by Bonferroni test, $n = 6$ in each group.

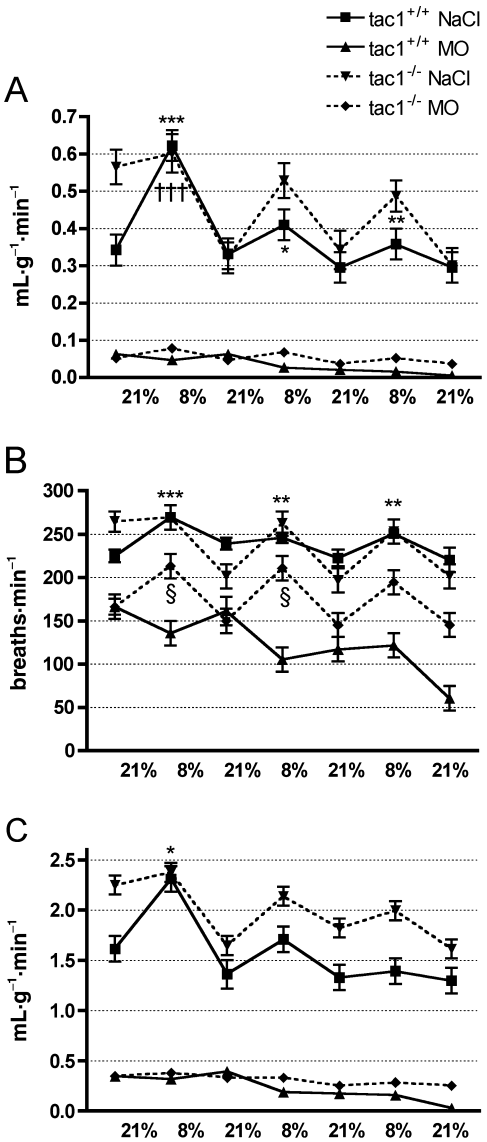

Figure 3 Effect of morphine on respiration parameters during normoxia and repeated hypoxia. (A) showing ventilation/body weight (VE/bw in mL·g^{-1}·min^{-1}); (B) frequency (*f* in breaths·min^{-1}) and (C) tidal volume/body weight (VT/bw in µL·g^{-1}). Morphine (MO) severely depressed ventilation in both *tac1*$^{+/+}$ and *tac1*$^{-/-}$ mice. However, transgenic animals displayed a significantly stronger hypoxic ventilatory response resulting from an increase in *f*. **P* < 0.05; ***P* < 0.01; ****P* < 0.001 significant difference between saline- and morphine-treated *tac1*$^{+/+}$. †*P* < 0.05; ††*P* < 0.01 significant difference between saline- and morphine-treated *tac1*$^{-/-}$ mice. §*P* < 0.05 significant difference between morphine-treated *tac1*$^{+/+}$ and *tac1*$^{-/-}$ animals (three-way ANOVA followed by Tukey test, *n* = 6 in each group).

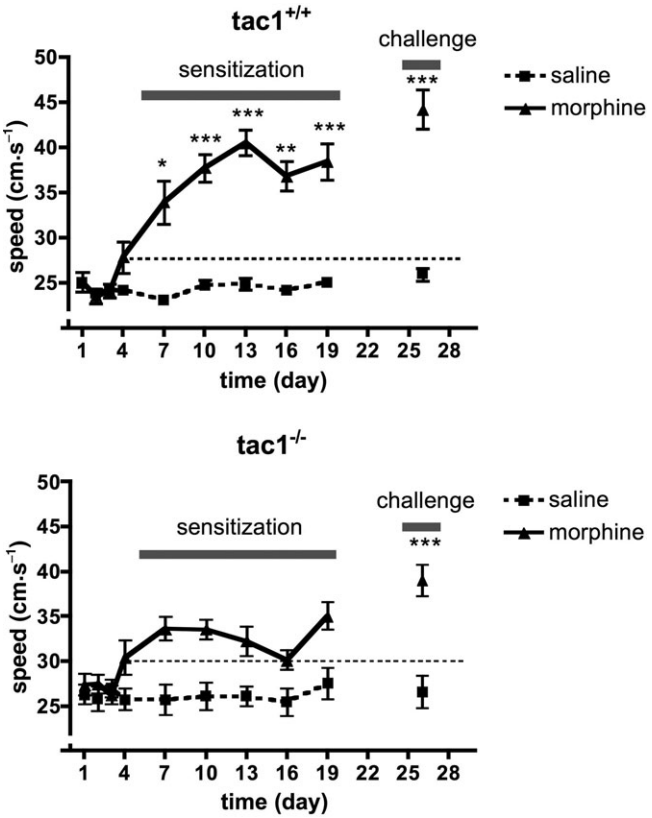

Figure 4 Effect of repeated morphine administration on the speed of wild-type and *tac1*$^{-/-}$ mice measured in open-field apparatus. The animals received saline in the first 3 days in each group, the morphine was injected first at day 4 (15 mg·kg^{-1} s.c.), and then twice daily with the same dose until day 19. After an 8 day break in the treatments the animals received again a single injection of saline or morphine at day 27. Control animals received saline during the experiment (NaCl). Sensitization to morphine effects was present from day 7 in wild-type, whereas only at day 27 in *tac1*$^{-/-}$ mice. **P* < 0.05; ***P* < 0.01; ****P* < 0.001 significant difference between first and following morphine treatments (one-way ANOVA followed by Bonferroni *t*-test, *n* = 10–16). Stacked line indicates the mean speed of animals after the first (acute) morphine treatment at day 4.

groups in wild-type ($F_{3,44}$ = 3.95; P < 0.05) and also in knockout strains ($F_{3,44}$ = 4.77; P < 0.01). Bonferroni *post hoc* comparisons revealed an increasingly robust preference for the morphine associated compartment with increasing drug doses in both strains (1.5 mg·kg^{-1}, P > 0.05; 3 mg·kg^{-1}, P < 0.05; 6 mg·kg^{-1}, P < 0.01) (Figure 5A).

Conditioned place aversion to naloxone

In order to evaluate the aversive effects of morphine withdrawal, we tested naloxone-precipitated place aversion in *tac1*$^{+/+}$ and *tac1*$^{-/-}$ morphine-dependent mice. The severity of psychical effects of morphine withdrawal was reduced in the null mutants, as shown by a significantly lower score values in *tac1*$^{-/-}$ mice (t_{21} = 2.71; P < 0.05) (Figure 5B).

Physical withdrawal signs

We finally investigated the physical withdrawal signs. Mice chronically treated with morphine showed characteristical behavioural changes after naloxone treatment in both genotypes. The severity of physical withdrawal signs was similar between the strains (Table 2).

Discussion

In this study, we addressed the role of the tachykinin neuropeptides SP and NKA as modulators of the pharmacological effects of morphine. Using mice with a genetic deletion of the *tac1* gene, we studied the effects of morphine on thermal pain

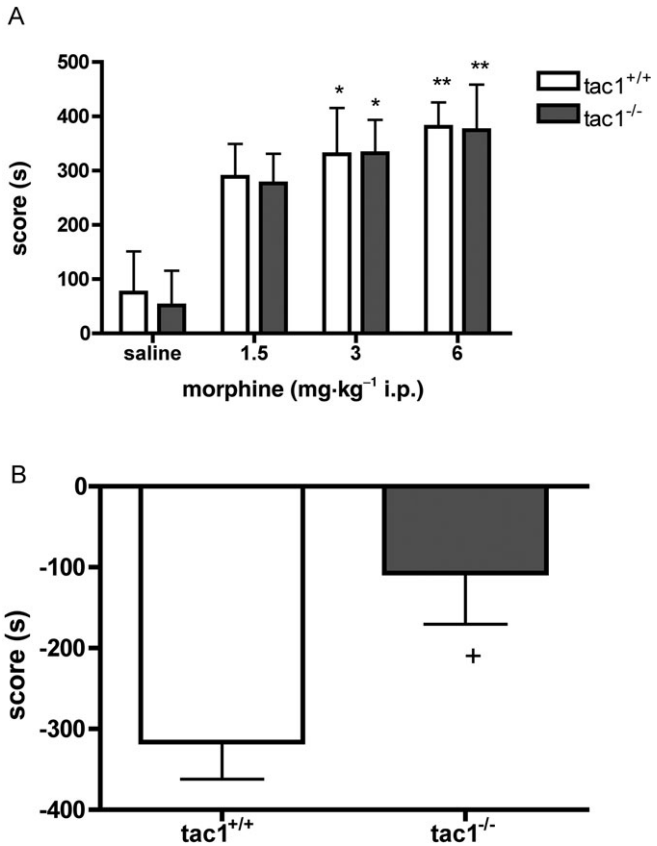

Figure 5 (A) Conditioned place preference induced by morphine treatment was similar between wild-type and *tac1*$^{-/-}$ animals. The score is calculated as difference between the time spent in the drug-associated compartment during the post- and pre-conditioning phase. *P < 0.05; **P < 0.01 difference between saline- and morphine-treated group with the same genotype (one-way ANOVA followed by Bonferroni t-test; n = 11 in each group). (B) Place aversion induced by a single naloxone injection in morphine-treated animals was significantly higher in *tac1*$^{+/+}$ as in *tac1*$^{-/-}$ strain. The score is calculated as difference between the time spent in the drug-associated compartment during the post- and pre-conditioning phase. +P < 0.05 difference between score values according to unpaired Student's t-test, n = 12 in both groups.

Table 2 Physical signs of withdrawal

Withdrawal symptom	tac1$^{+/+}$	tac1$^{-/-}$
Jumping (mean ± SEM)	48.5 ± 4.70	38.5 ± 5.29
Paw tremor (% animals)	40	30
Air chewing (% animals)	100	100
Soft stool (% animals)	30	20

The intensity of physical withdrawal signs precipitated with 30 mg·kg^{-1} naloxone in morphine-treated mice was similar in wild-type and *tac1* null mutant animals (n = 10 in both genotypes).

sensation, as well as some of clinically relevant side effects such as the suppression of intestinal motility and respiration, morphine reward, sensitization and somatic dependence. Generally, we found a moderate increase in the analgesic efficacy of morphine, whereas some of its side effects – suppression of HVR, affective withdrawal signs, development of sensitization – were reduced. The rewarding effect of morphine, severity of physical withdrawal symptoms and suppression of gut motility remained, however, unaltered in the null mutants. Taken together we conclude that the pharmacological profile of morphine was more favourable in animals with genetic deletion of *tac1*.

The *tac1* null mutants displayed similar pain sensitivity as wild-type mice in the mild thermal pain model, supporting the hypothesis that SP does not influence the sensitivity to moderate pain (Cao *et al*., 1998; Martin *et al*., 2004). We originally found a slightly decreased pain sensitivity in *tac1*$^{-/-}$ mice (Zimmer *et al*., 1998). It is thus likely that the originally described phenotype at low intensity pain conditions, which we observed on a mixed 129SV × C57BL/6 genetic background, is lost after further crossing to the CB57BL/6 genetic background. It is well known that the 129 and C57BL/6 mouse strains differ very substantially in animal models of pain (Mogil *et al*., 1999).

The higher sensitivity of *tac1*$^{-/-}$ animals to the analgesic effect of morphine was shown by a lower minimum effective dose and ED_{50} of morphine in this strain. A similar result was reported with this strain using the tail-flick test (Guan *et al*., 2005), a model of spinal analgesia. As hot-plate test reactivity involves supraspinal responses, our results suggest that the increased analgesic potential of morphine in the absence of SP and NKA is not restricted to the spinal level. The mechanism underlying the enhanced sensitivity of animals lacking *tac1* to the analgesic effects of morphine is not clear, but a disturbed balance (Gillman *et al*., 1981) between the opioid and tachykinin system activities in the null mutants could be responsible for this effect.

Morphine exerted similar effects on the intestinal system in *tac1*$^{-/-}$ and in wild-type mice, suggesting that there is no similar interaction between opioid and tachykinin systems in the intestinal tract as described in pain-sensory pathways. Although opioid and neurokinin receptor agonists have opposite effects on the intestinal activity, the tonic activity of tachykinins seems to be low. NK_1 receptor antagonists did not influence gastrointestinal motor functions in humans (Madsen and Fuglsang, 2008) or gastric mechanical activity in mice (Mule *et al*., 2006). Thus, in absence of functional antagonism between the opioid and tachykinin systems, chronic reduction of NK_1 and NK_2 receptor signalling does not appear to influence the activity of opioid receptors.

Morphine severely depressed ventilation both during normoxia and hypoxic challenge in wild-type animals, but in knockouts this effect was significantly reduced. Additionally, our study demonstrated several interesting aspects on respiratory control and the involvement of the tachykinin and opioid systems. First, mice lacking the *tac1* gene display a significantly elevated VE/g during basal conditions (Table 1). In contrast, pups lacking SP and NKA have a normal basal respiratory pattern at post-natal days 2–10 (Berner *et al*., 2007). This indicates a role for these neuropeptides also in basal respiration and the development of compensatory mechanisms. Alterations in 5-HT signalling (this amine is known to be co-released with SP) and/or the dopaminergic system, both normally activated during hypoxia, may be part of this compensatory mechanism. Second, morphine has a similar effect on *tac1*$^{+/+}$ and *tac1*$^{-/-}$ mice on ventilation during basal conditions. Hence, the tachykinin system does not seem

to be involved in the sensitivity to morphine during normoxic conditions. Finally, whereas wild-type animals withstood hypoxic challenges following morphine administration poorly, *tac1*$^{-/-}$ mice had a better HVR and outcome.

To our knowledge, the respiratory effect of morphine has been poorly described during hypoxia in rodents. The blunted HVR seen in wild-type animals following morphine treatment in this study demonstrates that the decreased respiratory response to hypercapnia following morphine is also relevant for hypoxia. In contrast, *tac1*$^{-/-}$ mice display a relatively normal HVR during intermittent hypoxia, which may be indicative of compensatory systems activated by hypoxia (Figure 3). Another explanation could be that endorphins, normally released during hypoxia, have a competitive μ-opioid receptor affinity and thereby reduces the inhibitory effect on the respiratory drive of morphine. Furthermore, endorphins themselves have a positive effect on respiratory drive when applied to the pre-Bötzinger complex (delineated by μ-opioid and NK_1 receptors) and other related areas in the brainstem (Lonergan *et al*., 2003).

The effect of lacking the *tac1* gene on the addictive potential of morphine was assessed by morphine-induced place preference, severity of the withdrawal symptoms and development of sensitization. In the sensitization experiment, morphine produced a similar increase in motor activity after the first treatment at day 4 in both strains (Figure 4), showing that the acute effect of morphine on the motility is not influenced by *tac1*. Sensitization to morphine after repeated injections was observed from day 7 in wild-type mice, which persisted even 6 days after terminating the drug treatment. In *tac1*$^{-/-}$ animals, however, increased sensitivity to morphine was not detected until day 26, thus sensitization to morphine was reduced in animals lacking *tac1*. It has previously been reported that tolerance, an important underlying mechanism of addiction, is lacking in *tac1*$^{-/-}$ mice (Guan *et al*., 2005). In this study we demonstrated that in *tac1* null mutant animals the behavioural sensitization to morphine was also significantly reduced. In contrast, the rewarding effect of morphine was unchanged in null mutants, as morphine induced a similar increase in the time spent with the drug-associated chamber in the place preference test in both strains.

In addition to the rewarding properties, the development of withdrawal symptoms after the cessation of drug administration contributes to the addictive potential of a drug. Importantly, we found that *tac1*$^{-/-}$ animals were less sensitive to the affective symptoms of morphine withdrawal shown by a diminished naloxone-induced place aversion in morphine-treated *tac1*$^{-/-}$ mice, whereas the severity of physical withdrawal symptoms was similar between mutant and wild-type mice. A possible reason of the difference in *tac1*$^{-/-}$ phenotype in the CPP and CPA models might be that C57BL6 mice (which represent the genetic background of the *tac1*$^{-/-}$ line) are very sensitive to the rewarding properties of morphine and less sensitive to the aversive effects of naloxone (Solecki *et al*., 2009). Therefore, a reduction in the SP/NK_1 signalling may influence CPA, but not CPP or the physical signs of withdrawal.

A secondary aim of the present study was to compare our results of the *tac1* knockout mice with data published about the morphine reactivity of *NK1* knockouts. It is known that differences in the genetic background (Bilkei-Gorzo *et al*., 2004; McCutcheon *et al*., 2008), in housing conditions and research environment (Crabbe *et al*., 1999; Bilkei-Gorzo *et al*., 2008) significantly influence the phenotype of the strain and often makes it difficult to compare the results between research groups. Furthermore, when comparing the phenotypes of the receptor knockout *NK1*$^{-/-}$ and the ligand knockout *tac1*$^{-/-}$ strains, important additional factors may contribute to the differences. Although both strains are genetic models of reduced SP/NK_1 receptor signalling, there are also considerable differences. In the *NK1* knockout mice, signalling through this receptor is completely abolished, whereas in *tac1*$^{-/-}$ mice it is only reduced, because the tachykinin peptide neurokinin B (NKB) is still expressed in *tac1*$^{-/-}$ animals. The expression of NK_1 receptors and *tac2*, gene encoding NKB, overlaps in brain areas involved in the physiological activity of morphine (Otsuka and Yoshioka, 1993; Duarte *et al*., 2006). NKB binds with two order of magnitude lower affinity to the septide-sensitive binding site of NK_1 receptors, compared with SP (Torrens *et al*., 2000) but it is probably high enough (K_i = 7.84 nM in mice) to maintain a reduced activity on the NK_1 receptors. Second, the lack of NKA in *tac1*$^{-/-}$ mice reduces also the NK_2 receptor signalling, although it probably does not influence the effects of morphine. Third, it was suggested that protachykinin is involved in the sorting of δ-opioid receptors and therefore a deletion of *tac1* might influence morphine effects, independent of effects on NK_1 receptor signalling (Guan *et al*., 2005). In a recent publication, however, very strong arguments were presented against a direct interaction between protachykinin and δ-opioid receptors (Scherrer *et al*., 2009). Although our study was not intended to investigate these conflicting hypotheses, one can expect similar or milder morphine reactivity in *tac1* compared with NK_1 knockout mice, if the reduced NK_1 receptor signalling is solely responsible for this phenotype, whereas a stronger phenotype in the *tac1*$^{-/-}$ strain would be expected if Guan's theory is right. Interestingly, the analgesic properties of morphine were increased in *tac1*$^{-/-}$ but not in *NK1*$^{-/-}$ animals (Table 3). Deletion of either *tac1* or the NK_1 receptor reduced the addictive properties of morphine, although the phenotype of the *tac1* null mutants was weaker as reported in the receptor mutants. Morphine was not rewarding in mice with a selective deletion of the NK_1 receptor, as shown by the lack of morphine place preference (Murtra *et al*., 2000) or self-administration (Ripley *et al*., 2002). Surprisingly, *tac1* null mutants had similar preference to the morphine associated compartments as wild-type

Table 3 Summary of effects of morphine in *tac1* and NK_1 null mutant animals

Effect of morphine on	$NK_1^{-/-}$	tac1$^{-/-}$
Hot-plate reactivity	Normal[1]	Increased
Tail-flick reactivity	Reduced[1]	Increased[4]
Place preference	Reduced[2]	Normal
Affective signs of withdrawal	Reduced[2]	Reduced
Physical signs of withdrawal	Reduced[2]	Normal
Sensitization	Reduced[3]	Reduced
Tolerance	Not tested	Reduced[4]

Some results in the Table are from earlier work as indicated: [1](De Felipe *et al*., 1998); [2](Murtra *et al*., 2000); [3](Ripley *et al*., 2002); [4](Guan *et al*., 2005).

animals. It was shown that amygdalar NK_1 receptor signalling is crucial for morphine place preference (Gadd *et al*., 2003). In *tac1* knockout animals however, NKB is expressed in the amygdala (Duarte *et al*., 2006), which could maintain a NK_1 receptor activity necessary for the development of morphine place preference.

Both the physical and affective withdrawal signs were reduced (Murtra *et al*., 2000) in *NK*$_1^{-/-}$ mice, whereas in *tac1*$^{-/-}$ animals, only the affective symptoms were diminished. This result suggests that the presence of SP/NKA and therefore an unimpaired NK_1/NK_2 receptor signalling is necessary for the affective, but not for the physical symptoms of morphine withdrawal, although the differences in the genetic background of the receptor and ligand null mutants could also be responsible for the dissimilarities in the phenotypes (McCutcheon *et al*., 2008; Solecki *et al*., 2009).

Sensitization to morphine after repeated administration was almost identically reduced in the *tac1*$^{-/-}$ and *NK*$_1^{-/-}$ strains using the same protocol. Hence, the effect of *tac1* deletion on the addictive properties of morphine is weaker than the null mutation of NK_1 receptors and the proposed effect of protachykinin on δ-opioid receptor sorting – if it exists – appears not to have any influence on this phenotype. The *tac1*$^{-/-}$ mice showed a higher sensitivity to the analgesic effect of morphine than NK_1 receptor knockouts, which may be a non-receptor mediated effect of the *tac1* deletion. However, in the light of recent results (Scherrer *et al*., 2009) we suppose that it rather represents an effect of the genetic background on the phenotype.

In conclusion, the results in the present study demonstrate an increased analgesic potential of morphine and a contrasting reduction in the respiration suppressor effects and in the addictive potential of morphine in *tac1* knockout mice. These results suggest that reducing the activity in tachykinin system may be a possible strategy to improve the pharmacological potential of morphine.

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Conflicts of interest

All the authors declare that they have no conflicts of interest.

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