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RESEARCH PAPER

Inhibitors of catechol-*O*-methyltransferase sensitize mice to pain

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BACKGROUND AND PURPOSE

Catechol-*O*-methyltransferase (COMT) inhibitors are used in Parkinson's disease in which pain is an important symptom. COMT polymorphisms modulate pain and opioid analgesia in humans. In rats, COMT inhibitors have been shown to be pro-nociceptive in acute pain models, but also to attenuate allodynia and hyperalgesia in a model of diabetic neuropathy. Here, we have assessed the effects of acute and repeated administrations of COMT inhibitors on mechanical, thermal and carrageenan-induced nociception in male mice.

EXPERIMENTAL APPROACH

We used single and repeated administration of a peripherally restricted, short-acting (nitecapone) and also a centrally acting (3,5-dinitrocatechol, OR-486) COMT inhibitor. We also tested CGP 28014, an indirect inhibitor of COMT enzyme. Effects of OR-486 on thermal nociception were also studied in COMT deficient mice. Effects on spinal pathways were assessed in rats given intrathecal nitecapone.

KEY RESULTS

After single administration, both nitecapone and OR-486 reduced mechanical nociceptive thresholds and thermal nociceptive latencies (hot plate test) at 2 and 3 h, regardless of their brain penetration. These effects were still present after chronic treatment with COMT inhibitors for 5 days. Intraplantar injection of carrageenan reduced nociceptive latencies and both COMT inhibitors potentiated this reduction without modifying inflammation. CGP 28014 shortened paw flick latencies. OR-486 did not modify hot plate times in *Comt* gene deficient mice. Intrathecal nitecapone modified neither thermal nor mechanical nociception.

CONCLUSIONS AND IMPLICATIONS

Pro-nociceptive effects of COMT inhibitors were confirmed. The pro-nociceptive effects were primarily mediated via mechanisms acting outside the brain and spinal cord. COMT protein was required for these actions.

Abbreviations

COMT, catechol-*O*-methyltransferase; DOPEGAL, 3,4-dihydroxyphenyl-glycolaldehyde; MPE%, percentage of the maximum possible effect; OR-486, 3,5-dinitrocatechol

Introduction

Catechol-*O*-methyltransferase (COMT) metabolizes catecholamines in glial cells and postsynaptic neurons in the brain (Männistö and Kaakkola, 1999). Much less is known about the location of COMT in the peripheral nerves but COMT exists in peripheral neurons, sensory ganglia and spinal cord although a majority of activity is extraneuronal, for example, in epithelial cells (Guldberg and Marsden,

1975; Karhunen *et al.*, 1996). Metabolic activity of COMT is subject to gene polymorphisms, such as the Val108/158Met polymorphism, leading to significant differences in COMT activity in human tissues (Weinshilboum and Raymond, 1977; Boudikova *et al.*, 1990). Polymorphisms, in which COMT activity is low, are related to several functional differences including increased sensitivity to pain and increased response to opioids (Akil *et al.*, 2003; Zubieta *et al.*, 2003; Kim *et al.*, 2004; 2006; Oswald *et al.*, 2004; Rakvåg *et al.*, 2005).

Only a few studies have been carried out using COMT inhibitors in experimental animal models of pain and the outcome of these studies is variable and seems to depend on the model and COMT inhibitor used. OR-486 (3,5-dinitrocatechol) and tolcapone, which penetrate the blood brain barrier (Nissinen *et al.*, 1988; Männistö and Tuomainen, 1991; Männistö *et al.*, 1992), shortened nociceptive latencies to thermal stimuli and thresholds of mechanical nociception in rats suggesting that low COMT activity is associated with enhanced nociception (Diatchenko *et al.*, 2005; 2006; Nackley *et al.*, 2007). On the other hand, a short-acting COMT inhibitor, nitecapone, with poor penetration of the blood brain barrier (Männistö *et al.*, 1988; Nissinen *et al.*, 1988), had an anti-hyperalgesic effect in diabetic rats (Pertovaara *et al.*, 2001). In COMT-deficient mice, the effects of morphine and stress-induced analgesia were reduced in the tail flick test, while the morphine response was enhanced in the hot plate test (Kambur *et al.*, 2008).

Peripheral COMT inhibitors are increasingly used in treatment of Parkinson's disease as adjuncts to *L*-DOPA therapy to alleviate mainly motor symptoms. COMT inhibitors protect a significant amount of *L*-DOPA from metabolism and prolong the action of *L*-DOPA, allowing a reduction of its doses (Männistö and Kaakkola, 1999). Pain is a prevalent symptom in Parkinson's disease (Beiske *et al.*, 2009). Thus it is relevant to know how COMT inhibitors could modulate pain and, if they do, whether these effects are due to central or peripheral mechanisms.

We have now taken the advantage of the availability of both brain-penetrating and peripherally restricted COMT inhibitors (Männistö and Kaakkola, 1999; Huotari *et al.*, 2002), and studied the effects of acute and repeated administration of COMT inhibitors on mechanical and thermal nociception, carrageenan-induced hyperalgesia and allodynia. Based on earlier studies, we hypothesized that administration of the centrally acting COMT inhibitor, OR-486, would increase nociception, as shown by a change in nociceptive responses, such as decreased reaction/withdrawal time in tests of thermal nociception and decreased mechanical nociceptive thresholds. We also hypothesized that if the pro-nociceptive effects of COMT inhibition are mediated by peripheral mechanisms, they would also be seen after the administration of nitecapone. The spinal effect was studied separately by intrathecal administration of a COMT inhibitor in rats. In addition, we also assessed the effects of an atypical COMT inhibitor CGP 28014 (Waldmeier *et al.*, 1990a,b), the effect of which on nociception is still unknown. This compound does not directly inhibit COMT activity but it decreases catecholamine metabolism, apparently by preventing the access of the substrates to the enzyme (Waldmeier *et al.*, 1990a,b; Männistö and Kaakkola, 1999). Finally, the effects of OR-486 on thermal nociception were studied in COMT deficient mice.

Our results confirmed the pro-nociceptive effects of COMT inhibitors and found these effects to be primarily mediated by action at peripheral sites.

Methods

Animals

All animal care and experimental procedures were approved by the institutional animal investigation committee and the provincial government of Southern Finland. A total of 196 male mice of the C57BL/6J background (Harlan, Horst, the Netherlands), aged 3–4 months and weighing 26–34 g were used. Of those 53 were used in the nociceptive tests after acute and repeated administration of COMT inhibitors, 30 were used in nociceptive tests after administration of the atypical COMT inhibitor, CGP 28014, and 88 in the enzyme activity measurements. Also 25 mice of the same genetic background with a deletion of COMT (COMT knockout; for a detailed description of these mice, see Tammimäki *et al.*, 2008) were used in the behavioural experiments. In intrathecal administration experiments, 15 adult male Wistar rats (Harlan, Horst, the Netherlands) weighing 200–300 g were used.

Animals were not pre-selected for nociceptive or behavioural experiments. The animals were housed in individual plastic cages in ambient temperature (22–24°C) and artificial lighting with a fixed 12 h light–dark cycle. All experiments were conducted during the light phase of the day. Pellet food and tap water were available *ad libitum*. The animals were habituated to handling and to the testing environment for four consecutive days before the experiments. The mice spent 10–15 min and the rat 30 min, every day in each apparatus and the order of habituation was the same as the testing order during experiments.

Catechol-O-methyltransferase activity assay

Catechol-*O*-methyltransferase activity was measured in a separate group of 88 mice that were killed 1 or 3 h after i.p. administration of vehicle or the compounds, and the liver, striatum, prefrontal cortical tissue and spinal cord were rapidly dissected and placed in pre-cooled plastic centrifuge tubes on dry ice. Samples were stored in –80°C until assayed. The COMT assay and subsequent chromatographic analysis were performed as described earlier (Nissinen and Männistö, 1984; Reenilä *et al.*, 1995). The protein concentration of the samples was determined with the Pierce protein assay kit based on the bicinchoninic acid method (Pierce, Rockford, IL, USA). The specific activity of COMT was expressed as pmol vanillic acid formed in 1 min per mg of protein in the sample. All tissues were not taken from all mice, and both time points were not studied from all tissues. There were 7–8 samples from the striatum, prefrontal cortex and liver at 1 h, and 13–16 striatal, 15–19 spinal cord and 5–6 liver samples at 3 h. In addition, we had 28 vehicle controls for the striatal samples and 14–17 for other tissues.

Design of nociceptive studies

In the main study, the mice were allocated into three parallel groups (saline and two COMT inhibitors) that were used in all assays. After habituation, nociceptive baselines were measured during four consecutive days. Then the mice received nitecapone (30 mg·kg^{-1}, i.p.; n = 16), OR-486 (30 mg·kg^{-1}, i.p.; n = 16) or vehicle (i.p.; n = 21) and nociceptive latencies were measured again 2 and 3 h after drug administration. Measurement points were selected based on pilot experiments and results of earlier studies (Diatchenko *et al.*, 2005).

Repeated (chronic) drug treatment consisted of a daily dose given for 6 days. On the fifth day, nociceptive latencies were measured again 2 and 3 h after the drug injection. On the sixth day, 1–2 min after the last drug injection, the animals received an intraplantar injection of carrageenan and nociceptive latencies were measured 3 h after this injection.

Two additional behavioural experiments were performed in mice and one in rats. In the first experiment, mice received CGP 28014, an atypical COMT inhibitor (Waldmeier *et al.*, 1990a,b; 30 mg·kg^{-1}, i.p.; n = 15) or vehicle (i.p.; n = 15) after habituation and baseline measurements, and paw flick latencies were measured 2 and 3 h after drug administration. On the next day the animals received the same treatment, and 1–2 min after the injection they were administered intraplantar carrageenan, and nociceptive latencies were measured 3, 4 and 5 h after injection. In the second experiment, COMT knockout mice (n = 25) were used. After habituation and baseline measurements, which were identical to the main experiment, the animals received OR-486 and the hot plate latencies were measured again 2 and 3 h after drug administration.

In the third experiment, 15 rats were used. After habituation, the baseline nociceptive thresholds to mechanical stimulation and hot plate latencies were measured. The rats were randomly assigned to groups that received intrathecal injection (10 µL) of nitecapone (200 µM, 600 µM or 1000 µM) or vehicle, and nociceptive responses were measured 1, 2 and 3 h after injection. After 2 days of no treatment (washout period), the baselines were measured again and the animals received another dose of nitecapone or vehicle. This was performed 1–2 times so that each animal received 2–3 different treatments. Thus, there were 11 animals in the nitecapone groups (200 and 600 µM) and 14 in the vehicle group. Because the dose of 1000 µM caused motor complications, it was given only to two rats.

Nociceptive measurements

Nociceptive tests included assessment of mechanic nociceptive thresholds (digital force gauge; Imada, DPS-1, Northbrook, IL, USA), paw flick (model DS20, Ugo Basile, Comerio, Italy), tail flick (model DS20, Ugo Basile) and hot plate (Harvard Apparatus, Kent, UK) tests. On each mouse, tests were always performed in this order with 1 min intervals. The baseline nociceptive thresholds and latencies to responses were measured twice each day with 1 h intervals during 4 days.

To assess mechanical nociceptive thresholds the animals were placed on the metal mesh covered with a Plexiglas dome and allowed to settle down for 1 min. When the animal was standing on both hind paws, the plantar surface of the hind paw was approached perpendicularly with a metallic monofilament with diameter of 0.2 mm for mice and 0.3 mm for rats. The paw was gently touched, and the force applied was steadily increased until the nociceptive behaviour, either a withdrawal, brisk shaking or licking of the paw, occurred. The force initiating the nociceptive response was recorded by digital force gauge attached to the monofilament as a measure of a threshold of mechanical nociception.

The temperature in the hot plate test was 52 ± 0.2°C, and to prevent tissue damage, a 60 s cut-off time was used. The intensity of the light beam in the tail flick and paw flick tests was set to 50 arbitrary units, which in average produced a response in 2–3 s in the pilot experiments. Cut-off times of 7 s (tail flick) and 10 s (paw flick) were used. In the carrageenan model, development of inflammation was

confirmed by measuring a diameter of the injected paw with digital vernier caliper (model CD-6"CP, Mitutoyo, Andover, UK).

In rats, the temperature of skin was measured before each round of behavioural measurements from plantar area of the hind paw with microprobe thermometer (Physitemp, model BAT-12; Physitemp Instruments Inc., Clifton, NJ, USA). After that mechanical nociceptive thresholds were measured from both hind paws using a digital force gauge. Then the hot plate latency was measured under conditions that were identical to those used with mice in the main experiment. Nociceptive baselines were measured twice before each drug administration with an interval of 1 min between the tests. After drug administration, nociceptive responses were measured 1, 2 and 3 h after the injection. At each time, mechanical nociceptive thresholds were measured twice from each paw followed by a single determination of the hot plate latency.

Procedure for intrathecal microinjections in rats

An intrathecal catheter made of a polyethylene (PE-10) tubing was inserted, under pentobarbital anaesthesia (50 mg·kg^{-1}, i.p.; Mebunat Vet 60 mg·mL^{-1}, Orion Pharma, Espoo, Finland), using a method described by Størkson *et al*. (1996), into the lumbar subarachnoid space and fixed through a layer of superficial muscles. Tubing was tunnelled rostrally and made to appear through the skin in the occipital region. On the day following the surgery, 10–15 µL of 2% lidocaine hydrochloride (Orion Pharma, Espoo, Finland), followed by 10–15 µL of saline was administered through the catheter using 50 µL Hamilton microsyringe to confirm the intraspinal location of the catheter. An immediate paralysis of hind limbs lasting 15–30 min was considered to indicate a correct location of the catheter. Only rats with properly located catheters and lacking neurological deficits from the catheter insertion were included in the study. After surgery, the rats were allowed to recover for 4 days before intrathecal administration of nitecapone. Nitecapone solution was administered intrathecally in volume of 10 µL using a 50 µL Hamilton microsyringe and flushed afterwards with 10 µL of saline.

Statistical analysis

Data analysis was performed using Prism 4.0 software (GraphPad Software, Inc., San Diego, CA, USA). In the text and figures the results are presented as means ± standard error of the mean (SEM) of n observations. $P < 0.05$ was considered as a limit of statistical significance in all tests.

A two-way analysis of variance (ANOVA) for repeated measurements was used for the statistical analysis. Treatment and time/inflammation were used as independent variables. Bonferroni test was used for the *post hoc* comparisons between the study groups.

In the acute and repeated administration studies, mechanical nociceptive thresholds are expressed in grams and results from the paw flick, tail flick and hot plate tests as percentage of the maximum possible effect [MPE% = (post – pre)/(cut-off – pre) × 100%). MPE% takes into account individual nociceptive baselines. In mice, the baselines were measured on four consecutive days in the beginning of the experiments, the averages of which were used as pre-drug values in the calculations of the MPE%. In rats, the baselines were measured before each drug administration to avoid the confounding effect of surgery on the nociceptive baselines. In the carrageenan study, results from the paw flick, tail flick and hot plate tests were expressed in seconds. Responses of the non-inflamed paw were used as controls in the paw flick and mechanical nociception tests. In the hot plate test, nociceptive latencies measured after induction of inflammation were compared with those measured after chronic administration of COMT inhibitors.

In the tests where the effects of the acute and repeated administrations of drugs were significant, additional analyses were performed to detect possible changes in the effect as a function of time. A paired two-tailed Student's *t*-test was used to compare nociceptive responses measured on the first and the fifth days of the drug treatment, separately for each treatment group at both time points (e.g. 2 and 3 h after drug administration). COMT activities were compared within the tissues with one-way ANOVA followed by Bonferroni test for *post hoc* comparisons, as appropriate.

Materials

Nitecapone [OR-462, 3-(3,4-dihydroxy-5-nitrobenzylidene)-2,4-pentanedione], OR-486 and CGP 28014 [N-(2-pyridone-6-yl)-N′,N′-di-n-propylformamidine] were synthesized by Dr Aino Pippuri (Orion Pharma, Espoo, Finland) using methods described earlier (Bäckström *et al*., 1989). For the systemic administration studies, the drugs were suspended in 0.5% carboxymethyl-cellulose (Fluka AG, Buchs SG, Switzerland) and administered in volume of 7.5 mL·kg^{-1} intraperitoneally (i.p.). The dose of COMT inhibitors was 30 mg·kg^{-1}. The same dose has been used in other behavioural animal studies (Diatchenko *et al*., 2005; Nackley *et al*., 2007). At this dose, nitecapone and OR-486 selectively and specifically inhibit COMT (Nissinen *et al*., 1988).

OR-486 (but not nitecapone) penetrates the blood-brain barrier and its administration leads to significant inhibition of COMT also in the brain (Nissinen *et al.*, 1988; Männistö and Kaakkola, 1999). Duration of action of nitecapone is 1–2 h, that of OR-486 several-fold longer (Nissinen *et al.*, 1988). The level of COMT inhibition is comparable to, or higher than that achieved after administration of the COMT inhibitors used in Parkinson's disease (Männistö and Kaakkola, 1999). For the intrathecal administration, nitecapone was dissolved in 30% ($w \cdot v^{-1}$) hydroxypropyl-β-cyclodextrin (Acros Organics, Geel, Belgium) dissolved in sterile physiological phosphate-buffered saline (pH 7.4) and administered in a volume of 10 µL into the lumbar subarachnoid space (see above).

Carrageenan (Sigma, St. Louis, MO, USA) was dissolved in physiological saline (0.9% NaCl, Natrosteril 9 $mg \cdot mL^{-1}$, Baxter, Vantaa, Finland) to give a 2% ($w \cdot v^{-1}$) solution and administered in a volume of 40 µL into the plantar region of the right hind paw.

Results

Effects of a single dose (acute administration) of COMT inhibitors on COMT activity

A single injection of OR-486 (30 $mg \cdot kg^{-1}$, i.p.) significantly reduced COMT activity in the striatum (Figure 1A, $P < 0.001$) and liver (Figure 1B, $P < 0.001$) 1 h after its administration. COMT activity was still reduced 3 h after administration of OR-486 in both tissues (Figure 1A and B; striatum, $P < 0.001$; liver, $P < 0.01$). OR-486 also reduced COMT activity in the prefrontal cortex, where the effects of the drugs were assessed 1 h after administration (Figure S1; $P < 0.001$) and in the spinal cord 3 h after drug administration (Figure S2; $P < 0.01$).

Nitecapone (30 $mg \cdot kg^{-1}$, i.p.) inhibited COMT activity in the liver 1 h after the drug administration (Figure 1B, $P < 0.001$) but not in the two brain areas studied or in the spinal cord (Figure 1A, and Figures S1 and S2). CGP 28014 (30 $mg \cdot kg^{-1}$, i.p.) did not affect the COMT activity in any of the tissues (Figure S3).

Catechol-*O*-methyltransferase activity of the group that had received OR-486 was also lower than that of the nitecapone group in the striatum 1 and 3 h after drug administration (1 h, $P < 0.001$; 3 h, $P < 0.01$) and in the liver 1 h after drug administration ($P < 0.001$).

Effects of acute administration of COMT inhibitors on nociception

Both nitecapone and OR-486 lowered the mechanic nociceptive thresholds (Figure 2A). There was a significant effect of treatment ($P < 0.001$), time ($P < 0.001$) as well as time–treatment interaction ($P < 0.001$). In the *post hoc* comparisons, thresholds of the nitecapone group were lower than those of the control group, 2 and 3 h after treatment

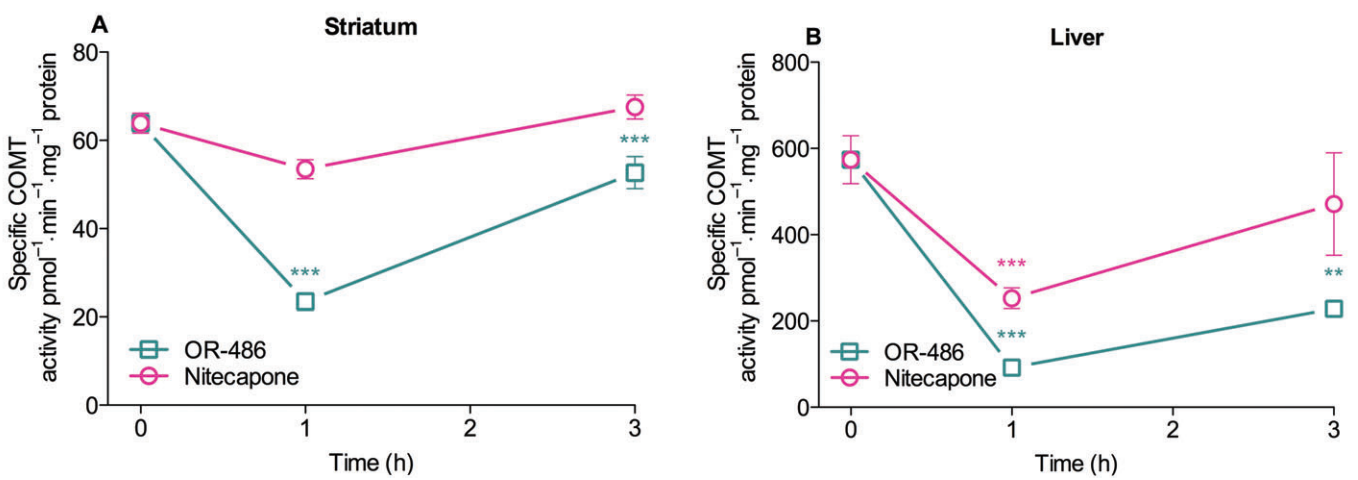

Figure 1

(A) Striatal catechol-*O*-methyltransferase (COMT) activity in male C57/BL6J mice 1 and 3 h after i.p. administration of 3,5-dinitrocatechol (OR-486) (30 $mg \cdot kg^{-1}$; 1 h, $n = 7$; 3 h, $n = 13$) or nitecapone (30 $mg \cdot kg^{-1}$; 1 h, $n = 8$; 3 h, $n = 16$). Baseline (0 h) represents COMT activity of vehicle (carboxymethylcellulose, CMC; $n = 21$) treated animals. $^{**}P < 0.01$; $^{***}P < 0.001$ versus vehicle group; one-way ANOVA followed by a Bonferroni test. COMT activity of the OR-486 group was also lower than that of the nitecapone group 1 and 3 h after drug administration (1 h, $P < 0.001$; 3 h, $P < 0.01$). (B) Hepatic COMT activity after the same i.p. treatment as above (OR-486: 1 h, $n = 7$; 3 h, $n = 6$; nitecapone: 1 h, $n = 8$; 3 h, $n = 5$; vehicle: $n = 12$). $^{**}P < 0.01$ versus vehicle group. COMT activity of the OR-486 group was also lower than that of the nitecapone group 1 h after drug administration ($P < 0.001$).

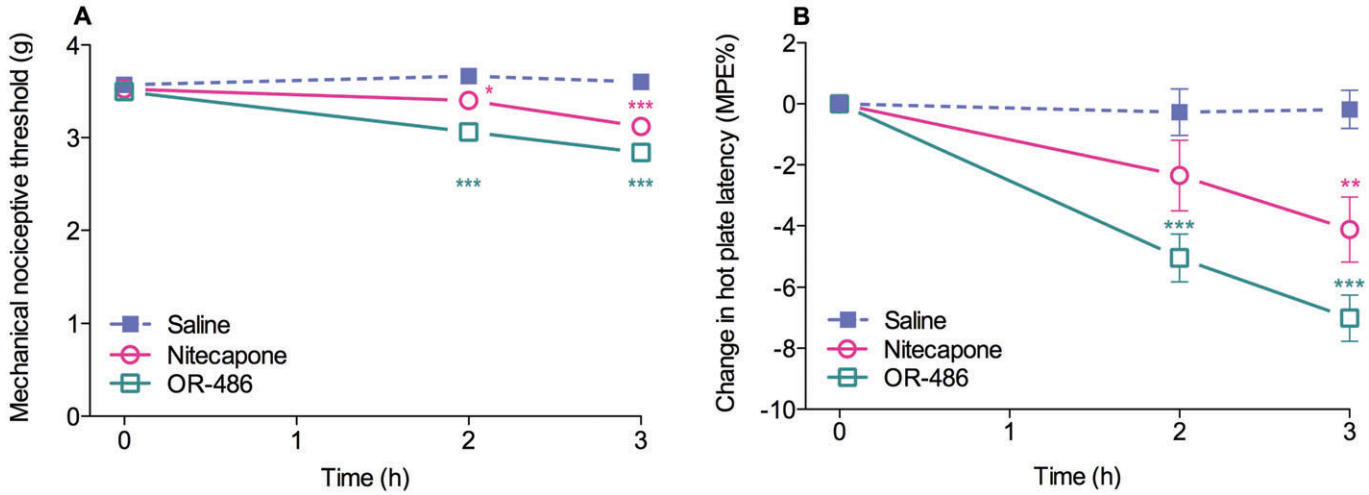

Figure 2

(A) Mechanical nociceptive thresholds after acute i.p. administration of 3,5-dinitrocatechol (OR-486) (30 mg·kg^{-1}; n = 16), nitecapone (30 mg·kg^{-1}; n = 16) or saline (n = 21). Results are expressed in grams. * P < 0.05; ** P < 0.01 versus vehicle group; two-way repeated ANOVA followed by a Bonferroni test. Thresholds of the OR-486 group were also significantly lower than of the nitecapone group at 2 and 3 h after treatment (at 2 h, P < 0.01; at 3 h, P < 0.05). (B) Change in nociceptive latencies in hot plate test after acute i.p. administration of OR-486 (30 mg·kg^{-1}; n = 16), nitecapone (30 mg·kg^{-1}; n = 16) or saline (n = 21). Results are expressed as percentages of the maximum possible effect (MPE%). Statistics: **P < 0.01; ***P < 0.001 versus vehicle group.

(P < 0.05 and P < 0.001 respectively). Thresholds of the OR-486 group were also lower than those of the control group, 2 and 3 h after administration of the inhibitor (P < 0.001 and P < 0.001 respectively). In the OR-486 group, the thresholds were also significantly lower than in the nitecapone group at 2 and 3 h after treatment (P < 0.01 and P < 0.05 respectively).

In the hot plate test, both COMT inhibitors decreased nociceptive latencies (Figure 2B). There was a significant effect of treatment (P < 0.001), time (P < 0.001) as well as interaction (P < 0.001). In the *post hoc* comparisons, latencies in the OR-486 group were significantly lower than in the control group 2 and 3 h after administration (P < 0.001 and P < 0.001 respectively). Also the latencies of the nitecapone group were shorter than those of the control group 3 h after drug administration (P < 0.01). In the hot plate test, the effect of nitecapone was not different from that of OR-486.

There were no differences between the groups in either the tail flick or the paw flick tests (data not shown).

Effects of chronic treatment (repeated administration) with COMT inhibitors on nociception

On the fifth day of the chronic treatment, the pronociceptive effect of COMT inhibitors on mechanical nociceptive thresholds was still present. There was a significant effect of treatment (P < 0.001), time (P < 0.001) as well as interaction (P < 0.001). In the *post hoc* comparisons, thresholds of mechanical nociception of both OR-486 and nitecapone groups were lower than those of the control group 2 and 3 h after administration (P < 0.001 for each comparison). There were no differences between the OR-486 and the nitecapone groups or between the baseline values of the groups (Figure 3A).

On the fifth day of nitecapone treatment, thresholds for mechanical nociception were significantly lower 2 h after nitecapone administration than thresholds measured at the corresponding timepoint after a single (acute) administration (Student's t-test, P < 0.05). Otherwise, on the fifth day of chronic treatment, thresholds did not differ from those measured after acute administration of the drugs.

On the fifth day of treatment, the pronociceptive effect of the COMT inhibitors in the hot plate test was still present (Figure 3B). When the groups were compared there was a significant effect of treatment (P < 0.001), time (P < 0.001) as well as interaction (P < 0.001). In the *post hoc* comparisons, the latencies of both OR-486 and nitecapone groups were significantly shorter compared with the control group 2 and 3 h after administration of the drugs (P < 0.01 or P < 0.001). There were no differences between the OR-486 and the nitecapone groups.

Two hours after drug administration, latencies of the chronically treated nitecapone group were

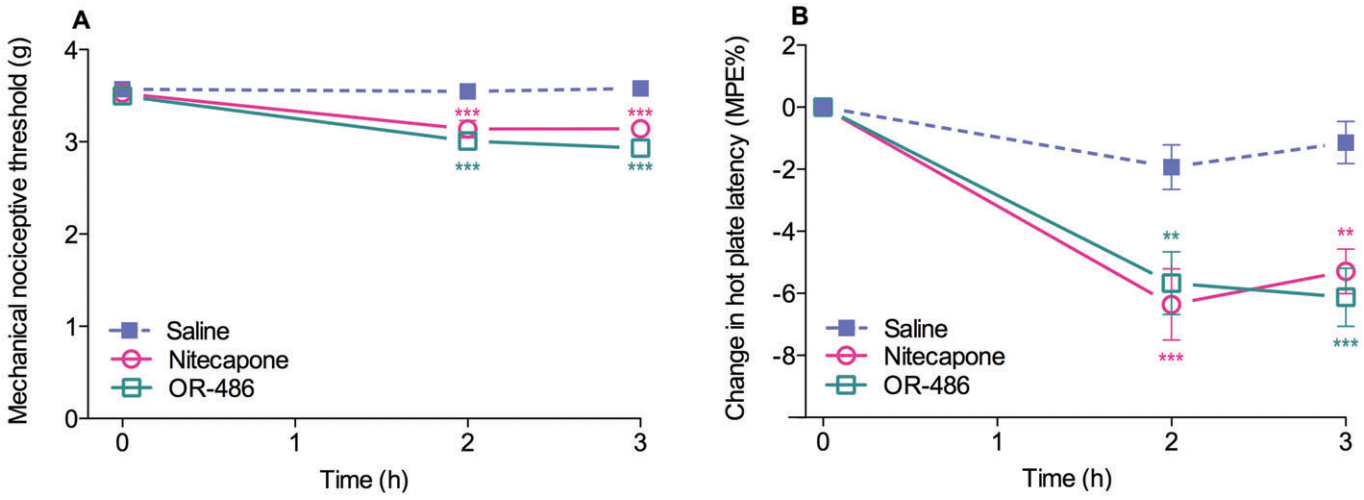

Figure 3

(A) Mechanical nociceptive thresholds after chronic (repeated) i.p. administration of 3,5-dinitrocatechol (OR-486) (30 mg·kg^{-1}; n = 16), nitecapone (30 mg·kg^{-1}; n = 16) or saline (n = 21). Results are expressed in grams. ***P < 0.001 versus vehicle group. (B) Thermal nociceptive latencies in hot plate test after repeated i.p. administration of OR-486 (30 mg·kg^{-1}; n = 16), nitecapone (30 mg·kg^{-1}; n = 16) or saline (n = 21). Results are expressed as percentage of the maximum possible effect (MPE%). Statistics: **P < 0.01; ***P < 0.001 versus vehicle group.

significantly shorter than after acute administration (Student's t-test, P < 0.01). Otherwise, the hot plate responses did not differ from those measured after acute administration of drugs.

In the tail flick test, there was a significant effect of treatment (P < 0.001) and interaction (P < 0.001) on the fifth day of treatment (data not shown). In the *post hoc* comparisons, the latencies of the nitecapone group were significantly shorter than the latencies of the control group, 2 and 3 h after administration of the drugs (P < 0.01 and P < 0.01 respectively). Also latencies of the OR-486 group were significantly shorter than those of the control group 2 h after administration of the drugs (P < 0.001). There were no differences between the OR-486 and the nitecapone group.

In the paw flick test, the effect of treatment did not reach the level of statistical significance (data not shown).

Effects of COMT inhibitors on carrageenan-induced inflammation

Carrageenan injection increased the diameter of the injected paw by about 50% compared with the control paw in all groups (effect of inflammation, P < 0.001), and there were no drug-related differences between the groups (data not shown).

Mechanical nociceptive thresholds of the injected paw in all groups were lower than thresholds of the control paw (effect of inflammation, P < 0.001; data not shown).

In the paw flick test, nociceptive latencies of the injected paw were in all groups shorter than latencies of the control paw (two-way ANOVA for repeated measurements, effect of inflammation; P < 0.001; Figure 4A). There was a difference between the treatment groups (P < 0.001) but the interaction was not significant. In the *post hoc* comparisons, the latencies of the nitecapone group were significantly shorter than those of the control group (inflamed paw, P < 0.001; control paw, P < 0.01). Latencies were also significantly shorter in the OR-486 group than in the control group (inflamed paw, P < 0.01; control paw, P < 0.05). There were no differences between the OR-486 and nitecapone groups.

In the hot plate test, carrageenan inflammation decreased nociceptive latencies in all groups when compared with the baseline on the fifth day (Figure 4B). When the groups were compared with two-way ANOVA for repeated measurements, there was a significant effect of treatment (P < 0.001) and inflammation (P < 0.001). Interaction was not significant (P > 0.05). In the *post hoc* comparisons, carrageenan latencies were significantly shorter in the nitecapone and OR-486 groups than in the control group (P < 0.05 and P < 0.001 respectively). There were no differences between the OR-486 and nitecapone groups.

The tail flick latencies did not change in any group, and there were no differences between the groups (data not shown).

Effects of the atypical COMT inhibitor CGP 28014

After acute administration of this inhibitor, paw flick latencies to response were shorter in the CGP

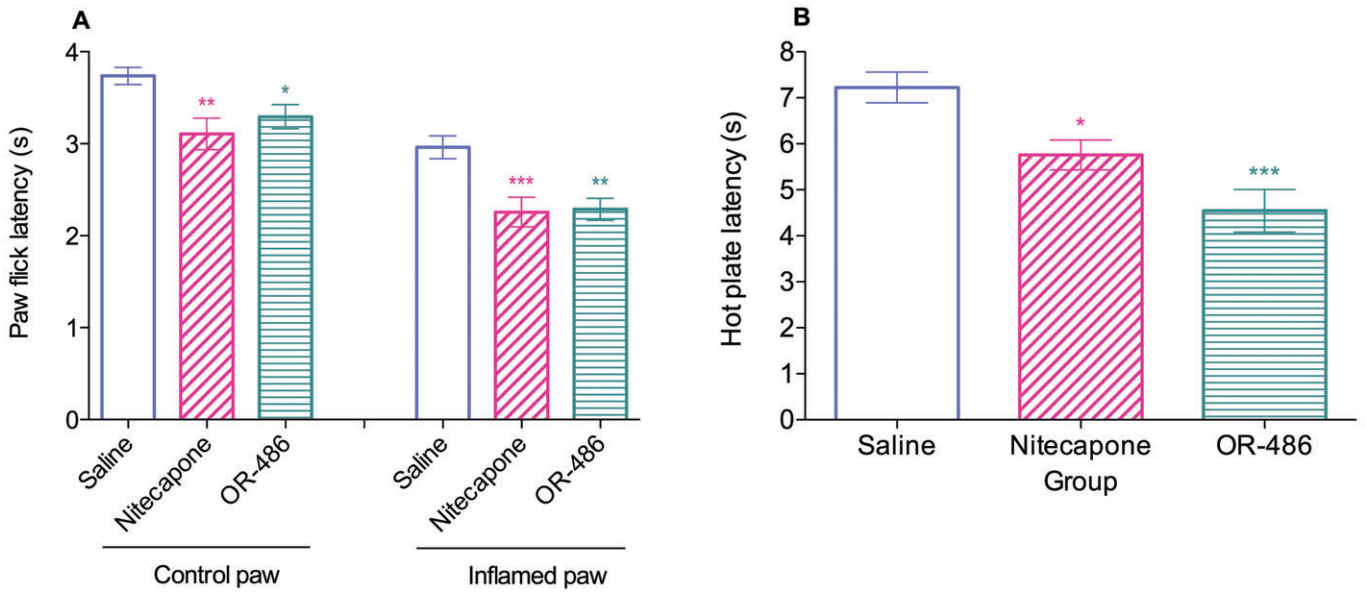

Figure 4

(A) Effect of repeated i.p. administration of 3,5-dinitrocatechol (OR-486) (30 mg·kg^{-1}; n = 16), nitecapone (30 mg·kg^{-1}; n = 16) and saline (n = 21) on paw flick responses 3 h after carrageenan injection in inflamed and non-inflamed paws. Results are expressed in seconds. *P < 0.05; **P < 0.01; ***P < 0.001 versus corresponding paw of the vehicle group. (B) Effect of repeated i.p. administration of OR-486 (30 mg·kg^{-1}; n = 16), nitecapone (30 mg·kg^{-1}; n = 16) or saline (n = 21) on carrageenan-induced nociception. Nociceptive latencies were measured with hot plate test 3 h after induction of inflammation and are expressed in seconds. *P < 0.05; ***P < 0.001 versus vehicle group.

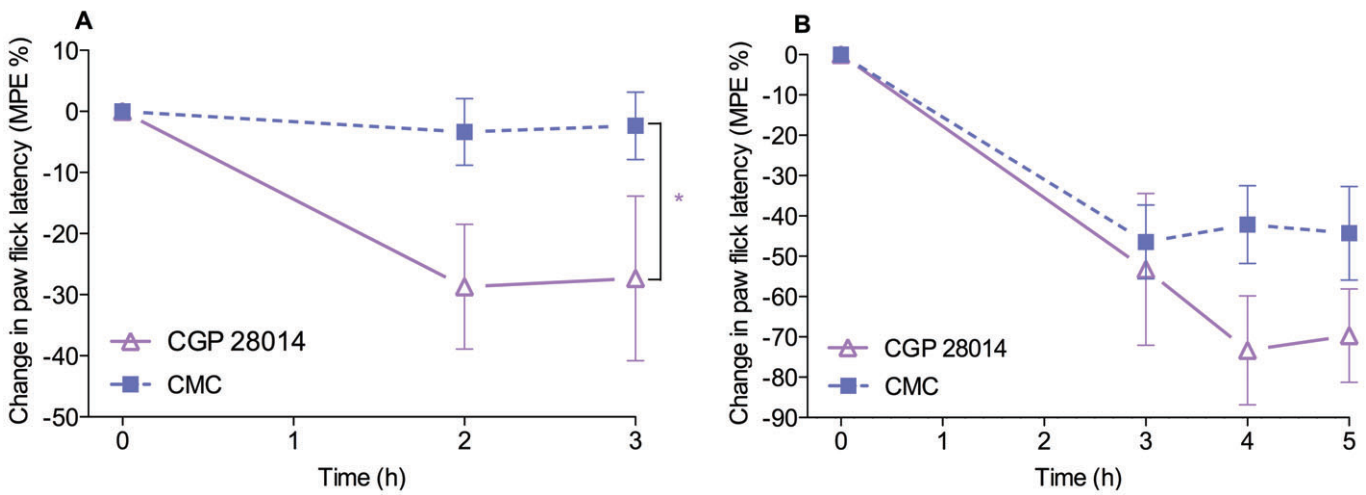

Figure 5

(A) Effect of acute i.p. administration of CGP 28014 (30 mg·kg^{-1}; n = 15), or vehicle (carboxymethylcellulose, CMC; n = 15) on paw flick responses 2 and 3 h after drug administration. *P < 0.05 versus vehicle group. (B) Effect of acute i.p. administration of CGP 28014 (30 mg·kg^{-1}; n = 15), or vehicle (CMC; n = 15) on paw flick responses 3, 4 and 5 h after carrageenan injection in inflamed paws. Results are expressed as percentage of the maximum possible effect (MPE%).

28014 group than in the control group (two-way ANOVA for repeated measurements, effect of treatment, P < 0.05) (Figure 5A). Carrageenan inflammation shortened paw flick latencies in both groups. The difference between the latencies of CGP 28014 and control groups remained also after carrageenan injection but it was not statistically significant (Figure 5B).

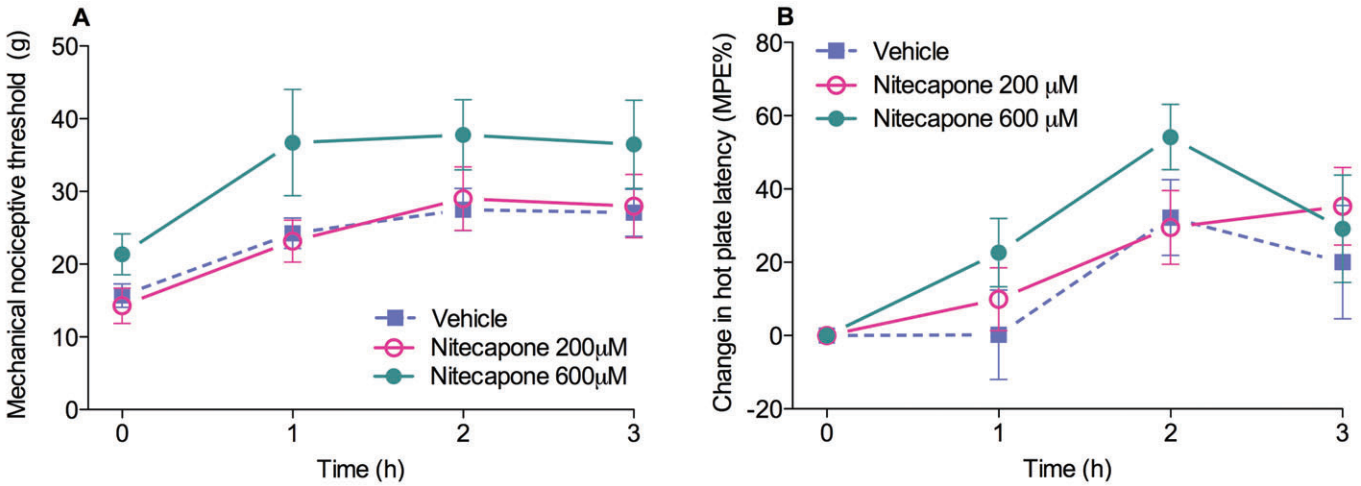

Figure 6

(A) Effect of intrathecal administration of 600 μM (n = 11) or 200 μM (n = 11) of nitecapone or vehicle (n = 14) on mechanical nociceptive thresholds in male Wistar rats. Thresholds are expressed in grams. There was a time effect (P < 0.05) but no treatment effect. (B) Effect of intrathecal administration of 600 μM (n = 11) or 200 μM (n = 11) of nitecapone or vehicle (n = 14) on hot plate latencies in male Wistar rats. Latencies are expressed as percentage of the maximal possible response (MPE%). There was a significant effect of time (P < 0.05) but no effect of treatment.

Effect of OR-486 on nociception in COMT knockout mice

Treatment with OR-486 did not change the hot plate latencies in COMT knockout mice (data not shown).

Effects of intrathecal administration of nitecapone on nociception in the rat

Mechanical nociceptive thresholds did not differ between the treatment groups (Figure 6A). There was a time effect (P < 0.05) but interaction was not significant. Similarly, the hot plate latencies did not differ between the treatment groups (Figure 6B). There was again an effect of time (P < 0.05) while interaction was not significant. Nitecapone did not affect the temperature of the skin at any time (data not shown). As the first few (n = 2) treatments with 1000 μM nitecapone caused motor impairment, no further experiments used this dose and the corresponding data is neither shown nor included in the statistical analysis.

Discussion

The main finding of the present study was that, regardless of their ability to penetrate the blood brain barrier, systemically administered COMT inhibitors increased mechanical and thermal nociception. Sensitization to nociceptive stimuli was not observed after direct intrathecal administration of nitecapone. This suggests that inhibition of COMT activity in the brain or the spinal cord is not needed for the pro-nociceptive effects of COMT inhibitors and that the increase in nociception was mediated by peripheral mechanisms. Presumably, COMT protein and a reduction of *O*-methylation of catecholamines are needed for this action.

In our study, a single (acute) administration of OR-486 inhibited COMT activity in the liver and brain tissue, and increased mechanical and thermal nociception in mice. This is in agreement with earlier studies where OR-486 had a pro-nociceptive effect in rats (Diatchenko *et al.*, 2005; Nackley *et al.*, 2007). Effects of peripheral COMT inhibitors have not been previously described in animal models of acute or inflammatory pain. We used nitecapone that does not penetrate the blood-brain barrier at low doses but, similarly to OR-486, temporarily inhibits COMT in peripheral tissues (Männistö *et al.*, 1988; Nissinen *et al.*, 1988). As expected, nitecapone, given i.p., did not inhibit COMT activity in the brain (Figure 1A) or spinal tissues (Figure S2). Pro-nociceptive effects of both COMT inhibitors remained stable up to the fifth day of the chronic treatment. In fact, 2 h after injection, these effects of nitecapone, shown as shortening of nociceptive latencies, were even more pronounced than after the acute administration and nitecapone was as effective as OR-486. Unexpectedly, even GGP 28104, which inhibited neither liver nor brain COMT activity (Figure S3A-C), was able to effectively reduce paw lick latencies as the two direct COMT inhibitors. The mechanism of action of CGP 28014 is not known

but it does not inhibit COMT enzyme *in vitro* (Waldmeier *et al*., 1990a). The actions of CGP 28014 depend on the route of administration. Intrastriatal administration releases dopamine, in a tyramine-like manner, without affecting levels of homovanillic acid (Steulet *et al*., 1993). After peripheral administration, the effect of CGP 28014 mimicked closely that of conventional COMT inhibitors suppressing formation of COMT-derived metabolites both in tissue homogenates and extracellular fluid, suggesting that an active metabolite was formed outside the brain (Waldmeier *et al*., 1990a; Steulet *et al*., 1993). CGP 28014 also potentiated the behavioural effects of *L*-DOPA as effectively as nitrocatechol-type COMT inhibitors (Waldmeier *et al*., 1990b; Törnwall and Männistö, 1993). This may indicate that it is the functional *O*-methylation, rather than the COMT activity itself, which is critical for modulation of pain.

Both OR-486 and nitecapone increased carrageenan-induced pro-nociception, apparent as shorter hot plate and paw flick latencies. This is in line with earlier studies which showed increased carrageenan-induced nociception in rats (Nackley *et al*., 2007). However, in the paw flick test, latencies in the non-inflamed paw were also decreased. Thus the pro-nociceptive effects of COMT inhibitors in the carrageenan model were due to a generalized increase in nociceptive sensitivity, rather than an enhancement of inflammation.

Three studies have been carried out previously in *Comt* gene modified mice (Kambur *et al*., 2008; Papaleo *et al*., 2008; Tammimäki *et al*., 2010). In one of these studies, pain sensitivity was decreased in mice over-expressing a high activity COMT variant (*COMT-Val-tg* mice; Papaleo *et al*., 2008). Correspondingly, COMT knockout mice, in which COMT activity is totally lacking showed altered opioid- and stress-induced analgesia and were more sensitive to thermal nociception in the tail flick test (Kambur *et al*., 2008). Similar, although less marked, pro-nociceptive effects were observed in mice lacking only the soluble form of COMT (*S-COMT* knockout mice; Tammimäki *et al*., 2010). In the study by Papaleo *et al*. (2008), COMT knockout mice, in which COMT activity is totally lacking (homozygous mice) or decreased to about 50% (heterozygous mice), showed increased pain sensitivity in the tail flick test. However, in our recent study, responses of COMT knockout mice did not differ from those of their wild type littermates under basal conditions (Kambur *et al*., 2008). This could be due to compensatory mechanisms developed in response to the enzyme deficiency.

To exclude other pharmacological effects of the nitrocatechol-type COMT inhibitors, besides COMT inhibition, we performed some nociception studies in COMT knockout mice. In these mice, OR-486 (30 $mg \cdot kg^{-1}$, i.p.) did not further modify hot plate latencies, indicating that its pro-nociceptive effects were dependent on the presence of COMT.

These results may have implications in clinical situations. A peripherally acting COMT inhibitor, entacapone, is widely used as an adjunct drug to the *L*-DOPA treatment of Parkinson's disease. Also, a common genetic COMT polymorphism can alter COMT activity as much (*c*. 40%) as COMT inhibitors (Chen *et al*., 2004; Nackley *et al*., 2006). In the current study, we showed that COMT inhibitors, even when not able to suppress COMT activity in the brain, produced pro-nociceptive effects. Increased pain sensitivity may not necessarily be observable in the absence of nociceptive stimuli (e.g. under normal conditions) but low COMT activity may enhance pain related to pathological conditions, such as Parkinson's disease. To our knowledge, the effects of COMT inhibitors on pain have not been characterized in human subjects. On the other hand, the Val/Met polymorphism of the *Comt* gene has received some attention. Low COMT activity, caused by the Met/Met allele, has been associated with increased pain sensitivity in experimental pain studies (Zubieta *et al*., 2003) and in osteoarthritis-related pain (van Meurs *et al*., 2009), together with an increased risk for developing a chronic pain condition in retrospective epidemiological studies (Diatchenko *et al*., 2005; 2006). Our present results are compatible with those earlier studies.

There are several hypotheses of how the inhibition and/or decrease of COMT activity would affect nociception. COMT is expressed in the prefrontal cortex and several other brain regions as well as in spinal and peripheral structures, such as superficial laminae of the dorsal horn of the spinal cord and ganglia of the primary sensory neurons, involved in processing of nociception (Karhunen *et al*., 1996; Hong *et al*., 1998). Initially, it was suggested that low COMT activity enhanced morphine analgesia via dopamine-triggered compensatory changes in the endogenous opioid system in the brain (Zubieta *et al*., 2003). This hypothesis later received some support from other studies in human subjects (Berthele *et al*., 2005; Rakvåg *et al*., 2005; Nikoshkov *et al*., 2008). Also in our earlier studies, opioid antinociception was changed in COMT knockout mice (Kambur *et al*., 2008), but not after an acute decrease of COMT activity caused by administration of brain-penetrating COMT inhibitors (O. Kambur and P.T. Männistö, unpublished results, 2009). This supports the suggestion that enhancement of the morphine response is not due to inhibition of COMT and

consequent changes in catecholamine dynamics *per se*, but rather to compensatory changes in the level of protein synthesis, such as an increase of opioid receptor synthesis.

However, in terms of the pro-nociceptive effects of COMT inhibitors, changes in opioidergic transmission do not explain all of the effects of COMT inhibition and therefore other or supplementary mechanisms have been suggested (Nackley *et al.*, 2007; Kambur *et al.*, 2008). It has been shown that some of the effects of COMT inhibition are blocked by antagonists of β_2- or β_3-adrenoceptors, favouring a catecholaminergic link in the nociceptive pathway (Nackley *et al.*, 2007). The location of those receptors and the corresponding site of action of COMT inhibitors are not known. Inhibition of COMT activity in the brain does not seem to be needed for the pro-nociceptive effects of COMT inhibitors, as nitecapone increased nociception but did not alter COMT activity either in the brain or in the spinal cord (Figure 1A, Figure S2). In fact, inhibition of spinal COMT activity seems to have anti-nociceptive effects (Jacobsen *et al.*, 2010). Also in our study, intrathecal nitecapone increased thresholds of mechanical nociception and thermal nociceptive latencies compared to the baseline, although the effect of treatment did not reach the level of statistical significance when compared to the vehicle-treated group (Figure 6A and B). Taken together this suggests that pro-nociceptive effects of COMT inhibitors are mediated via peripheral mechanisms and activation via β_2 or β_3 adrenoceptors has been suggested as a mechanism (Nackley *et al.*, 2007).

As to a general mechanism of action of nitecapone, OR-486 and CGP 28014, our conclusion is as follows. Certainly, the ability to inhibit *O*-methylation of catechols is necessary. The presence of COMT protein is needed, since no further effect was seen in COMT knockout mice. After peripheral administration, all compounds clearly inhibited formation of COMT-derived metabolites, although increase of dopamine itself has not been seen without *L*-DOPA administration. Therefore, we think that a common link could be a shift of metabolism of catecholamines to MAO-catalysed products. Indeed, recently, a monoamine oxidase-dependent catecholamine metabolite, 3,4-dihydroxyphenyl-glycolaldehyde (DOPEGAL) has received some attention. Under normal circumstances, DOPEGAL is metabolized by aldehyde reductase and further by COMT. If COMT activity is compromised, DOPEGAL may accumulate and generate reactive oxygen species, inflammation and, ultimately, neuronal damage (Marchitti *et al.*, 2007). A report of hyperalgesia caused by DOPEGAL (Dina *et al.*, 2008), however, has been withdrawn because of impurities found in the batch used (Dina *et al.*, 2009), leaving open any interpretation of the pro-nociceptive effect of low or absent COMT activity.

In conclusion, acute administration of OR-486 increased mechanical and thermal nociception in mice and these effects persisted after 5 days of chronic treatment. OR-486 also increased the pro-nociceptive effects of carrageenan-induced inflammation. In COMT deficient mice, OR-486 failed to influence pain-related behaviour. Nitecapone, a peripherally acting COMT inhibitor which does not inhibit COMT activity either in the brain tissue or in the spinal cord, caused a similar, although less pronounced, increase in nociception as OR-486. Thus, the pro-nociceptive effects of COMT inhibitors do not depend on inhibition of COMT in the brain or spinal cord, and are primarily mediated via peripheral, COMT protein dependent mechanisms.

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Conflict of interest

None.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Prefrontal catechol-*O*-methyltransferase (COMT) activity in male C57/BL6J mice 1 h after intraperitoneal administration of 3,5-dinitrocatechol (OR-486) (30 mg·kg^{-1}; n = 7), nitecapone (30 mg·kg^{-1}; n = 7) or vehicle (carboxymethylcellulose, CMC, n = 7). ***P < 0.001 versus vehicle group (one-way ANOVA and a Bonferroni test).

Figure S2 Spinal cord catechol-*O*-methyltransferase (COMT) activity in male C57/BL6J mice 3 h after intraperitoneal administration of 3,5-dinitrocatechol (OR-486) (30 mg·kg^{-1}; n = 15), nitecapone (30 mg·kg^{-1}; n = 19) or vehicle (carboxymethylcellulose, CMC, n = 17). **P < 0.01 versus vehicle group.

Figure S3 (A) Striatal catechol-*O*-methyltransferase (COMT) activity in male C57/BL6J mice 1 h after intraperitoneal administration of CGP 28014 (30 mg·kg^{-1}; n = 7) or vehicle (carboxymethylcellulose, CMC; n = 7). No significant differences. (B) Prefrontal COMT activity after the same treatment as above (CGP 28014, n = 7; vehicle, n = 7). No significant differences. (C) Hepatic COMT activity after the same treatment as above (CGP 28014, n = 7; vehicle, n = 7). No significant differences.

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