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Catechol-*O*-methyltransferase and pain in rodents and humans

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ACADEMIC DISSERTATION

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To my parents...

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ABSTRACT

Acute **pain** is an important warning signal, however, neuropathic pain and often chronic pain, lack a physiological function. Pain is a major clinical challenge and especially chronic and neuropathic pain are difficult to treat. On individual level, pain causes occupational and functional disability, suffering, and impairs quality of life. On a macro level pain and its direct and indirect consequences cause multi-billion expenses. Genetic factors and mechanisms underlying susceptibility to chronic pain have recently raised significant scientific interest. *COMT*-gene, which codes for catechol-*O*-methyltransferase (COMT), is subject for genetic polymorphic variation and *COMT* polymorphisms modulate pain and opioid analgesia in humans. The effects of COMT on pain and opioid responses were studied in rodents and humans. In mice, **COMT deficiency** was associated with altered stress- and morphine-induced analgesia reflecting weakened capacity of endogenous pain modulation and changes in opioidergic transmission. In normal mice, **COMT inhibitors** reduced thresholds of mechanical nociception and shortened thermal nociceptive latencies and thus increased nociceptive sensitivity in models of acute and inflammatory pain. Pronociceptive effects were COMT-dependent. In the spinal nerve ligation model of **neuropathic pain** in rats nitecapone decreased nociceptive symptoms - cold and mechanical hyperalgesia and allodynia.

In **humans**, genetic variation of *COMT* gene was associated with pain phenotypes. The associations were strongest for the experimental pain phenotypes but also clinical pain phenotypes, such as acute postoperative pain, showed associations (uncorrected $p=0.006-0.007$) with three single nucleotide polymorphisms (SNPs). The strongest effect was observed in the SNP located in the 3'UTR-region of *COMT*, rs887200, pointing to importance of this region in regulation of nociceptive phenotype and confirming the results in rodents. Together, these results confirm the role of COMT in pain and opioid responses. The antiallodynic effects of COMT inhibitors should be further studied in neuropathic pain since the safety and efficacy of current therapies are not satisfactory. In humans, mutations in *COMT* gene affect pain. The predictive value of individual SNPs, however, is limited and several SNPs of *COMT* as well as other genetic factors should be included in the same analysis or treatment algorithms possibly utilizing a haplotypic approach. Finally, the effects of most SNPs associated with pain phenotypes on COMT expression and activity are not known and should be explored in further studies.

ABBREVIATIONS

Aa, amino acids
AC, axillary clearance
ACE, angiotensin I converting enzyme
ANOVA, analysis of variances
APS, haplotype with predicted low pain sensitivity
ASIC, acid-sensing ion channel
AUC, area under the curve
Bp, basepairs
CNS, central nervous system
COMT, catechol-*O*-methyltransferase
DAT, dopamine transporter
DLPT, dorsolateral pontine tegmentum
DNIC, diffuse noxious inhibitory controls
DOPEGAL, 3,4-dihydroxyphenyl-glycolaldehyde
GABA, γ -aminobutyric acid
HPS, haplotype with predicted high pain sensitivity
HUS, Hospital District of Helsinki and Uusimaa
IL-12, interleukin 12
i.p., intraperitoneal
i.t., intrathecal
i.v., intravenous
KO, knockout mice
L-DOPA, *L*-3,4-dihydroxyphenylalanine
LITAF, lipopolysaccharide-induced TNF factor
LPS, haplotype with predicted low pain sensitivity
MAO, monoamine oxidase
MB-COMT, Membrane-bound COMT
MPE%, percentage of the maximum possible effect
mRNA, messenger ribonucleic acid
NAT, noradrenaline transporter
NCF, nucleus cuneiformis
NRS, numerical rating scale

PACU, post anaesthesia care unit
PAG, periaqueductal gray
PCA, patient-controlled analgesia device
RVM, rostral ventromedial medulla
s.c., subcutaneous
SEM, standard error of the mean
SNL, spinal nerve ligation
SNP, single nucleotide polymorphism
STAI, state and trait anxiety inventory
TMD, temporomandibular joint disorder
TNF- α , tumor necrosis factor alpha
TRP, transient receptor potential channel
UTR, untranslated region
VAS, visual analogue scale
WDR, wide-dynamic range neurons
WT, wild-type mice

LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following publications, herein referred by their Roman numerals (I-IV), and some unpublished data:

- I. **Kambur O**, Männistö PT, Viljakka K, Reenilä I, Lemberg K, Kontinen VK, Karayiorgou M, Gogos JA, Kalso E (2008). Stress-induced analgesia and morphine responses are changed in catechol-*O*-methyltransferase-deficient male mice. *Basic & Clinical Pharmacology & Toxicology*, 103, 367–373.
- II. **Kambur O**, Talka R, Ansah OB, Kontinen VK, Pertovaara A, Kalso E, Männistö PT (2010). Inhibitors of catechol-*O*-methyltransferase sensitize mice to pain. *British Journal of Pharmacology*, 161, 1553–1565.
- III. **Kambur O**, Männistö PT, Pusa AM, Käenmäki M, Kalso EA, Kontinen VK (2011). Nitecapone reduces development and symptoms of neuropathic pain after spinal nerve ligation in rats. *European Journal of Pain*, 157, 732-740.
- IV. **Kambur O**, Kaunisto MA, Tikkanen E, Leal SM, Ripatti S, Kalso EA (2013). Effect of *COMT*-gene variants on experimental and acute post-operative pain in humans. *Anesthesiology* [submitted]

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1 INTRODUCTION

Pain is a sensation generated by an organism as a response to a stimulus able to cause tissue damage. Thus, pain has a clear evolutionary value by promoting the survival of an individual. Pain, however, can result from damage, malfunctioning or alterations in the sensory system, and be present even without adequate stimulation. It is a common symptom of several pathological conditions and is encountered by virtually every human individual (with some exceptions) at some point of their lives. Severe or chronic pain can cause suffering, occupational and functional disability and have a devastating impact on human lives (Wall *et al.*, 2006). Moreover, chronic pain is difficult to treat. This holds true especially for neuropathic pain, which is caused by a damage or malfunctioning of the nervous system and affects *ca.* 6.9-8.2% of the population (Bouhassira *et al.*, 2008; Bridges *et al.*, 2001; Costigan *et al.*, 2009; Dieleman *et al.*, 2008; Taylor, 2006; Torrance *et al.*, 2006). In neuropathic pain pharmacological therapy produces usually only a partial pain relief and its effectiveness is limited by several factors such as severe adverse effects that can exclude/prevent effective dose (Dworkin *et al.*, 2007; Farrar *et al.*, 2001; Finnerup *et al.*, 2005,2010).

The pathophysiology of pain is still incompletely understood. Pain is known to have a strong genetic component, as genetic background affects the prevalence and intensity of symptoms of various painful conditions as well as the effectiveness and adverse effects of analgesics. Much effort has been put to the research of individual genes underlying clinical phenotypes. If such genes could be identified, it would offer a possibility to identify patients with increased risk of developing a chronic pain condition or altered response to treatment, and offer them more individualized treatment in an earlier stage. However, only few individual genes underlying pain phenotypes have been identified so far. *COMT*-gene, which encodes for catechol-*O*-methyltransferase (COMT), is one of those that has received a significant amount of attention (Belfer and Segall, 2011; Kambur and Männistö, 2010; Segall *et al.*, 2010,2012).

The current works have been carried out to evaluate and characterize the significance of COMT in animal and human models of experimental and clinical pain.

2 REVIEW OF THE LITERATURE

2.1 Catechol-*O*-methyltransferase

2.1.1 Gene

In humans, the *COMT* gene is located in chromosome 22 band q11.21 (Grossman *et al.*, 1992; Winqvist *et al.*, 1992). *COMT* gene contains six exons of which the first two are non-coding. The translation is initiated in the third exon, which contains codons for initiation of the membrane bound and soluble isoforms. Two separate promoters control the expression of two partially overlapping transcripts. The 1.5 kb transcript is expressed constitutively whereas transcription of the 1.3 kb specimen is regulated in tissue-specific manner (Tenhunen *et al.*, 1993,1994; Tenhunen and Ulmanen, 1993). Translation of the 1.3 kb transcript results in soluble COMT (S-COMT) and the 1.5 kb transcript can be translated into both, membrane-bound COMT (MB-COMT) and S-COMT by the leaky scanning mechanism of translational initiation (Kozak, 1989; Tenhunen and Ulmanen, 1993).

2.1.2 Protein

COMT protein has at least two isoforms: soluble (S-COMT) and membrane-bound (MB-COMT) (Lundström *et al.*, 1991; Salminen *et al.*, 1990). The isoforms differ in their expression pattern and in both humans and rodents S-COMT is the prevalent isoform in most of the tissues except for the human brain, where MB-COMT is more (≥ 2.5 -fold) abundant than S-COMT (Hong *et al.*, 1998; Tenhunen *et al.*, 1994; Tenhunen and Ulmanen, 1993). It appears that MB-COMT, however, can take over about 50% of the function of S-COMT, even in peripheral tissues if S-COMT is insufficient or lacking, as in the case of S-COMT-mutant mice (Käenmäki *et al.*, 2009). It has been suggested that in humans MB-COMT is present in two distinct forms, which could be demonstrated with enzyme-linked immunosorbent assay (ELISA) and Western blotting (Tunbridge *et al.*, 2006). Even though the same band (39 kDa) of the putative MB-COMT

isoform has been shown also in one earlier study, it was interpreted as an artefact (Chen *et al.*, 2004) and the existence of the isoform has not been confirmed.

In addition to S- and MB-COMT also other COMT-isoforms may exist. According to the ENSEMBL-database the *COMT* gene has 11 predicted transcripts of which 8 are protein coding, one mediates nonsense-mediated decay, one retains intron and one processes transcript (**Table 1**) (Ensemble database, Feb 2012). In total, 8 alternatively spliced variants of COMT protein have been identified (Tunbridge *et al.*, 2007).

Table 1. *COMT* gene transcripts.

NAME	LENGTH (bp)	PROTEIN ID	LENGTH (aa)	BIOTYPE
COMT-010	354	No protein product	-	Processed transcript
COMT-009	546	No protein product	-	Retained intron
COMT-008	724	ENSP00000387695	152	Protein coding
COMT-005	871	ENSP00000403958	223	Protein coding
COMT-007	1319	ENSP00000207636	199	Nonsense mediated decay
COMT-001	1339	ENSP00000385150	271	Protein coding
COMT-003	1457	ENSP00000384654	221	Protein coding
COMT-002	1548	ENSP00000385917	271	Protein coding
COMT-201	2035	ENSP00000416778	221	Protein coding
COMT-004	2217	ENSP00000383966	235	Protein coding
COMT-011	2437	ENSP00000354511	271	Protein coding

Abbreviations: bp, basepairs; aa, amino acids.

2.1.3 Distribution and function

In the brain, COMT is present in glial cells and postsynaptic neurons but seems to be lacking from presynaptic dopaminergic neurons (Kaakkola *et al.*, 1987; Karhunen *et al.*, 1995a; Karhunen *et al.*, 1995b; Rivett *et al.*, 1983a; Rivett *et al.*, 1983b). In the nociceptive system, COMT is expressed in superficial laminae of the dorsal horn of the spinal cord and sensory ganglia (Karhunen *et al.*, 1996), as well as in supraspinal sites (Männistö and Kaakkola, 1999; Myöhänen *et al.*, 2010). Cell populations expressing COMT in the spinal cord and peripheral ganglia have not been characterized.

COMT O-methylates a wide range of substrates containing a catechol structure (Axelrod *et al.*, 1958; Axelrod and Tomchick, 1958; Guldborg and Marsden, 1975; Nissinen *et al.*, 1988). Its substrates include catecholamines dopamine, adrenaline and noradrenaline, catecholestrogens, drugs such as L-DOPA and other molecules carrying a catechol structure.

In the brain, COMT complements presynaptic amine transporters (dopamine transporter, DAT; noradrenaline transporter, NAT) and monoamine oxidase (MAO) in elimination of catecholamines, particularly in some brain areas but their quantitative role varies in different brain structures (Männistö and Kaakkola, 1999). As COMT has been shown to be mainly an intracellular protein, it needs a mechanism to carry its substrates inside the cell, *i.e.* it requires transport such as uptake₂ which transports catecholamines to glial and postsynaptic cells (Männistö *et al.*, 1992; Myöhänen *et al.*, 2010; Trendelenburg, 1990; Wilson *et al.*, 1988). Recently it has been suggested that significant proportion of MB-COMT may be localised to the cell membrane with its catalytic domain oriented towards the extracellular side, and therefore it can inactivate extracellular catecholamines regardless of transport mechanisms (Chen *et al.*, 2011a; see in more details below).

In mice, both isoforms were expressed in all tissues in the study by Myöhänen *et al.* (2010). In comparison to peripheral tissues, expression of MB-COMT was more pronounced in the brain tissue, in particular in the hippocampus, in the cerebral cortical areas and the hypothalamus. Both isoforms were shown to be intracellular. MB-COMT was not associated with plasma membranes in the brain. Both isoforms were largely expressed in microglial cells, astroglial cells and intestinal macrophages. COMT was also expressed by some neurons, including pyramidal neurons, cerebellar Purkinje and granular cells and also striatal spiny neurons. It was mainly lacking from long projection neurons. S-COMT deficient mice also lacked nuclear COMT, which indicates the nuclear localization of S-COMT.

In rats, it has recently been shown that in cortical neurons MB-COMT is located in cell bodies but also axons and dendrites (Chen *et al.*, 2011a). It was also shown to be co-localized with markers of pre- and postsynaptic structures, lipid rafts and secretory vesicles. Surprisingly, C-terminals, which contain catalytic domains of MB-COMT, were located on the extracellular side of the cell membrane and MB-COMT was shown to inactivate also extracellular catecholamines (Chen *et al.*, 2011a).

In the striatum, the dopamine signal is primarily terminated by presynaptic uptake₁ mediated by a DAT (Cass *et al.*, 1993; Eisenhofer *et al.*, 2004; Giros *et al.*, 1996; Moron *et al.*, 2002). DAT rapidly uptakes most of the released dopamine from the synapse after which it is either packed to storage vesicles by vesicular monoamine transporter 2 or metabolized to 3,4-dihydroxyphenylacetic acid (DOPAC) by MAO. Even complete lack of COMT activity in *Comt* knockout mice does not increase the extracellular dopamine level in the striatum (Huotari *et al.*, 2002). Instead, in the prefrontal cortex, and some other brain areas where the density of DAT is low (Matsumoto *et al.*, 2003; Sesack *et al.*, 1998), dopaminergic transmission seems to be regulated by COMT and uptake by the NAT (Di Chiara *et al.*, 1992; Mazei *et al.*, 2002; Moron *et al.*, 2002; Mundorf *et al.*, 2001; Tanda *et al.*, 1997; Yamamoto and Nototney, 1998). In the prefrontal cortex, lack of COMT activity in *Comt* knockout mice increases dopamine levels in the extracellular fluid (Käenmäki *et al.*, 2010). This was confirmed by voltametric studies, in which the elimination time of prefrontal dopamine was doubled (Yavich *et al.*, 2007).

COMT isoforms have functional differences. MB-COMT binds catechol substrates with much higher affinity than S-COMT. The reaction kinetics of these isoenzymes are different and S-COMT has a higher enzymatic capacity (Lotta *et al.*, 1995; Männistö and Kaakkola, 1999; Roth, 1992). S-COMT and MB-COMT are also to some extent spatially differentially expressed (Myöhänen *et al.*, 2010). On a functional level, a lack of S-COMT-activity induces neurochemical (Käenmäki *et al.*, 2009) and behavioural (Tammimäki *et al.*, 2010) effects in S-COMT mutant mice. In general the effects, however, are slight and occur in a sex- and tissue-dependent manner. After *L*-DOPA administration, lack of S-COMT-activity does not affect the levels of *L*-DOPA in plasma or peripheral tissues (Käenmäki *et al.*, 2009; Tammimäki *et al.*, 2010). When S-COMT-activity is lacking, MB-COMT is able to compensate about 50% of its function, even in peripheral tissues (Käenmäki *et al.*, 2009). Thus, in general, S-COMT seems to play only a limited role in the inactivation of catecholamines whereas MB-COMT may be functionally more significant.

Little is known about alternatively spliced variants of COMT described by Tunbridge *et al.* (2007). One variant, which is transcribed into COMT variant truncated from the C-terminal has recently been shown to be functionally different from normal COMT (Meloto *et al.*, 2012). *In vitro* truncated S- and MB-COMT are less stable, but S-COMT metabolizes noradrenaline more efficiently, while its metabolic efficacy for catechol substrate is poorer than that of normal

COMT variant. Also longer COMT isoforms may exist as rs165599, which is located after the 3' polyadenylation site shown by Tenhunen *et al.* (1994), is present in some longer EST sequences in the GenBank database. mRNA species containing rs165599 have been confirmed in the brain, suggesting that polyadenylation sites alternative and more 3' to the initially shown one, are being used (Bray *et al.*, 2003; GenBank, <http://www.ncbi.nlm.nih.gov/Genbank>).

Metabolic activity of COMT is subject to genetic variation of which Val^{108/158}Met polymorphism is the best known (Lotta *et al.*, 1995; Männistö and Kaakkola, 1999; Nackley *et al.*, 2006; Weinshilboum and Raymond, 1977; Weinshilboum *et al.*, 1979). According to Chen *et al.* (2004), such polymorphisms can substantially, about 40%, decrease the enzymatic activity of COMT. Such polymorphisms have been shown to be related to differences in physiological and pathophysiological processes (see below).

In addition to attenuation of COMT-activity by Met-allele of Val^{108/158}Met (rs4680), several other polymorphisms, described and implicated in pathological states, have been shown to modulate enzymatic activity of COMT in molecular, cellular, or tissue level (Bray *et al.*, 2003; Nackley *et al.*, 2006; Tsao *et al.*, 2011). Haplotypes consisting of rs4680 and synonymous SNPs located 5' to rs4680 (*e.g.* rs4633-rs4818-rs4680) have been shown to alter secondary structure of COMT mRNA consequently changing its stability and efficiency of protein translation (Nackley *et al.*, 2006). These haplotypes have often been referred to as predicted high, average and low pain sensitivity haplotypes (HPS, APS and LPS, respectively), based on the studies by Diatchenko *et al.* (2005,2006). CCG-haplotype (HPS), containing rs4680G coding for valine, resulted in longest and most stable stem-loop structure in rs4680 proximity showing lowest rate of expression and enzymatic activity, as compared to other haplotypes including CGG (LPS), which also contains rs4680G. In the TCA-haplotype (APS), rs4818C did not reduce the level of protein expression but showed reduced level of COMT-activity due to the methionine coded by rs4680A. This is in line with the findings that also rs4633 located at the 5' end of mRNA near the ribosomal binding site modulates protein expression *in vitro*. Its T-allele which is APS haplotype-specific increases translational efficiency and protein expression in tissue-specific manner, in some tissues to the level of LPS or even higher (Tsao *et al.*, 2011). Also SNPs rs737865 located in the first intron of the MB-COMT transcript and rs165599 modulate expression of COMT mRNA via *cis*-acting mechanisms at least in the brain tissues (Bray *et al.*, 2003). Rs737865 C-allele is associated with decreased allelic expression of rs4633C-rs4680G transcripts. The effect is likely brain/CNS-specific and not mirrored by gross changes in peripheral COMT activity, as rs737865 is located

within the first intron of the MB-COMT, which contributes little to the peripheral COMT activity. As to rs165599, variants containing G-allele show lower relative expression and are overrepresented in schizophrenic patients. The mechanism of these effects and possible role of alternatively spliced variants remain to be investigated (Bray *et al.*, 2003; GenBank, <http://www.ncbi.nlm.nih.gov/Genbank>).

2.1.4 COMT inhibitors

COMT-inhibition. Nitecapone, OR-486 and other COMT-inhibitors (**Fig. 1**) decrease COMT activity in the peripheral tissues and to some extent in the CNS. In behavioural animal studies, a COMT-inhibitor dose of 30 mg/kg has been generally used (Diatchenko *et al.*, 2005; Nackley *et al.*, 2007). At this dose, both nitecapone and OR-486 selectively, specifically and strongly inhibit COMT (Nissinen *et al.*, 1988). Duration of action of nitecapone is *ca.* 3 hrs (**III**), while that of OR-486 is longer and some of the newer compounds can inhibit COMT activity even for 24 hrs (Nissinen *et al.*, 1988; Nissinen and Männistö, 2010). OR-486 and, to some extent, nitecapone, penetrate the blood-brain barrier and lead to a temporary inhibition of COMT in the brain (Nissinen *et al.*, 1988; Nissinen and Männistö, 2010) (**III**).

OTHER EFFECTS. Nitrocatechol-structured inhibitors of COMT, such as nitecapone, are also potent antioxidants. Nitecapone scavenges reactive oxygen species and nitric oxide radicals and prevents lipid peroxidation (Männistö and Kaakola, 1999; Marcocci *et al.*, 1994a,b; Nissinen *et al.*, 1995; Suzuki *et al.*, 1992). As antioxidative effects occur already at clinically relevant concentrations, they can at least partially attribute to the beneficial effects of COMT in some pathological states. In a rat model of diabetes, nitecapone improves various symptoms such as kidney function (Pertovaara *et al.*, 2001). It also exhibits a protective effect on gastric lesions which lasts longer than 6 hrs, and the concomitant stimulatory effect on the release of prostaglandin E2 can last more than 12 hrs (Aho and Lindén, 1992).

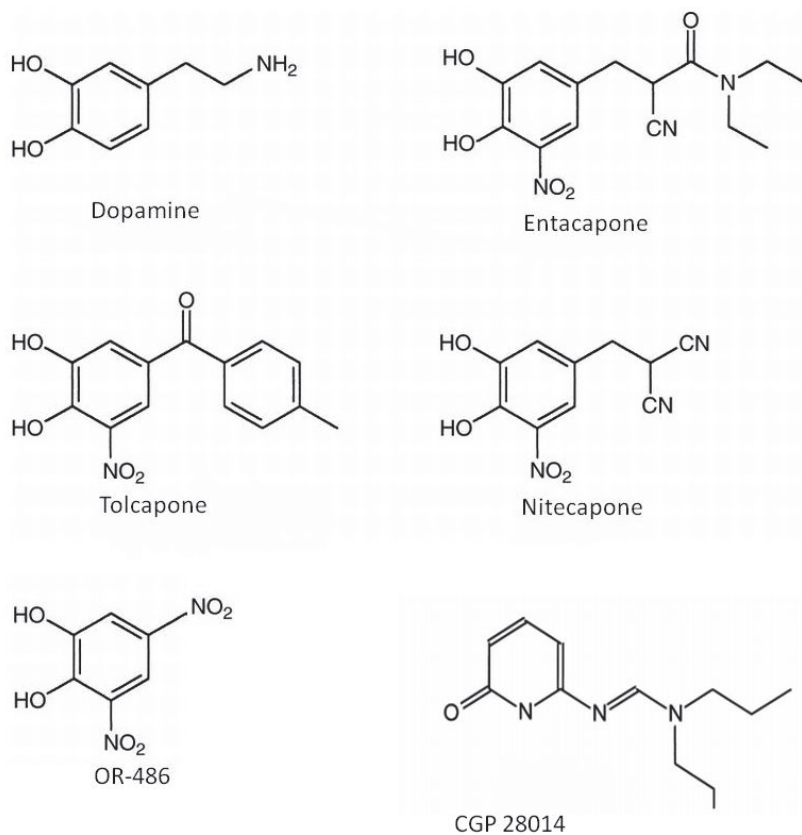


Fig.1. Chemical structures of dopamine and COMT inhibitors used in pain studies.

OTHER USES, ANIMALS. Tolcapone, but also entacapone, have beneficial effects in animal models of depression (forced swimming test and learned helplessness paradigm) when combined with *L*-DOPA (Männistö *et al.*, 1995). However, in another animal model of depression (chronic mild stress-induced anhedonia), also tolcapone alone has alleviated depressive behaviour (Moreau *et al.*, 1994). The effects of COMT inhibitors on learning and memory have also been studied. Tolcapone has been shown to improve working and spatial memory performance in animal models, but the effect was slight and test-specific (Khromova *et al.*, 1997; Liljequist *et al.*, 1997). Tolcapone also improved executive memory processes in rats (Lapish *et al.*, 2009).

CLINICAL USE. Peripheral COMT inhibitors are increasingly used in the treatment of Parkinson's disease as adjuncts to *L*-DOPA therapy that mainly alleviates motor symptoms. COMT inhibitors protect a significant amount of *L*-DOPA from metabolism, thus prolonging its

action and allowing a reduction of its doses (Männistö and Kaakkola, 1999). In patients suffering from Parkinson's disease entacapone increases the mean "on" time and correspondingly reduces the time, frequency and severity of the "off" periods time (Mizuno *et al.*, 2007; Pellicano *et al.*, 2009; Rinne *et al.*, 1998). Entacapone increases the availability of *L*-DOPA increasing its effect by 1/3, which allows reduction of required daily *L*-DOPA dose (Rinne *et al.*, 1998). Symptoms were rapidly improved in 69% of patients receiving also entacapone, and the effect was sustained until the end of the study (Larsen *et al.*, 2003). Moreover, positive therapeutic effects have been shown in elderly Parkinson patients with more severe symptoms and experiencing *L*-DOPA related wearing-off effect (Pellicano *et al.*, 2009). When compared with dopaminergic agonists, entacapone was also more effective than cabergoline showing quicker onset of therapeutic effect in conjunction with *L*-DOPA (Deuschl *et al.*, 2007). Entacapone also provides additional therapeutic value in patients receiving dopaminergic agonist therapy (Fenelon *et al.*, 2003). Entacapone is well tolerated and during a 3-year follow-up study, only 14% of the patients discontinued the treatment due to adverse effects (Larsen *et al.*, 2003). Its main adverse events include diarrhoea, insomnia, dizziness, nausea, increased dyskinesias, urine discoloration, aggravated parkinsonism, and hallucinations (Larsen *et al.*, 2003; Mizuno *et al.*, 2007; Rinne *et al.*, 1998). Increase of dopaminergic adverse events, mainly dyskinesias, occurred mostly in the beginning of the treatment and could be alleviated by reducing the *L*-DOPA dose (Larsen *et al.*, 2003; Rinne *et al.*, 1998).

Other COMT inhibitors, tolcapone (Ebersbach and Storch, 2009; Factor *et al.*, 2001; Koller *et al.*, 2001) and nitecapone (Ferreira *et al.*, 2010) have been similarly effective in treating symptoms of Parkinson's disease when combined with *L*-DOPA. Tolcapone has shown more pronounced therapeutic effect while having similar tolerability. However, it has potentially severe hepatic adverse effects, which have limited its use (Borges, 2003,2005; Ebersbach and Storch, 2009; Factor *et al.*, 2001).

In addition to *L*-DOPA adjunct therapy, several other indications have been suggested for tolcapone. In clinical settings it has been studied in a small open study in major depressive disorder patients (Fava *et al.*, 1999). It was found to be effective, but its use has not gain popularity due to adverse effects, and a lack of properly conducted double-blinded randomized studies (Fava *et al.*, 1999). Tolcapone has also been shown to improve cognition and cortical information processing in healthy human subjects (Apud *et al.*, 2007) and parkinsonian patients

(Gasparini *et al.*, 1997), and has been suggested to improve cognitive deficits associated with schizophrenia (Apud and Weinberger, 2007). However, these indications remain disputed.

2.2 Nociception and pain

2.2.1 Nociception in the periphery and primary nociceptors

Stimuli of intensity high enough to potentially cause tissue damage, are initially detected by primary sensory nociceptive neurons, which can be categorized into two different types, A δ - and C-fibers (**Fig. 2**) (Albrecht and Rice, 2010; Reichling and Levine, 1999; Wall *et al.*, 2006). Axons of A δ -fibers are thicker than C-fibers and they are myelinated resulting in higher conduction velocity of action potentials. The cell bodies of these first neurons are located in dorsal root ganglia or trigeminal root. First neurons are unipolar and the distal branches of their axons terminate at the surface of the skin, joints, muscle tissue, or viscera. The second branch of the axon enters the spinal cord or, in case of trigeminal nerves, the brainstem at the level of the pons.

FUNCTION. Stimuli of different modalities such as heat, mechanical, acid etc., applied to the peripheral endings of primary nociceptors, activate specialized proteins, such as ion channels of transient receptor potential channel (TRP) or acid-sensing ion channel (ASIC) family (Nilius and Owsianik, 2011; Wemmie *et al.*, 2013). Activation leads to excitatory ion currents that cause depolarization of the nerve ending and generation of action potentials. Action potentials are then propagated by voltage-gated ion channels along the axon to the CNS.

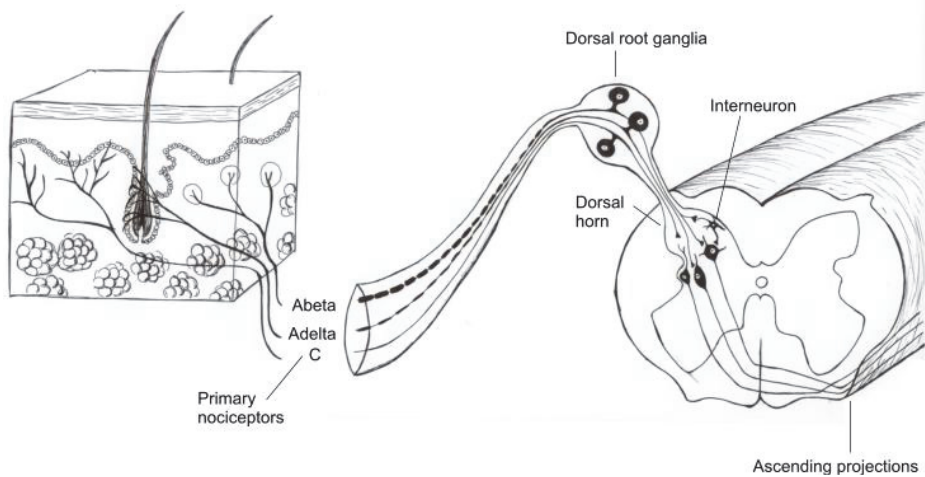


Fig. 2. Nociception in the periphery and spinal cord.

The functional state of nociceptive neurons varies and not all of the nociceptive neurons are active. Many of them remain in an inactive “silent” state under physiological conditions, but, for example during inflammation, they can become activated by chemical messengers, such as hormones and neurotransmitters.

PRIMARY NOCIREPTORS. Primary nociceptors are a heterogeneous group of sensory afferent neurons, which respond to nociceptive stimulation (Cain *et al.*, 2001; Perl, 1996). Primary nociceptors can be grossly classified to C-fibers and A-fibers. Whereas some nociceptors are specialized, most of them are polymodal and respond to different types of stimuli. Both types of nociceptors are usually described as mechano-heat sensitive, but most also respond to chemical stimulation. So-called “silent nociceptors” (30% of C-fibers and 50% of A-fibers) are functionally more passive and mechanically insensitive. They either do not respond or have very high threshold ($>60 \text{ g/mm}^2$) to mechanical stimulation. Some of the silent nociceptors, however, respond to chemical stimulation.

2.2.2 Nociception in the spinal cord

Spinal cord receives inputs from primary nociceptors, other primary afferents carrying information from the periphery, and descending projections from the brain (Todd, 2010; Wall *et al.*, 2006). Primary nociceptors originating from skin, muscle, joints, and viscera terminate and arborize at different

laminae of the dorsal horn. The connection pattern is highly organized and it depends on the type of afferent fibers and the modality of their receptive fields. Thinly myelinated and slow fibers represent the majority of nociceptors and synapse at superficial layers of the dorsal horn. Primary nociceptors make synaptic connections with several types of neurons. They synapse with projection neurons ascending anteriorly to various brain regions (1) and axons of projection neurons (posteriorly) descending from different brain regions that modulate processing of nociceptive information at the spinal level (2). First-order nociceptors also synapse with interneurons (3), whose axons remain within the spinal cord in the same segment or project to other segments, and terminals of other primary sensory afferents that supply non-nociceptive information to the spinal cord (4). Primary nociceptors also make functional connections directly and indirectly with other neuronal and non-neuronal components of the spinal cord (5). These include neurons of autonomous and motor nervous system and glial cells, which mediate various responses to nociceptive stimulation but are also involved in modulation of nociception.

Primary nociceptors make excitatory glutamatergic synapses with projection neurons, thus providing flow of nociceptive information to the brain (Todd, 2010; Wall *et al.*, 2006). Also, synaptic connections of primary nociceptor terminals and interneurons, are mostly excitatory. However, a large variety of other neurotransmitters are used in spinal synapses. Interneurons can be divided according to their function into excitatory interneurons releasing glutamate, and inhibitory interneurons releasing γ -aminobutyric acid (GABA) or glycine. Both types of interneurons have various integrative and modulatory functions and are important processors of nociceptive information in physiological and pathological states. For example, discharges of tactile afferents can activate nociceptive neurons, which under normal circumstances are suppressed by inhibitory interneurons. Suppression can be blocked by spinal administration of GABA-A or glycine antagonists leading to tactile allodynia. The malfunctioning of the inhibitory interneurons is one of the underlying factors of clinical tactile allodynia.

2.2.3 Inflammation, nerve injury, and modulation of spinal and peripheral nociception

MODULATION OF PERIPHERAL NOCICEPTION. Primary nociceptors are highly dynamic entities whose structure and function integrate changes in their environment, which are reflected in their action (Basbaum *et al.*, 2009; Wall *et al.*, 2006). Environment can modulate primary nociceptors by different mechanisms and involve for example, inflammatory processes. Tissue injury, which often

accompanies trauma or other pathological states, can cause inflammation which modulates the properties and function of primary nociceptors contributing to pain and hyperalgesia (Basbaum *et al.*, 2009; Linhart *et al.*, 2003; Petho and Reeh, 2012; Schumacher 2010; Uçeyler *et al.*, 2009; Verri *et al.*, 2006). During inflammation, immune and other cells release various inflammatory mediators, such as arachidonic acid metabolites, bradykinin, ATP, NO and other small molecules, inflammatory cytokines and chemokines, growth factors, and also classical neurotransmitters such as noradrenaline.

Inflammatory mediators act on peripheral terminals of nociceptive neurons. Several types of polymodal nociceptors express inflammatory mediator receptors, including G-protein coupled receptors, ligand-gated ion-channels, cytokine receptors and receptor tyrosine kinases. Activation of these receptors activates different second messenger cascades, which alter molecular and structural properties of primary nociceptors via phosphorylation, methylation, changes in gene expression, and other mechanisms. Changes in structural and molecular properties of primary nociceptors can activate and sensitize nociceptive terminals and change their response properties. They can decrease the activation threshold to stimuli of one or several modalities, and increase responsiveness to suprathreshold stimulation. Altered functional properties of primary nociceptors are critical in several chronic pain states.

Inflammation can also affect the spinal nociceptive system by releasing the same inflammatory mediators in the spinal cord, or indirectly via altered discharge activity of primary nociceptors and consequent release of neurotransmitters in the spinal cord (Linley *et al.*, 2010).

MODULATION OF SPINAL NOCICEPTIVE FUNCTIONS. Spinal neuroplastic changes can cause various sensory and other consequences including clinically significant symptoms, such as secondary hyperalgesia and tactile allodynia. In the spinal cord, different types of primary afferents are synaptically connected with second order nociceptive neurons and other cells. They release a variety of chemicals, which mediate transmission of nociceptive information to other spinal and supraspinal structures. Connections also mediate different spinal responses including motor and autonomic responses. Connections are highly organized and topographically distinguishable. However, they are also extremely dynamic. Increased or altered activity of primary nociceptors is followed by changes in the amount or composition of chemical substrates they release. Chemicals can be released by other spinal cells but also by peripheral tissues in synaptic or paracrine manner. Peripheral substrates can access the spinal cord by crossing blood-cerebrospinal fluid-barrier utilizing different transport mechanisms or disruption of the barrier caused by trauma or other pathologies. Chemicals activate whole plethora of receptors and secondary messenger cascades in other cells. This causes changes in

intracellular milieu of target cells, modulates intracellular components, regulates gene expression, and modulates the activity of other cells (*e.g.* glial cells). These activity-dependent neuroplastic changes temporarily or permanently alter processing of nociceptive information and other functions of spinal cells. For example, repetitive stimuli of constant intensity applied on C-fibers causes pattern of action potentials which lead to progressive, frequency-dependent facilitation of the responses of wide-dynamic range neurons (WDR) which is referred as wind-up (for review, see Coste *et al.*, 2008; Herrero *et al.*, 2000).

NERVE INJURY. Neuropathic pain has been defined as “pain initiated or caused by primary lesion or dysfunction of nervous system”, or more recently as “pain arising as a direct consequence of a lesion or disease affecting the somatosensory system” (Merskey and Bogduk, 1994; Treede *et al.*, 2008).

Clinical manifestation of neuropathic pain consists of a well characterized and defined set of clinical symptoms which can arise from numerous pathological states and involves different pathobiological mechanisms. Neuropathic pain affects *ca.* 6.9-8.2 % of the population and it can be caused by several factors such as trauma, viral infections, chemotherapy, or metabolic disorders that lead to damage or malfunctioning of the nervous system (Bouhassira *et al.*, 2008; Bridges *et al.*, 2001; Costigan *et al.*, 2009; Davis, 2007; Dieleman *et al.*, 2008; Taylor, 2006; Torrance *et al.*, 2006; Zimmermann, 2001).

Neuropathic pain manifests as spontaneous burning pain in the absence of stimuli, sensitization to both noxious stimuli (hyperalgesia), pain sensation caused by innocuous stimuli of different modalities (allodynia), and other sensory symptoms (Costigan *et al.*, 2009; Jensen and Finnerup, 2007; Woolf and Mannion, 1999).

Transition **from nerve injury to neuropathic pain** is not self-evident. Nerve injury leads to clinical neuropathic pain symptoms only in a fraction of patients. Prevalence of the symptoms varies greatly and depends on different factors including location, type, and extent of damage to the nervous system and type and size of the damaged nerves. Also, age and gender of the patient, genetic and epigenetic factors play a role. Moreover, acute nociceptive symptoms elicited by the injury and their treatment and emotional and cognitive factors affect the occurrence of neuropathic pain symptoms (Costigan *et al.*, 2009).

Neuropathic pain can involve different pathophysiological **mechanisms**, the importance of which varies between different neuropathic pain conditions, underlying factors and phase of the disease (Bridges *et al.*, 2001; Costigan *et al.*, 2009). Mechanisms include peripheral sensitization, ectopic impulse generation and transduction, recruitment of low-threshold A β -fiber to nociceptive transmission, central

sensitization, disinhibition of nociceptive neurons and structural changes. Plastic changes can be detected upstream in the spinal cord and even in the brain even if damaged nerve(s) would be in the periphery (Zimmermann, 2001). As pathophysiology of neuropathic pain may involve different mechanisms, several drugs affecting different ion channels or neurotransmitter systems are used and have shown efficacy in its **treatment** (Cruccu, 2007). Drugs acting through different mechanisms are often co-administered to achieve adequate pain relief (Dworkin *et al.*, 2007). However, neuropathic pain is often difficult to treat, as pharmacological therapy produces usually only partial pain relief, and adverse effects are often intolerable preventing optimal dosing (Costigan *et al.*, 2009; Farrar *et al.*, 2001).

2.2.4 From nociception to pain – the role of the brain

FROM SPINAL CORD TO THE BRAIN. A subpopulation of neurons of the spinal cord projects to the brain supplying it with sensorimotor information. Groups of projection neurons originating from the same area of the spinal cord, and directly or indirectly, connecting it to the same brain region have been conceptualized as ascending nociceptive tracts. Consequently, ascending nociceptive tracts can be classified or named according to the brain regions where projection neurons terminate. Spinothalamic tract connects the spinal cord with several thalamic regions (**Fig. 3**) and is the main nociceptive tract, whereas spinobulbar tract connects with the brainstem, and spinohypothalamic with the hypothalamic region. Also other tracts have been named (*e.g.* spinoreticular, spinocervical, spinoparabrachial and spinoamygdalar tracts and postsynaptic dorsal column pathway), but their classification and nomenclature are not unambiguous and may vary among different sources (Wall *et al.*, 2006; Willis, 2007). It must be noted that despite anatomical resemblance and close location, the neurons originating from the same area of the spinal cord can be differentially regulated, project to different sites, and serve different purposes.

Regardless of the tract, the neurons carrying nociceptive information originate mostly from superficial (lamina I) layers of the dorsal horn, but also from deeper (laminae IV-V) layers, from the intermediate zone and medial ventral horn (laminae VII-VIII) (spinothalamic tract and other) (**Fig. 3**). Axons of projection neurons cross the spinal cord and ascend contralaterally or bilaterally, depending on the brain area they target. Axons of projection neurons of spinothalamic tract ascend mainly in lateral or anterior funiculus.

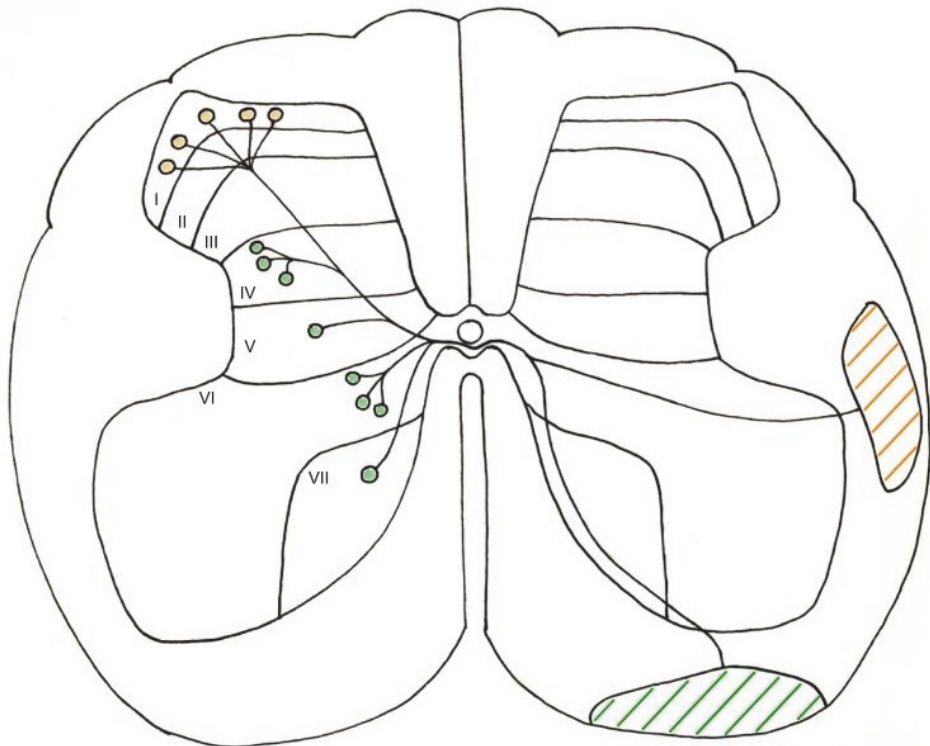


Fig. 3. Spinal ascending nociceptive projections. In the figure, spinal origin and route of lateral and anterior spinothalamic tracts are highlighted with green and orange colours, respectively.

In the brain, the projection neurons terminate in thalamic, spinobulbar, or hypothalamic regions, which provide different types of nociceptive responses. Thalamus is the main gateway of nociceptive and other sensory information, and spinal projections terminating here process the greatest part of ascending nociceptive information and contribute to the largest part of final pain experience. Spinothalamic cells terminate in different regions of the thalamus *e.g.* ventral medial nucleus, ventral caudal part of the medial dorsal nucleus and ventral posterior nucleus, which process and further transfer the nociceptive information to other brain regions. In the spinobulbar projections, axons target the regions of catecholamine cell groups (A1-A7; incl. locus caeruleus, A6), parabrachial nucleus, periaqueductal gray (PAG) and reticular formation of the brain stem. In these areas, the nociceptive information is integrated with the homeostatic processes and processes regulating the behavioural state. Nociceptive information is further transferred to forebrain and is also used to modulate neuronal activity at spinal and forebrain levels, which can modulate pain experience. Hypothalamic projections

could be involved in autonomic, neuroendocrine, and emotional dimensions of nociception, but understanding their role, especially in primates and humans, requires further studies.

FROM NOCICEPTION TO PAIN – processing in different brain areas. From the thalamus, nociceptive information is further propagated to other brain structures modulating their function. Brain areas activated by nociceptive stimulation can be visualised and studied by *in vivo* imaging techniques, and are often referred to as the pain matrix. Activation of different brain regions can be seen after application of experimental pain stimuli (Shenoy *et al.*, 2011) in different pain conditions, such as fibromyalgia (Gracely and Ambrose, 2011) or neuropathic pain (Chen *et al.*, 2008), but also in other pathological conditions in which pain symptoms are present, such as in Parkinson's disease (Stoessl, 2009). Some of the brain activation may result from other stimuli such as the activation of somatosensory cortex by touch, or other situational factors. However, nociceptive stimulation also induces brain activation, which is nociception-specific and can be distinguished from other signals (Chen *et al.*, 2011b; Chen *et al.*, 2012). Activation patterns of anterior cingulate cortex, prefrontal cortex, primary and secondary somatosensory cortices, insular cortex, amygdala, thalamus and PAG are relatively consistent (Chen *et al.*, 2008; Wall *et al.*, 2006). Across the studies, however, there is some variation and activation patterns may depend on the modality and duration of stimuli, experimental or underlying pathological conditions and patient group.

WHAT DOES THE BRAIN ACTIVATION MEAN OR CAN TELL US? Activation of the brain area can reflect its involvement in processing of nociception. Activation of certain parts of nociceptive circuitry can cause pain. For example, electrical stimulation of medial parietal operculum or posterior insula, can cause pain in the absence of nociceptive stimulation (Mazzola *et al.*, 2011). Even though activation of other brain areas does not cause pain, they may be required for more coordinated activation or be crucial for some aspects of pain. Correspondingly, attenuation of processing in some of nociceptive brain areas by means of stimulation, such as transcranial magnetic stimulation, or neurosurgery can in some cases alleviate pain symptoms and is sometimes used as a treatment in otherwise intractable pain (Wall *et al.*, 2006).

Functionally, brain areas activated by nociceptive stimuli are neurophysiological correlates of the subjective psychological pain experience. They integrate nociceptive input with other internal and external information, giving the pain its dimensions – aspects, finally resulting in wholesome pain experience. From neuropsychological perspective, the experience of pain can be conceptualized into sensory-discriminative and affective-motivational components. Sensory-discriminative component

encompasses localization of the stimuli, discrimination of its intensity and quality. Affective-motivational component contributes to negative hedonic quality and emotional reaction, general activation or arousal, stimulus selective attention, the drive to terminate the stimulus causing the sensation, leading to withdrawal or verbal reaction (Treede *et al.*, 1999). Additionally, a cognitive component could be considered to be an entity on its own.

Pain is processed by brain areas and networks that also have other psychological or neurological functions. In addition to pain, activated brain areas can often process other types of stimuli or other types of emotional, cognitive, motivational etc. information. The brain area coding for location and intensity of tactile stimulation, for example, could process both innocuous and noxious touch, and the area coding for unpleasantness of pain could also be activated in other unpleasant experiences. Thus nociceptive brain circuitry is partially shared and overlapping with other brain processes. If nociceptive and other brain functions co-occur and are processed simultaneously, **simultaneous processing** can interfere with pain (Chan *et al.*, 2012; Legrain *et al.*, 2005, 2012; Lorenz and Garcia-Larrea, 2003; Seminowicz and Davis, 2007). Such interference or modulation can be caused by expectation, distraction, emotional context, cognitive, situational or other factors and attenuate pain if it recruits processing networks and decrease resources required/reserved for nociceptive processes. This has been utilised by different mind-body therapies but also some of the actions of analgesic drugs can be partially explained by their effects on these brain areas (Bushnell *et al.*, 2013). Modulation, however, is not inevitably inhibitory but can also be facilitatory and increase pain. Nociceptive circuits can, at least partially, also be activated in the absence of nociceptive stimulation, for example when images of pain are being shown or memorized (Shimo *et al.*, 2011).

The degree and extent of activation of neural nociceptive networks by noxious or innocuous stimuli vary and is shaped by current and earlier pain experiences (Bushnell *et al.*, 2013). Activation may be more pronounced or recruit wider brain circuitry in chronic pain patients (Shimo *et al.*, 2011). Thus, brain does not only integrate and process nociceptive information, but is also shaped by nociceptive information. Chronic pain can alter neuronal connections of the brain and cause structural changes and reorganization, which can be seen by imaging techniques in clinical pain states (Henry *et al.*, 2011). Such changes can be maladaptive and strengthening of nociceptive connections can increase severity of the pain symptoms. Plasticity of the brain, on the other hand, can be utilized in the clinic: amount of non-pain-related information received and processed by the brain can be increased by for example sensorimotor training, which reduces and even reverses structural changes and pain symptoms (Napadow *et al.*, 2012).

DOWNSTREAM MODULATION OF PAIN. Although the experience of pain is shaped by co-occurring cerebral processes, the main mechanism of pain modulation remains downstream regulation of nociceptive input (Tracey and Mantyh, 2007). Modulation of ascending nociceptive transmission is carried out by a descending projection network including frontal lobe, anterior cingulate cortex, insula, amygdala, hypothalamus, PAG, nucleus cuneiformis (NCF), dorsolateral pontine tegmentum (DLPT) and rostral ventromedial medulla (RVM) (**Fig. 4**) (Ossipov *et al.*, 2010; Tracey and Mantyh, 2007). One of the best described and the most important neural circuits is the PAG region of the midbrain, which is connected to the RVM projecting to the spinal cord. PAG receives inputs from several cortical and other brain areas including rostral anterior cingulate cortex, hypothalamus and amygdala. PAG projects to the RVM. RVM is formed by nucleus raphe magnus, nucleus reticularis gigantocellularis and some other brain areas, and it projects to spinal and medullary dorsal horns. This circuit directly and indirectly modulates transmission and processing of nociceptive information flowing from the periphery by altering characteristics of electrical impulse trails ascending to the brain, thus altering nociceptive input received by the brain.

Downstream regulation is crucial from the clinical perspective: regulatory networks are involved in endogenous modulation of pain and are mediating the therapeutic effects of several pharmacological and non-pharmacological treatments. The dysfunction of these networks can manifest as clinical pain states or undermine pain therapies by reducing their efficacy or by causing side effects. Modulation of nociception can be inhibitory or facilitatory (Tracey and Mantyh, 2007). Both types of modulation have several functional, evolutionary, pharmacological and clinical implications. Both systems seem to be simultaneously active and functioning, feeding and regulating each other. Thus, the resulting nociceptive status of the organism is dynamic and determined by functional status of both of these systems.

INHIBITORY MODULATION. Descending inhibition of pain is involved in a naturally activated control system, namely conditioned pain modulation (in earlier literature referred to as diffuse noxious inhibitory controls, DNIC) (Yarnitsky, 2010), whereby the response of WDR neurons in the dorsal horn (for review, see Le Bars, 2002) and trigeminal nuclei (Dallel *et al.*, 1999) to C-fibre activation is inhibited by the application of noxious stimuli to remote body areas. Descending inhibition of pain can also occur after exposure to environmental stressors, which modulate nociceptive responses in animals (Yamada and Nabeshima, 1995). Modulation is stressor- and modality-specific and can involve different neurotransmitter systems (Lapo *et al.*, 2003; Lewis *et al.*, 1980; Mitchell *et al.*, 1998; Mogil *et al.*, 1996; Yamada and Nabeshima, 1995), and it has been used in different pain studies as a scientific tool. Some

stressors (such as 3 min swim in 32°C water) activate the endogenous opioid system inhibiting nociceptive responses for a short period of time, which can be prevented by administration of naloxone (Lapo *et al.*, 2003; Lewis *et al.*, 1980; Mitchell *et al.*, 1998; Mogil *et al.*, 1996; Yamada and Nabeshima, 1995).

FACILITATORY MODULATION. Activation of certain brain areas and pathways can facilitate the nociceptive transmission (McNally, 1999). Facilitation involves different anatomical structures and relies on inhibition of neurons of endogenous analgesic circuits or direct facilitation of neurons of ascending nociceptive tracts. Facilitatory circuits can be classified based on several factors, and from nociceptive and functional point of view, facilitatory circuits can be antianalgesic, inhibiting analgesia, or hyperalgesic, increasing basal pain sensitivity. Antianalgesic and hyperalgesic components can occur but also be present independently from each other.

2.3 COMT and nociception: animal studies

Effects of COMT on nociception have been characterized in a few experimental animal studies using COMT inhibitors or mice with modified *Comt* gene. COMT inhibitors are pronociceptive in models of acute and inflammatory pain in rodents and the effects have been shown in different modalities of nociceptive stimuli (Diatchenko *et al.*, 2005; Nackley *et al.*, 2007). The effects were blocked by administration of adrenergic $\beta_{2/3}$ inhibitors. In models of neuropathic pain, however, nitecapone and OR-486 have shown profound antiallodynic and antihyperalgesic properties in behavioural and electrophysiological experiments (Jacobsen *et al.*, 2010; Pertovaara *et al.*, 2001; for details, see Kambur and Männistö, 2010). A first generation COMT inhibitor, tropolone, has also caused lethal interactions with morphine, but such effect should rather be attributed to the non-specific effects of tropolone that are lacking from more specific and selective COMT inhibitors (Davis *et al.*, 1979a,b) (Kambur and Männistö, 2009, *unpublished data*).

In *COMT* gene modified mice, overexpression of a high-activity *COMT* variant decreases nociceptive sensitivity, whereas knockout of *COMT* increased pain sensitivity (Papaleo *et al.*, 2008; Walsh *et al.*, 2010). Also a lack of S-COMT induced subtle sex-dependent changes of nociceptive phenotype suggesting different roles for COMT isoforms, which is in line with their expression pattern and effects on catecholamine metabolism (Tammimäki *et al.*, 2010). An

extensive study comparing genotypic differences with gene expression in a genome-wide analysis of 29 inbred mouse strains proved that genomic variation affect expression of *Comt1* which is *cis*-regulated and mainly determined by insertion of SINE-element in the 3' untranslated region (UTR) (Segall *et al.*, 2010). SINE-insertion increased expression and activity of Comt1 by about 20%, which was verified in transfected cell line, and the increase in COMT activity was accompanied by reduced nociceptive sensitivity. Expression of *Comt* mRNA and COMT activity in inbred mouse strains (C57 and DBA) have also been shown to correlate with morphine responses (Grice *et al.*, 2007).

2.4 Effect of genetic variation in *COMT* gene on human pain

2.4.1 Experimental pain

Val^{108/158}Met. There are several human studies assessing the association of genetic polymorphisms in *COMT* gene and pain. The most studied is the common SNP rs4680, often referred to as Val^{108/158}Met. In SNP rs4680 a non-synonymous change of G to A occurs, resulting in a replacement of valine by methionine at codon 158. Such replacement increases thermolability of the enzyme and decreases COMT activity (Lotta *et al.*, 1995; Männistö and Kaakkola, 1999). The effect of this polymorphism on pain was first suggested by Zubieta *et al.* (2003) in a small and ethnically heterogeneous cohort (Zubieta *et al.*, 2003). Pain was caused by injection of hypertonic saline to the masseter muscle and the amount of saline needed to achieve a predefined pain level was used as an indicator of pain sensitivity. Homozygous Met/Met (A/A) carriers were the most sensitive, whereas volunteers lacking the A-allele (G/G, Val/Val carriers) were the least sensitive, and the heterozygotes (A/G, Val/Met) showed intermediate pain sensitivity.

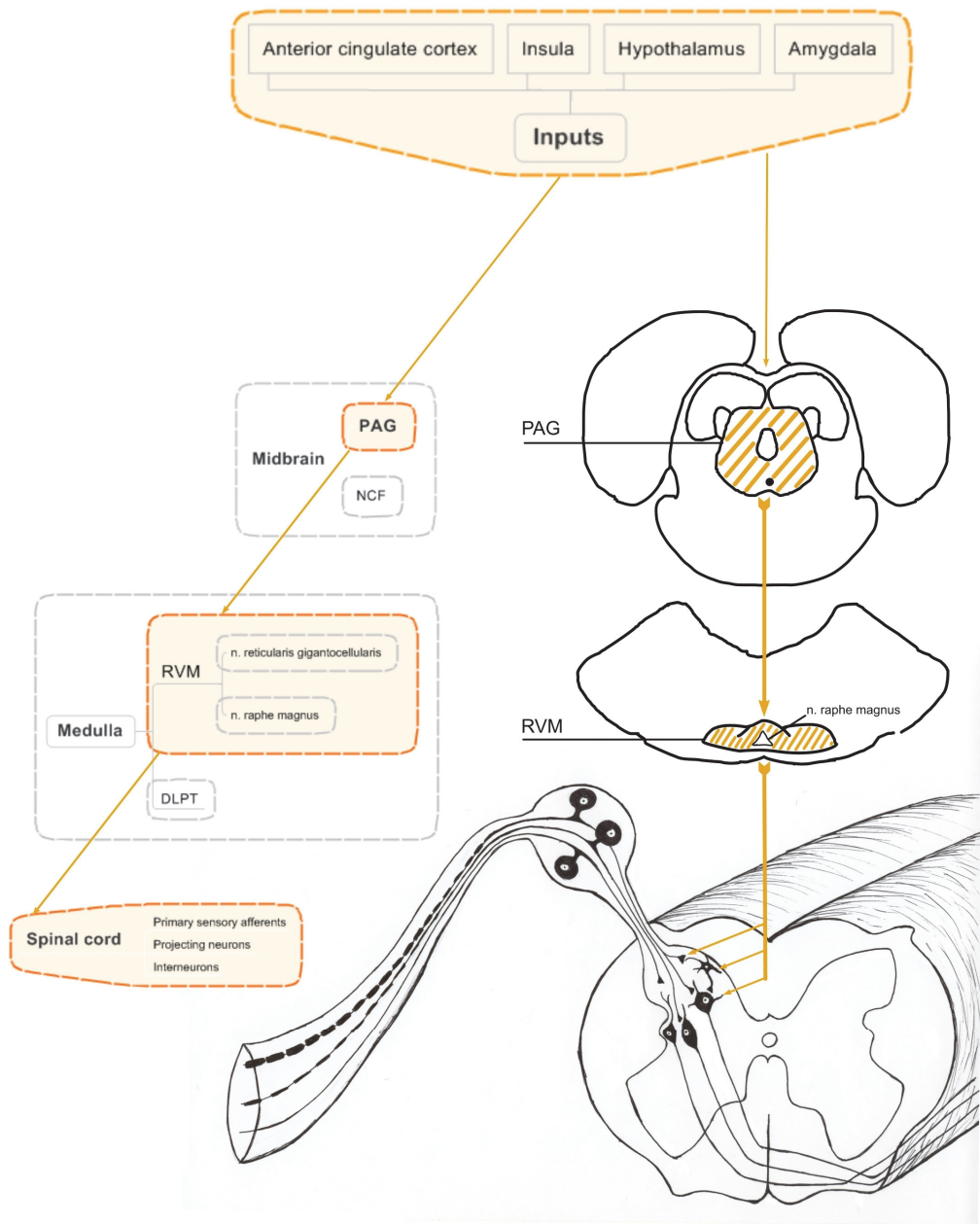


Fig. 4. Descending modulation of pain. In the figure, main parts of descending modulatory pathways are highlighted with orange colour. PAG - periaqueductal gray, NCF - nucleus cuneiformis, RVM - rostral ventromedial medulla, DLPT - dorsolateral pontine tegmentum.

Several other studies using different nociceptive models and patient/volunteer samples, however, failed to show an association between SNP rs4680 and experimental pain caused by nociceptive stimulation of different modality and/or duration (Birklein *et al.*, 2008; Diatchenko *et al.*,

2005,2006; Jensen *et al.*, 2009; Kim *et al.*, 2004a; Potvin *et al.*, 2009). In a large cohort of healthy females, Val^{108/158}Met genotype was associated with the rate of temporal summation of heat pain, but neither with other pain measures (pressure pain threshold, threshold and tolerance of thermal and ischemic pain), nor with summed pain scores (Diatchenko *et al.*, 2005, 2006). In another study, SNP rs4680 affected the cold pain threshold when the effect of the confounding SNP in gene coding for FAAH (rs4141964 causing T>C) was taken into account, and carriers of the variant *COMT* genotype (Val/Val) were less sensitive to cold pain than non-carriers (Met/Met) (Lötsch *et al.*, 2009a). However, if the confounding genotype was not taken into account, the results lost their significance. Thus, it seems that SNP rs4680 may affect pain as assessed in different experimental pain paradigms involving hypertonic saline, temporal summation of the heat pain or sensitivity to cold pain. The effect of *COMT* genotype, however, has varied among different studies and seems to be prone to confounding effects of other genetic factors (Lötsch *et al.*, 2009a) or other determinants of pain such as psychological phenotype (George *et al.*, 2008a), gender (Kim *et al.*, 2006b) which should be paid attention at and included in data analysis.

OTHER SNPs and HAPLOTYPES. In addition to rs4680, the effect of five other SNPs in the *COMT* gene (rs2097903, rs6269, rs4633, rs4818, rs165599) and their combination haplotypes on pressure, thermal and ischemic pain and temporal summation of thermal pain have been evaluated in the same cohort (Diatchenko *et al.*, 2005, 2006). The SNPs rs6269 and rs4818 were associated with pain ratings accounting for 6% (rs6269) and 7% (rs4818) of variation in the pain sensitivity. The effect of haplotypes on pain sensitivity was greater than that of any individual SNP. Three major haplotypes constructed in the study encompassed 96% of the genetic variation in the *COMT* gene and determined *COMT* activity that correlated with pain sensitivity. A high *COMT* activity was associated with a low sensitivity to pain and vice versa, and a correlation was first shown when pain was assessed by *Z*-scores, which are a combined outcome measure incorporating pressure pain thresholds, thermal pain thresholds and tolerance, temporal summation of thermal pain, ischemic pain threshold and tolerance (Diatchenko *et al.*, 2005). Among the individual pain measures, the associations were strongest for the threshold and tolerance of the thermal pain whereas other nociceptive variables showed the same trend, while lacking statistical significance (Diatchenko *et al.*, 2006). The rate of temporal summation of heat pain (which was associated with the Val^{108/158}Met genotype) did not differ between haplotype combinations. Thus it seems, that the Val^{108/158}Met SNP while contributing for variation in a temporal summation of pain and some other nociceptive variables, is less significant than other SNPs or haplotypes of *COMT* gene, which are more important determinants of a resting

nociceptive sensitivity. Also in another study a low COMT-activity haplotype (constructed using SNPs rs4633 and rs4818) was associated with an increased intensity of acute experimental muscle pain (but had no effect on other secondary outcomes including a muscle torque production and a self-report of upper-extremity disability) as assessed by visual analogue scale (VAS) (George *et al.*, 2008a). The effect was seen in volunteers with high pain catastrophising scores, suggesting an interaction between *COMT* gene and psychological phenotype. Finally, in a study assessing the effects of 13 *COMT* SNPs (rs5746846, rs2020917, rs933271, rs5993882, rs740603, rs4646312, rs165722, rs6269, rs4633, rs4818, rs4680, rs174699, rs165728) in a large (n=735) cohort of healthy volunteers, SNPs rs4646312 and rs6269 were associated with cold pain sensitivity. The effect was gender specific and seen exclusively in females (Kim *et al.*, 2006b).

2.4.2 Clinical pain

The effect of genetic variation in *COMT* on clinical pain has been evaluated by numerous studies in patients with diagnosed clinical, mostly chronic pain conditions using different approaches. These studies have assessed the prevalence of different *COMT* variants in different patient groups, risk of developing chronic pain condition or pain symptoms in different genotypes or impact of variants on pain symptoms.

CHRONIC PAIN STUDIES. There are many studies evaluating the effect of *COMT* on symptoms in different chronic pain conditions (for references, see Kambur and Männistö, 2010; Tammimäki and Männistö, 2012). In several conditions, such as temporomandibular joint disorder (TMD), fibromyalgia, migraine or headache, *COMT* genotype seems to play a role, since variants with a low predicted COMT activity can increase their incidence or symptoms. At least in TMD, pronociceptive effect of a low COMT activity genotype was reversed by propranolol, a non-selective β -receptor antagonist, supporting involvement of adrenergic $\beta_{2/3}$ -receptors in mechanism of pronociceptive effects (Tchivileva *et al.*, 2010). Variation in *COMT* seems to modulate not only pain intensity, but also other aspects of pain conditions. *COMT* genotype may contribute to psychological consequences of pain. For example, in fibromyalgia pain induced more pronounced attenuation of positive mood in Met/Met-carriers (Finan *et al.*, 2010). Also other symptoms of pain conditions, such as fatigue, disability, and morning stiffness in fibromyalgia (Vargas-Alarcon *et al.*, 2007) as well as nausea and vomiting in migraine attacks (Park *et al.*, 2007), can be modulated by *COMT*. In general Met/Met-genotype has been associated with

more severe symptoms. In some of the chronic pain conditions, such as in neuropathic or cancer pain, the evidence for the modulatory role of *COMT* is lacking.

There is a significant variation among chronic pain studies evaluating the effect of *COMT* variants and some of the studies failed to replicate the effect of *COMT*, especially in smaller cohorts or more heterogenic patient groups. Unfortunately, the vast majority of such studies fail to report estimates of their statistical power, making it difficult to evaluate if these studies were even capable of detecting the gene effect. This also suggests that for reliable evaluation of gene-effects in future studies, more attention should be paid to sufficient cohort size, control for confounding variables and overall quality of studies. It must be noted that the effect of *COMT* on pain is not only dependent on the pain condition, but also on psychological factors, ethnic background and sex which can interact with the gene effect or act as independent confounders (Fijal *et al.*, 2010; George *et al.*, 2006a,b; Slade *et al.*, 2007; Vargas-Alarcon *et al.*, 2007). Failure to account for such factors may explain lack of significance in some of the studies.

ACUTE PAIN STUDIES. There are only few studies assessing the effect of *COMT* genotype on acute pain. *COMT* has been reported to modulate pain symptoms in arthroscopic, orthopaedic, cardiac, and oral surgery patients (Ahlers *et al.*, 2013; Henker *et al.*, 2013; Mamie *et al.*, 2013; for review, see Kambur and Männistö, 2010). Rs4633-rs4818 haplotypes with low *COMT* activity were associated with higher pre- and postoperative pain in arthroscopic surgery patients, and the effect was modulated by psychological phenotype (high pain catastrophising scores) (George *et al.*, 2008b). In postoperative pain ratings after oral surgery, surprisingly, SNP rs740603 showed the strongest effect and A/A-carriers were less sensitive and had lower pain ratings, even though the association was weak (Kim *et al.*, 2006a). In orthopaedic, abdominal and cardiac surgery patients, rs4680A (Met) was associated with higher pain ratings (Ahlers *et al.*, 2013; Henker *et al.*, 2013; Mamie *et al.*, 2013). Similarly, GCGG-haplotype containing rs4680 has been associated with postoperative pain (Henker *et al.*, 2013). In some pain conditions, the effect of *COMT* variation may be at least partially reversible by adrenergic β -receptor blockade, since propranolol reduced the composite pain index in TMD patients with a low *COMT* activity haplotype in gene-dependent manner (Tchivileva *et al.*, 2010).

Compared with experimental pain, acute clinical pain is much more variable. In a clinical setting, pain is caused by inflammation, injury, or surgery which vary in their extent, location and stage. Consequently, origin and intensity of nociceptive stimuli vary. Since they both correlate with pain intensity, they are important sources of variation. The pain, underlying pathology, and

comorbidities are always treated due to ethical and legislative reasons. Treatment can also interfere with intensity of pain and represents another major source of variation. Pain measurements themselves can be important confounders, as patients are often assessed, due to practical reasons, at different time points, in differing conditions, and by different personnel. Intensity of acute pain is most reliably assessed in a standardized manner (such as VAS or NRS) and directly, since it is less prone to confounding factors than indirect measures. Pain is also a subjective measure and, therefore, anchors of NRS and VAS-scales (worst imaginable pain) are by no means objective. Controlling for confounders usually requires a prospective study design allowing uniform inclusion criteria, more homogenous cohorts, and standardised measurement protocols. This is laborious, which has led to small sample sizes in most of the studies assessing effect of *COMT* on acute clinical pain. However, despite of small sample sizes and higher amount of variation, several studies have shown association between acute clinical pain and genetic polymorphisms in *COMT* (Ahlers *et al.*, 2013; George *et al.*, 2008b; Henker *et al.*, 2013; Kim *et al.*, 2006a; Mamie *et al.*, 2013).

2.4.3 Conclusions from human studies

Several studies on chronic and acute pain show a significant effect of genetic variation in *COMT* on pain. The effect seems to depend on phenotype as seen from results of chronic pain studies suggesting differential role of *COMT* in different pain conditions, which is logical considering heterogeneity of pain pathologies. Also, the role of catecholamines and other factors are phenotype-dependent. Effect of *COMT* may depend on genotype, as seen from different results from different SNPs and haplotypes. Especially assessment and interpretation of effects of individual SNPs seems problematic. First of all, existing data indicate limited explanatory value of each individual SNPs alone. A vast majority of the studies assessed Val^{108/158}Met, leaving variation in the rest of the gene completely unaddressed. Val^{108/158}Met, despite being extensively characterized and having confirmed molecular mechanism of action, is not the only determinant of *COMT* function. Also, several other SNPs play a role, and have been shown to be more important than Val^{108/158}Met in several acute and chronic pain studies. If their effect is not taken into account in statistical analyses, they can act as confounders undermining the results, and leading to unjustified conclusions. What comes to other *COMT* SNPs, their effects have been assessed only in a tiny fraction of studies and in most of them, their effects on *COMT* activity

have not been verified. Also other SNPs except Val^{108/158}Met, are not sole determinants of COMT function. Their effect depends on other SNPs, which form their immediate environment together with other genetic machinery (see below). Thus, the effects of multiple putatively effective SNPs have to be addressed simultaneously. Such an assessment is, however, difficult from statistical point of view.

From **statistical perspective**, several clinical pain studies assessing multiple SNPs showed significant associations. However, when multiple hypotheses are tested the risk of type I error and false positives increases. Since type I error is generally considered important, it is typically addressed by correction for multiple comparisons. Such corrections, however, decrease statistical power. Indeed, in studies assessing *COMT* SNPs and clinical pain some of the effects did not reach the level of statistical significance after corrections. Corrections for multiple comparisons increase type II error, which in the case of *COMT* SNPs would mean a lack of statistically significant association when the effect of SNP on pain phenotype is real. Whereas type I error has often been addressed, type II error has been grossly overlooked and appropriate power analysis, describing detection limits and sensitivity of used statistical approach, are mostly non-existent. As in the case of *COMT*, its effects have often been shown in other pain studies or even seen in the same study in different phenotypes thus addressing the risk of type I error and getting positive association due to random variation, such approach seems overconservative.

Evaluating ***COMT* haplotypes** appears to be the best way to assess the effect of COMT on pain and take into account multiple *COMT* SNPs. Several acute and chronic studies showed that low COMT-activity haplotypes increase pain, supporting involvement of COMT. Finally, the effect may also depend on non-genetic confounding factors, which need to be taken into account in both, chronic and acute pain studies.

2.4.4 *COMT* genotype and effect of opioids

In several studies, *COMT* genotype has been shown to affect opioid responses in experimental and clinical settings. In general, genetic variants with a low *COMT* activity enhance opioid analgesia but also adverse effects, at least in cancer and postoperative pain patients (see Kambur and Männistö, 2010 for detailed description; Henker *et al.*, 2013; Jensen *et al.*, 2009; Matsuoka *et al.*, 2012; Rakvåg *et al.*, 2005, 2008; Reyes-Gibby *et al.*, 2007; Ross *et al.*, 2008; Zubieta *et al.*, 2003). The effects are also seen in genetic variants whose effect on *COMT* activity is not known. Finally, effects of *COMT* on opioid analgesia and adverse effects are modulated by polymorphisms in other genes. In analgesic effect, interaction was reported with polymorphism in *OPRM1*-gene (Reyes-Gibby *et al.*, 2007), whereas in adverse effects interaction was seen with multidrug-resistance1 gene (Ross *et al.*, 2008). Thus, gene-gene interactions need to be taken into account in further studies addressing effects of *COMT* on opioid responses. Finally, the modulatory effect of *COMT* has mostly been shown in cancer pain patients treated with morphine. Despite of that, it could be speculated that *COMT* would similarly modulate the effects of other μ -receptor agonists, as well as other patient groups. In future studies, however, one could expect that in heterogeneous patient groups (such as Lötsch *et al.*, 2009b) where intrinsic non-genetic variation is high, sample size must be increased to get statistically significant results. In accordance with this, in a recent multicenter study, *COMT* SNPs rs4680 and rs4646312 were associated with differences in the median opioid dose in cancer patients (Klepstad *et al.*, 2011). Differences were significant in a large sample despite heterogeneity in pathology of cancer, nociceptive symptoms, opioid medication, and other issues. However, the differences did not reach the level of statistical significance in a smaller validation sample. Besides analgesia, *COMT* genotype may modulate other effects of morphine as well, such as central adverse effects (Ross *et al.*, 2008). Genotype affected “central adverse effects” as well as “drowsiness and confusion or hallucinations”, especially in patients with moderate or severe symptoms. Significant associations were seen in haplotype 1, which is present in 10% of the population, and in SNPs in intron 1 which defines the haplotype. SNP rs740603 showed the strongest association with the protective effect, and G-carriers were less likely to have adverse effects than non-carriers. Also T-allele of SNP rs174680 showed a weak association. Effect of *COMT* genotype was modified by a SNP rs2032582 (Ala⁸⁹³Ser/Thr) in multidrug resistance-1 gene (G-allele). *COMT* genotype did not affect other, non-CNS-mediated, adverse effects or cognitive status (Rakvåg *et al.*, 2008; Ross *et al.*, 2008).

3 AIMS OF THE STUDY

The general aim was to study the role of COMT in pain using *Comt* gene modified animals and COMT inhibitors in experimental models of pain, and to study the clinical relevance of *COMT* polymorphisms in human pain in a large cohort of patients who were exposed to experimental and postoperative pain and genotyped for the *COMT* gene.

MODULATION OF NOCICEPTION AND PAIN BY COMT

The specific aims were:

1. To characterize the effects of an inborn COMT deficiency on experimental acute nociception and opioid responses in mice.
2. To measure the effects of COMT inhibitors on experimental acute and inflammatory pain in normal mice.
3. To find out whether nitecapone has antinociceptive effects in neuropathic pain in rats.
4. To study the effect of genetic variation in *COMT* gene on experimental and clinical postoperative pain in patients scheduled for breast cancer surgery. This includes assessment of *COMT* SNPs and haplotypes in experimental pain, acute postoperative pain and opioid requirements.

4 MATERIALS AND MAIN METHODS

4.1 Animal and biochemical experiments

4.1.1 General

MICE. Male *Comt* gene disrupted mice were used in studies **I** and **II**. *Comt* gene disrupted animals were originally generated by Gogos *et al.* (1998). In the study **I** homozygous mice (full *Comt* knockout mice), heterozygous mice and their wild-type littermates, aged 8–10 months at the beginning of the experiments, were used (**I**). Also in the study **II** 25 *Comt* knockout mice (for a detailed description of these mice, see Tammimäki *et al.*, 2008) were used in the behavioural experiments (**II**).

Mice were bred in the National Laboratory Animal Center (Kuopio, Finland), breeding heterozygous males and females produced by regularly enriching the homozygous animals with C57BL/6J mice (**I**). The mice were genotyped using Southern blotting as described in Huotari *et al.* (2002) (**I**). The *Comt* deficient mice show no gross phenotypes, compared to the wild-type mice (Gogos *et al.*, 1998; Huotari *et al.*, 2002). They breed normally, their lifespan is normal and they have normal diurnal rhythm (Gogos *et al.*, 1998; Haasio *et al.*, 2003).

In addition, in the study **II** a total of 196 male wild-type mice of the C57BL/6J background (Harlan, Horst, the Netherlands), aged 3–4 months and weighing 26–34 g were used.

RATS. Rats were used in studies **II** and **III**. Intrathecal infusions were done in 15 adult male Wistar rats (Harlan, Horst, the Netherlands) weighing 200–300 g (**II**). In the study **III** eighty-six male Wistar rats, (Harlan Laboratories, Horst, The Netherlands) weighing 140–350 g in the beginning of the experiment were used (14 in a pilot experiment, 48 in the behavioural tests and 24 specifically in the COMT activity measurements). The Wistar strain was chosen to allow

comparison of the results with the earlier diabetes model study (Pertovaara *et al.*, 2001). Rats of this strain have been reported to develop neuropathic pain symptoms after the spinal nerve ligation (SNL) model of neuropathic pain (Lee *et al.*, 1997; Lovell *et al.*, 2000), which was also verified in a pilot experiment in our own laboratory. Animals were randomly allocated to treatment groups so that in each cage the number of animals assigned to each treatment group was approximately equal.

HOUSING. The animals were housed in group cages in groups of 3-4 (**III**). To allow recovery after the SNL surgery the animals were transferred to single cages and returned to home cages 2–4 days after the surgery. In other studies the animals were housed in individual cages (**I** and **II**) at an ambient temperature of 21–24°C (**I**, 21–23°C; **II** and **III**, 22–24°C). Artificial lighting followed a fixed 12-hr light-dark cycle (**I**, **II** and **III**). All experiments were conducted during the light phase of the day (**I**, **II** and **III**). All animals had free and continuous access to food pellets and drinking fluid (tap water) which were available *ad libitum* (**I**, **II** and **III**).

SELECTION and HABITUATION. The animals were not pre-selected for nociceptive or behavioural experiments (**I**, **II** and **III**). After the surgery (**II** and **III**), however, only the animals in which surgery was successful/showing appropriate clinical/behavioural phenotype were used in nociceptive measurements. The animals were habituated to the testing environment and handling for 2-4 consequent days before the experiments (**I** and **II**, 4 days; **III**, 2-4 days). The animals spent 10–30 min in each of the testing apparatus (except the tail flick) and the order of habituation was the same as the testing order during experiments (**I**, **II** and **III**). Rats were also habituated again during the experiment to each test setting for 15 min before the actual measurements (**II** and **III**).

4.1.2 Nociceptive measurements in rodents

Nociceptive measurements in mice (**I** and **II**) and rats (**II** and **III**) included assessment of latencies of thermal nociception (**I** and **II**) with hot plate, tail flick and paw flick tests, measurement of mechanical nociceptive thresholds (**II** and **III**) and cold allodynia responses (**III**).

HOT PLATE TEST (Eddy and Leimbach, 1953; Le Bars *et al.*, 2001; O’Callaghan and Holzman, 1975; Woolfe and Mac-Donald, 1944) (Harvard Apparatus, Kent, UK) was applied in both mice (**I** and **II**) and rats (**II**) in identical manner. Briefly, the animal was placed on the top of the hot plate covered by plexiglass cylinder (diameter) within which it was allowed to move freely. The temperature in the hot plate was 52 ± 0.2 °C. When the first nociceptive behaviour, either a withdrawal and brisk shaking or licking of the hind paw or jumping, occurred the animal was removed from the test and the latency to response was recorded. To prevent tissue damage, a 60 s cut-off time was used (**I** and **II**).

TAIL FLICK TEST (Hardy, 1953; Hardy *et al.*, 1957; Le Bars *et al.*, 2001) was applied in studies **I** and **II** (model DS20, Ugo Basile, Comerio, Italy). The mouse was gently restrained by wrapping in the soft towel and its tail was placed in the device on the top of the photocell. The device produced infrared radiant light (100 W bulb, 15 V) which was focused on the tail skin by parabolic aluminium mirror. The intensity of the light beam was set to 50 arbitrary units what in average produced a response in 2–3 s in pilot experiment (**I** and **II**). When the animal withdrew its tail from the source of thermal stimulation, *e.g.* flicked its tail from the light source, light reached the photocell and stopped the built-in timer. The time from the beginning of the exposure to the actual response was recorded as tail flick latency. The cut-off time of 7 s was used to avoid tissue damage (**I** and **II**).

PAW FLICK TEST (Hargreaves *et al.*, 1988; Le Bars *et al.*, 2001; Yeomans and Proudfit, 1994) was used in the study **II** (model DS20, Ugo Basile, Comerio, Italy). In the test the animal was placed in the transparent plexiglass box which was placed on the top of the glass shield. Animal was allowed to freely move and settle down. Once stationary, the infrared light beam was projected on the plantar surface of the hind paw. The intensity of the light beam was set to 50 arbitrary units, which in average produced a response in 2–3 s in the pilot experiments. When the animal reacted by withdrawing and briskly shaking or licking the hind paw ipsilateral to stimulation the timer was automatically stopped and the time from the beginning of the stimulation to the first nociceptive response was recorded. Cut-off time of 10 s was used to prevent the tissue damage.

MECHANICAL NOCICEPTIVE THRESHOLDS were assessed in mice (**II**) and rats (**II** and **III**) with digital force gauge (Imada, DPS-1, Northbrook, IL, USA). The test is similar to traditional von Frey test (Le Bars *et al.*, 2001). In the test the animals were placed on the metal

mesh covered with an individual Plexiglas dome. They were allowed to settle down for 1 min and when the animal was standing on both hind paws and the weight was equally distributed between the paws, the plantar surface of the hind paw was approached perpendicularly with a metallic monofilament. 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 paw was approached slowly and contact was made for 1 s to standardize the time-course of stimulation. 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 force just sufficient to produce a response was applied to find out the lowest force leading to response – the nociceptive threshold. The monofilament with diameter of 0.2 mm was used in mice experiments (II) whereas 0.5 mm diameter was used in neuropathic rats (III) and 0.3 mm in normal, non-neuropathic rats (II).

COLD ALLODYNIA was assessed with acetone test (III) (Choi *et al.*, 1994). In the test a drop of acetone was applied to the plantar surface of the paw five times on both hind paws. The brisk withdrawal, shaking or licking of the foot was considered a nociceptive response and number of positive responses was recorded. In the results the ratio between positive responses and trials was used.

4.1.3 Carrageenan inflammation

Inflammatory pain was assessed in mice after the administration of different COMT inhibitors, OR-486, nitecapone or CGP 28014 (II). After administration of drugs the animals were briefly anaesthetized and received an intraplantar injection of carrageenan (Winter and Flataker, 1965a; 1965b; Winter *et al.*, 1962) to the plantar area of the hind paw. Nociceptive responses were assessed 3 h after induction of inflammation in animals receiving OR-486 or nitecapone and 3, 4 and 5 h after induction in animals receiving CGP 28014.

4.1.4 Neuropathic pain

The SNL model of neuropathic pain introduced by Kim and Chung (1992), was used (III). The animals were anaesthetized with isoflurane (Baxter Oy, Helsinki, Finland) diluted in air (4.5% for induction and 2–3% for maintenance) and the left L5 and L6 spinal nerves were exposed by

removing a small piece of the paravertebral muscle and part of the left spinous process of the L5 lumbar vertebra. The L5 and L6 spinal nerves were carefully isolated from the L4 spinal nerve and other surrounding tissues, and tightly ligated with 6-0 silk (Perma-Hand Seide, Ethicon, Belgium). After ligation, the muscle and the adjacent fascia were closed with sutures (Vicryl-Rapid 3/0, Ethicon, Belgium) and the skin with metal clips. To detect any complications of surgery the rats were closely observed daily, and animals showing motor complications produced by unintended damage of L4 spinal nerve, loss of weight or any other abnormal signs were immediately sacrificed.

4.1.5 Biochemical assays

COMT ASSAY. COMT activity was measured after administration of COMT inhibitors in different tissues in mice (**II**; liver, striatum, prefrontal cortical tissue and spinal cord) and rats (**III**; intact rats: striatum and liver, SNL rats: striatum, prefrontal cortex, spinal cord L1–2, L3–4 and L5–6 areas and sensory ganglia from the lumbar area). In mice, tissues were dissected 1 and 3 h and in rats 0.5 – 24 h after drug administration. Tissues were rapidly dissected, cooled in plastic tubes on dry ice and stored in -80°C until the COMT assay. COMT assay was performed as described earlier (Nissinen and Männistö, 1984; Reenilä *et al.*, 1995). Samples containing COMT were incubated with MgCl₂ (5 mM), *S*-adenosyl-*L*-methionine (200 mM) and 3,4-dihydroxy benzoic acid (500 mM) in phosphate buffer (100 mM, pH 7.4) at 37°C.

Enzymatic reaction gave rise to reaction products - vanillic and isovanillic acid, which were analysed using high-performance liquid chromatographic (HPLC) system with electrochemical detection. Sample dilutions were injected into the mobile phase (Na₂HPO₄ - EDTA – methanol) flowing through the column (RP-18) in which compounds being studied were separated. Compounds were detected with coulometric detector (Waters Spherisorb, Milford, MA, USA) and retention time was used to identify and peak height to quantify the reaction products. COMT activity was expressed as picomoles (pm) of vanillic acid formed in one min of enzymatic reaction per mg of protein in the sample. To assess the protein concentration of the samples, Pierce protein assay kit, which is based on bicinchoninic acid method, was used (Pierce, Rockford, IL, USA) (**II** and **III**).

4.1.6 Drugs

OPIOIDS. Morphine hydrochloride (**I**) (10 mg/kg; University Pharmacy, Helsinki, Finland) was dissolved in physiological saline (0.9% NaCl, Natrosteril 9 mg/ml, Baxter, Vantaa, Finland) (**I**). Morphine and saline were administered subcutaneously (*s.c.*) in a volume of 0.1 ml/10 g of body weight (**I**). Morphine tolerance (**I**) was produced with a morphine pellet (National Institute of Drug Abuse, Bethesda, MD, USA). Pellets were implanted *s.c.* for 5 days and each pellet contained 75 mg of morphine base. Implantation was performed during the anaesthesia. Morphine withdrawal (**I**) was produced with 1 mg/kg of naloxone (The DuPont Merck Pharmaceutical, Wilmington, DE, USA), which was administered *s.c.* in a volume of 0.025 ml/10 g.

ANAESTHESIA. For the implantation of morphine pellet (**I**) the animals were anaesthetized with a *s.c.* injection of a mixture of midazolam (5 mg/ml, Dormicum[®], Roche, Bâle, Switzerland) and fentanyl–fluanisone (fentanyl 0.2 mg/ml, fluanisone 10 mg/ml, Hypnorm[®], Janssen Pharmaceutica, Beerse, Belgium) solution. Each animal received 0.4 mg of fentanyl citrate, 1.25 mg of fluanisone, and 0.63 mg of midazolam per 10 g of body weight. Intrathecal catheters (**II**) were set under pentobarbital anaesthesia (50 mg/kg, intraperitoneally - *i.p.*; Mebunat Vet 60 mg/ml, Orion Pharma, Espoo, Finland).

INTRATHECAL MICROINJECTIONS. An intrathecal catheter made of a polyethylene (PE-10) tubing was inserted into the lumbar subarachnoid space as described by Størkson *et al.* (1996), and fixed through a layer of superficial muscles. To confirm the intraspinal location of the catheter lidocaine hydrochloride (Orion Pharma, Espoo, Finland) was administered through the catheter. An immediate paralysis of hind limbs lasting 15–30 min was considered an indicator of correct location of the catheter. Only rats with properly located catheters and lacking neurological deficits or other complications from the catheter insertion were included in the study. After surgery, the rats were allowed to recover before intrathecal administration of nitecapone for 4 days.

COMT inhibitors. Nitecapone (**II** and **III**) [OR-462, 3-(3,4-dihydroxy-5-nitrobenzylidene)-2,4-pentanedione], OR-486 (**II**) and CGP 28014 [N-(2-pyridone-6-yl)-N',N'-di-n-propylformamidin] (**II**) were synthesized by Dr. Aino Pippuri (Orion Pharma, Espoo, Finland) using methods described earlier (Bäckström *et al.*, 1989). For the systemic administration studies

(II and III), the COMT-inhibitors were suspended in 0.5% (w/v) carboxymethylcellulose (CMC) (Fluka AG, Buchs SG, Switzerland) and administered *i.p.* in volume of 7.5 ml/kg for mice and 3.3 ml/kg for rats. The dose of COMT inhibitors was 30 mg/kg. For the intrathecal administration (II), nitecapone was dissolved in 30% (w/v) hydroxypropyl- β -cyclodextrin (Acros Organics, Geel, Belgium) dissolved in sterile physiological phosphate-buffered saline (pH 7.4) and administered in a volume of 10 μ l into the lumbar subarachnoid space.

CARRAGEENAN. Carrageenan (II) (Sigma, St. Louis, MO, USA) was dissolved in physiological saline (0.9% NaCl, Natrosteril 9 mg/ml, Baxter, Vantaa, Finland) to give a 2% (w/v) solution and administered in a volume of 40 μ l into the plantar region of the right hind paw.

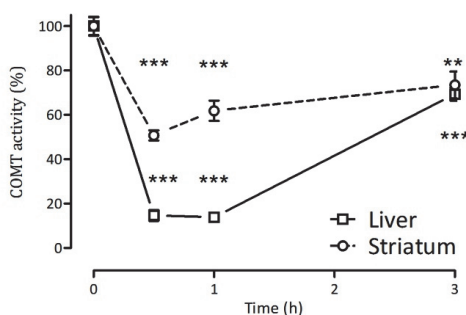


Fig. 5. COMT activity – rats. COMT activity in the liver and striatum of male Wistar rats after acute administration of nitecapone (30 mg/kg, *i.p.*). Tissue samples were collected 0.5 (n=7), 1 (n=6) and 3 h (n=3) after nitecapone administration. COMT activity of the untreated control group (n=8) is plotted at 0 min. Activity is normalised to that of the control group (mean activity = 100%). Asterisks indicate a difference as compared with the control group (1-way ANOVA: $p < 0.0001$; Bonferroni-correction: **, $p < 0.001$; ***, $p < 0.0001$).

COMT inhibitor considerations. The purity of the compounds was checked by thin layer chromatography and nuclear magnetic resonance, and it was always better than 99%. The efficacy/biological activity of the COMT inhibitors used in behavioural studies (II and III) was verified by *ex vivo* measurements of COMT activity in rats (Fig. 5) and mice (Fig. 6).

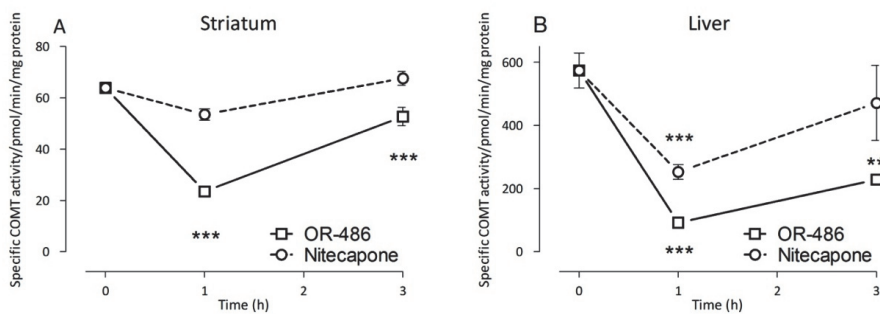


Fig. 6. COMT activity – mice. (A) Striatal catechol-*O*-methyltransferase (COMT) activity in male C57/BL6J mice 1 and 3 h after *i.p.* administration of 3,5-dinitrocatechol (OR-486) (30 mg/kg; 1 h, n=8; 3 h, n=13) or nitecapone (30 mg/kg; 1 h, n=8; 3 h, n=16). Baseline (0 h) represents COMT activity of vehicle treated animals (n=21). ** $p < 0.01$; *** $p < 0.001$ vs. vehicle group; 1-way ANOVA followed by a Bonferroni test. (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). ** $p < 0.01$ vs. vehicle group.

4.2 Humans

4.2.1 General

SUBJECTS. Study **IV** reports the data of 1000 female patients treated for breast cancer. For this sample 1149 patients of the 1536 eligible consecutive patients with unilateral non-metastasized breast cancer who enrolled for surgical treatment at the Unit for Breast Surgery at the Women's Hospital, Helsinki University Central Hospital, between August 2006 and December 2010, were asked to participate in the study. The specific types of surgery were modified radical mastectomy or breast conserving mastectomy (resection), with or without sentinel biopsy and/or axillary clearance (AC). The study cohort is described in detail in Kaunisto *et al.* (2013).

INITIAL CLINICAL ASSESSMENT. Following informed consent acquisition, medical and medication demographic history was taken. The patients filled in questionnaires about depression (Beck Depression Inventory; Beck *et al.*, 1961) and anxiety (State and Trait Anxiety Inventory, STAI) (Spielberger *et al.*, 1983). Both have been validated for this particular group of patients and they have been used in previous studies (Tasmuth *et al.*, 1996).

ANAESTHESIA. The patients were premedicated with diazepam 2.5-15 mg and paracetamol 1 g orally. The patients were anaesthetized with *i.v.* infusions of remifentanyl (0.05-0.25 µg/kg/min), and propofol (2-3 mg/kg for induction and 50-100 µg/kg/min for maintenance). Rocuronium was used to achieve sufficient muscle relaxation and to facilitate tracheal intubation. During the closure of skin, fentanyl (1 µg/kg, *i.v.*) was given and the infusion of remifentanyl was stopped. At the same time neostigmine (2.5 mg) with glycopyrrolate (0.5 mg) were administered *i.v.* to reverse the neuromuscular block. Blood specimen was drawn for DNA isolation and banking during anaesthesia to avoid unnecessary injections.

4.2.2 Pain measurements in humans

EXPERIMENTAL PAIN. All tests were carefully explained to the patients before the experiment. Contact heat pain was assessed using the 16 mm x 16 mm thermode of the TSA-II NeuroSensory Analyzer (Medoc Ltd., Ramat Yishai, Israel). A temperature of 43°C and 48°C was exposed, in this order, once each, for 5 s on the antebrachium contralateral to surgery. Patients assessed the intensity and unpleasantness of pain with a 0 to 10 Numerical Rating Scale (NRS) at the end of the test. Zero represented “no pain”, and 10 “maximal pain imaginable”.

Cold pain was measured by immersing the hand to cold (3°C) water bath (JULABO, U.S.A.) for the maximum of 90 s or for less depending on for how long the patient could tolerate the test. Time to withdrawal, and pain intensity (NRS) and unpleasantness were measured at withdrawal and every 15 s during the test. Cold pain test was not performed to the first 100 patients studied (n=900). Those patients who withdrew their hand before the analyzed time point were coded as having the maximum NRS value (n=118 at 15 s, n=353 at 30 s).

POSTOPERATIVE PAIN ASSESSMENT and administration of oxycodone. In the post anaesthesia care unit (PACU) the patients were asked to rate their pain intensity at rest and during motion using NRS. Motion pain was assessed by asking the patient to raise the arm ipsilateral to surgery up to 90°. Patients were titrated with *i.v.* oxycodone by the research nurse who asked about the pain intensity every 5 min and administered oxycodone in doses of 1-3 mg until adequate analgesia (NRS<4/10) was achieved. After this pain intensity was asked every 15 min until the patient needed the next dose of oxycodone. The required quantity of oxycodone was

recorded. For the ward the patients were provided with a patient-controlled analgesia device (PCA) for up to 20 hrs. The total amount of oxycodone consumed after the operation, including both, the titration at the PACU and PCA consumption was recorded. Any incidents of adverse events were recorded.

SNP SELECTION and GENOTYPING. A total of 22 SNPs were selected for genotyping from a 40 kb region covering the *COMT* gene as well as the 5'UTR and 3'UTR regions. The SNP selection was based on both the phase 2 and phase 3 data of the HapMap database (<http://www.hapmap.org>). The SNPs that captured most of the genetic information (tagging SNPs) were selected using the Tagger program included in the Haploview 4.1 software (Daly Lab, MIT/Harvard Broad Institute). The SNPs were chosen to capture all known HapMap SNPs having a minor allele frequency >10% in the Caucasian population with pair-wise $r^2 \geq 0.9$. Some additional SNPs were selected from the dbSNP database (<http://www.ncbi.nlm.nih.gov/projects/SNP>) based on their position on the exonic areas.

DNA was extracted from peripheral blood using the Autopure LS automated DNA purification instrument (Genra Systems, Inc., Minneapolis, MN, USA). The 22 SNPs were genotyped using the Sequenom MassARRAY system and the iPLEX Gold Single Base Extension chemistry (Sequenom, San Diego, CA, USA) (Jurinke *et al.*, 2002) in a multiplex format. This method has excellent success (>95%) and accuracy (100%) rates (Lahermo *et al.*, 2006).

4.3 Ethics

Animal experiments were performed according to the guidelines of local authorities, The International Association for the Study of Pain (Zimmermann, 1983) and Guide for the Care and Use of Laboratory Animals by the National Institutes of Health (<http://grants.nih.gov/grants/olaw/Guide-for-the-Care-and-Use-of-Laboratory-Animals.pdf>).

All experiments were approved by the provincial government of Southern Finland (**I-III**).

In the clinical study (**IV**), the patients were fully informed of the procedures and potential risks and the study was explained to them in detail before the enrolment. Participants were fully apprised of their right to withdraw from the study at any time. Written informed consent was obtained from each subject participating in the study by a research nurse or a physician. Surgery

and all other treatments were part of the patients' normal clinical care and were carried out under the supervision of the physician in charge. The research protocol had been approved by the coordinating ethics committee (136/E0/2006) and the ethics committee of the Department of Surgery (Dnro 148/E6/05) of the Hospital District of Helsinki and Uusimaa (HUS). All reasonable precautions have been taken to prevent loss of confidentiality. All specimens and clinical data files have been coded, and subject names or identifying information are not to be released.

4.4 Data analysis

In data analysis of experimental animal studies (**I-III**), parametrical tests (Student's *t* test, analysis of variances, ANOVA) with suitable *post hoc* tests were used when the criteria for parametrical analysis were met. In assays where cut off-time was used, data was not normally distributed, not continuous or variances were not equal, corresponding non-parametrical analysis were used. In the text and figures, the results are presented as means \pm standard error of the mean (SEM) of *n* observations. $P < 0.05$ was considered as a limit of statistical significance in all tests. Area under the time-effect curve (AUC) has been calculated using trapezoidal rule.

In the human study (**IV**), genotype distributions in the whole sample were tested for the Hardy-Weinberg equilibrium applying the χ^2 method. The Haploview 4.0 program was used to determine the pair-wise LD between the SNPs (r^2 -value) and to identify haploblock structures using the confidence interval algorithm. In association analyses all phenotypes were adjusted for potential confounding factors *i.e.* residual phenotypic scores after regressing out covariate effects were used. All phenotypes were adjusted for age, body mass index (BMI), presence of preoperative chronic pain condition, and anxiety (STAI) score. Postoperative variables were also adjusted for the type of surgery performed. These factors have been shown to modulate the pain phenotypes of our study (Kaunisto *et al.*, 2013). In addition to the phenotypes used in **Study IV** also cold pain intensity at 45 s, intensity of postoperative motion pain upon awakening, time of administration of first oxycodone and intensity of postoperative pain at rest before the administration, amount of oxycodone administered in PACU and amount of oxycodone required to achieve satisfactory analgesia were analysed for associations with *COMT* SNPs and haplotypes, and cold pain intensity at 60-90 s and heat pain intensity at 43°C for associations with *COMT* SNPs. Association between the *COMT* genotypes and pain phenotypes was tested using linear

regression analysis. Additive, dominant and recessive models were all considered. Additive model presumes the genotype effect on studied phenotype is r for heterozygous variant carriers, $2r$ for homozygous carriers, and 0 for non-carriers (Lewis, 2002). Dominant model presumes that the genotype effect is dominant and its effect on phenotype is r in both, homozygous and heterozygous variant carriers. Recessive model presumes recessive genotype effect, which requires both variant alleles and thus homozygous carriers are compared to the rest of the population. Analyses were performed with PLINK software (Purcell *et al.*, 2007). In the haplotype analysis homozygous haplotype carriers were compared with heterozygous carriers and non-carriers. In Study **IV**, the additive model was used. The data was complemented by additional analyses using dominant and recessive models, which are also shown in this summary (Kambur and Kalso, 2012, *unpublished data*). As some of the studied quantitative traits were not normally distributed, permutation procedures were used to obtain empirical p-values and p-values adjusted for performing multiple tests in order to control for the family-wise error rate. This method takes into account the correlation due to LD between the studied SNPs and thus provides a less stringent correction than using a Bonferroni correction to control for multiple testing. The level of statistical significance was set at an experiment-wise corrected $p=0.05$. The associations were calculated with PLINK software using the $\max(T)$ permutation option in which produces 10000 permutations are being performed for each SNP. Results were not adjusted for the number of examined phenotypes. In the results, in addition to naive empirical p-values shown in the tables and corrected p-values described in the text, also β -values have been reported. β is the variable which reflects the magnitude and direction of the effect of genotype in each analysis. If β is negative, phenotype values of study group, which is minor allele or haplotype carriers, are smaller as compared with control group. Magnitude of β reflects size of the effect – difference between the groups and is proportional to the unit size of compared phenotype. Thus, β should be interpreted in the context of the analysed phenotype. In some cases associations were verified by ANOVA or other parametrical tests. In addition, interactions between rs165774 and rs887200 and LPS/APS/HPS-haplotypes were assessed by epistatic interaction analysis which is based on linear regression, using PLINK software.

5 RESULTS

5.1 *Comt* knockout mice and stress- and opioid-induced analgesia (I)

In mice, a knockout of *Comt* gene decreased endogenous (**Fig. 7**) and opioid-induced (**Fig. 8**) analgesia as measured by latency to spinal withdrawal reflex in the tail flick test. It also increased opioid-induced analgesia as measured by latency to more complex nociceptive behaviour, *e.g.* licking or brisk shaking of the hind paw, in the hot plate test (**Fig. 8**).

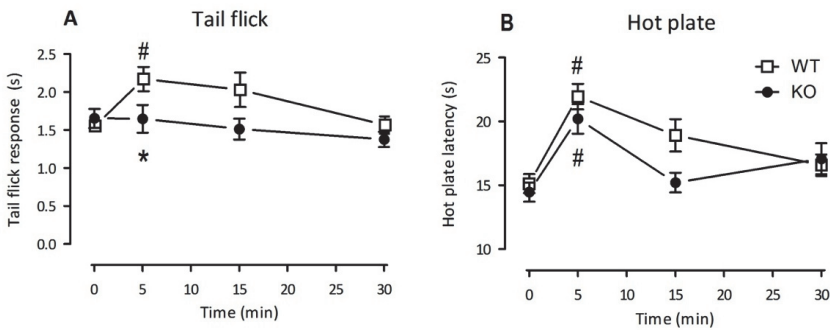


Fig. 7. Stress-induced analgesia in wild-type (WT; n=20) and *Comt* knockout (KO; n=17) mice. Stress-induced analgesia was measured with tail flick (A) and hot plate (B) tests. Asterisk (*) indicates a statistically significant difference as compared with the respective baseline ($p < 0.0001$), whereas hash (#) indicates a statistically significant difference between the knockout and wild-type mice at respective time points ($p < 0.05$).

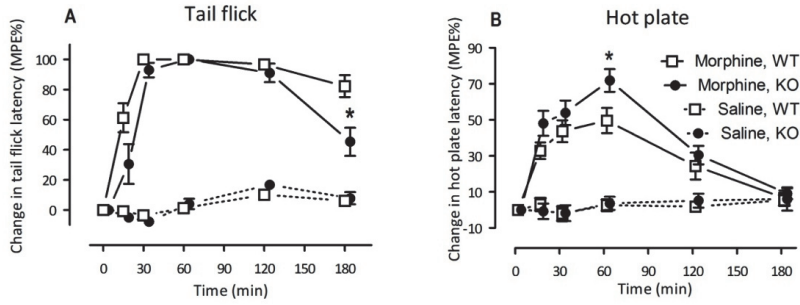


Fig. 8. Antinociceptive effect of morphine in *Comt* knockout (KO) and wild-type (WT) mice. Nociception was assessed with the tail flick (A) and hot plate (B) tests before and after administration of morphine (10 mg/kg *s.c.*, n=10-13) and vehicle (n=4-6). Results are presented as percentages of the maximum possible effect (MPE%). The symbols indicating the knockout group are moved slightly to the right to allow all symbols to be visible. Asterisk (*) indicates a statistically significant difference as compared with the wild-type ($p < 0.05$).

5.2 COMT inhibitors and acute and inflammatory pain (II)

OR-486, increased thermal and mechanical nociception after acute (Fig. 2) and repeated (Fig. 9) peripheral (*i.p.*) administration in models of acute nociception in mice.

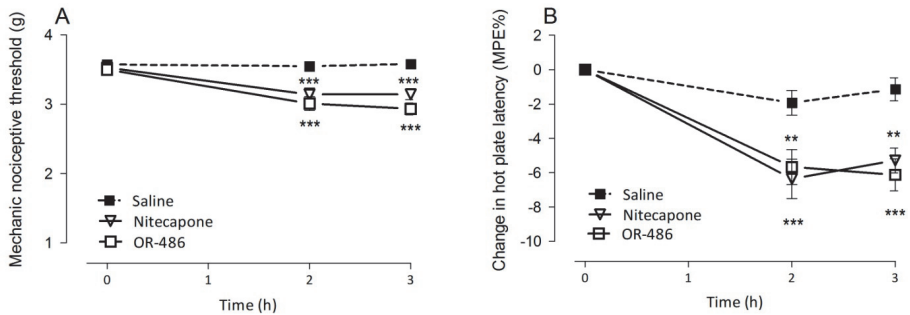


Fig. 9. A. Mechanic nociceptive thresholds after repeated administration of OR-486 (30 mg/kg, *i.p.*, n=16), nitecapone (30 mg/kg, *i.p.*, n=16), or vehicle (n=21). Results are expressed in grams. Statistics: *** $p < 0.001$ vs. vehicle group (Bonferroni-correction). **B.** Baseline nociceptive latencies in the hot plate test after repeated administration of OR-486 (30 mg/kg, *i.p.*, n=16), nitecapone (30 mg/kg, *i.p.*, n=16), or vehicle (n=21). Results are expressed as percentages of the maximum possible effect (MPE%). Statistics: ** $p < 0.01$; *** $p < 0.001$ vs. vehicle group (Bonferroni-correction).

The same effects were seen after acute (**Fig. 2**) and repeated (**Fig. 9**) (*i.p.*) administration of nitecapone. Both inhibitors increased the pronociceptive effects of carrageenan inflammation by an order of magnitude that was similar to that in the non-inflamed paws used as controls (**Fig. 10**). Similar effects were also seen after *i.p.* administration of CGP 28014. Nitecapone, however, was pronociceptive only after peripheral administration and did not increase nociception when administered *i.t.* to rats.

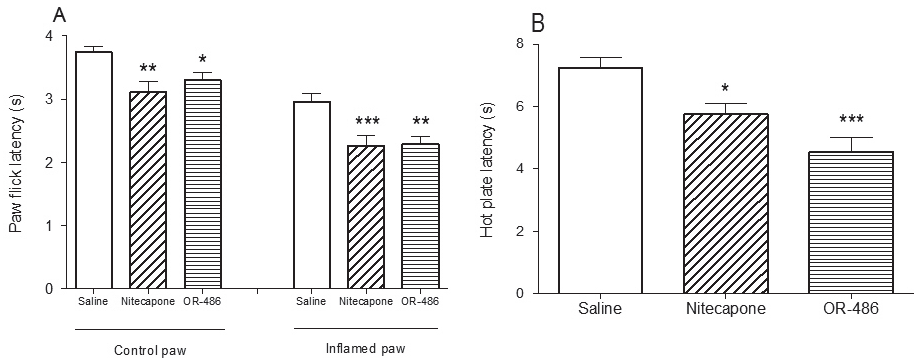


Fig. 10. Effect of repeated administration of OR-486 (30 mg/kg, *i.p.*, n=16), nitecapone (30 mg/kg, *i.p.*, n=16) and vehicle (n=21) on paw flick (A) responses in inflamed and non-inflamed paws, and hot plate (B) responses 3 h after induction of carrageenan inflammation. Statistics: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. corresponding paw of the vehicle group (A) or vehicle group (B) (Bonferroni-correction).

5.3 COMT inhibitors and neuropathic pain (III)

In a model of neuropathic pain, repeated nitecapone treatment (30 mg/kg, *i.p.*, once a day), reduced nociceptive symptoms in rats after SNL for a long period of time far exceeding the duration of COMT inhibition (**Fig. 11** and **12**). It reduced both cold allodynia and mechanical hyperalgesia. Nitecapone treatment was effective when started before the operation (**Fig. 11**) but also when initiated only 2 days after the operation (**Fig. 12**), when neuropathic symptoms were already developed.

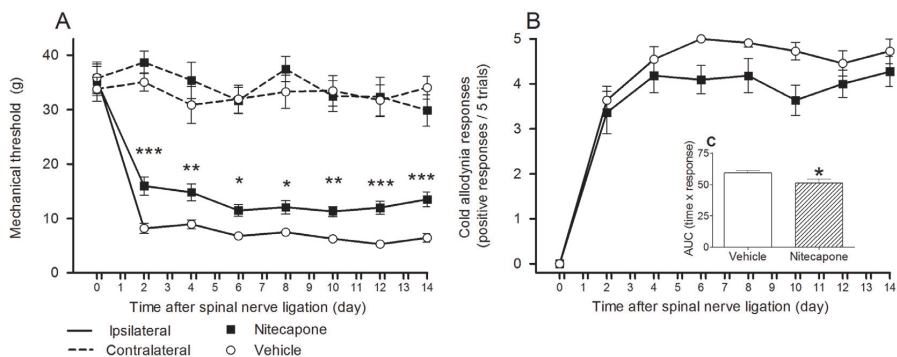


Fig. 11. Effect of nitecapone (30 mg/kg, *i.p.*, n=11) or vehicle (n=11) pretreatment on (A) thresholds for mechanical stimuli and (B) responses to cold stimuli after spinal nerve ligation. Times of administration are indicated by the minor ticks on the x-axis. Results are expressed in grams (A) and as a number of positive responses out of 5 trials (B). Cold allodynia responses of treatment groups over time were compared using the areas under the time-effect curves (C, AUC, days 2-14). Asterisks indicate a statistically significant difference between the treatment groups, A: 2-way RM ANOVA: effect of treatment, $p < 0.001$; Bonferroni-correction: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; C: Mann-Whitney U-test: *, $p < 0.05$.

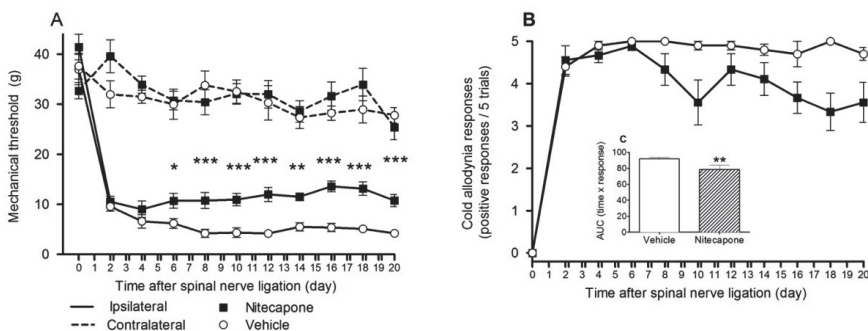


Fig. 12. Effect of nitecapone treatment on (A) thresholds for mechanical stimuli and (B) responses to cold stimuli after spinal nerve ligation. Nitecapone (30 mg/kg, *i.p.*, n=9) or vehicle (n=10) treatment was started on the second day after surgery and continued once daily for 20 days. Results are expressed in grams (A) and as a number of positive responses out of 5 trials (B). Times of administration are indicated by the minor ticks on the x-axis. Treatment groups over time were compared using the areas under the time-effect curves (C, AUC, days 4-20). Asterisks indicate a difference between the treatment groups (A: 2-way RM ANOVA: effect of treatment, $p < 0.001$; Bonferroni-correction: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; C: Mann-Whitney U-test: *, $p < 0.01$).

5.4 Genetic variation in *COMT* and pain sensitivity in humans (IV)

5.4.1 Patients: demographic description and experimental and clinical pain

The demographic data, types of breast cancer surgery, results of the experimental pain tests and postoperative pain and need of oxycodone are shown in **Table 2**.

Table 2. Demographic and nociceptive description of patients.

PHENOTYPE	AVERAGE	STANDARD DEVIATION	n
Age (yr)	57.0	9.3	1000
BMI	25.4	4.3	1000
Breast conserving surgery with SNB			420
Breast conserving surgery with AC			206
Mastectomy with SNB			140
Mastectomy with AC			234
Heat pain intensity (43°C)	0.7	1.4	992
Heat pain intensity (48°C)	3.5	2.4	990
Cold pain intensity (15s)	5.0	2.5	782
Cold pain intensity (30s)	6.2	2.4	546
Cold pain intensity (45s)	6.9	2.4	387
Cold pain intensity (60s)	7.1	2.2	297
Cold pain intensity (75s)	7.1	2.2	240
Cold pain intensity (90s)	7.3	2.2	216
Cold pain tolerance (total time, s)	46.4	29.5	900
Rest pain before 1st oxycodone dose	5.0	1.5	959
Motion pain before 1st oxycodone dose	5.0	1.5	952

Intensity of pain was assessed with a numerical rating scale (NRS 0-10). Abbreviations: SNB - sentinel node biopsy, AC - axillary clearance.

5.4.2 Genotyped SNPs and haploblocks

In addition to analysed SNPs (**Table 3**), haplotype analysis was performed (Kambur and Kalso, 2012, *unpublished data*). Three haploblocks were formed (**Fig. 13**). Five haplotypes having a frequency above 1% could be constructed within block 1, and six haplotypes within blocks 2 and 3.

Table 3. Genotyped *COMT* SNPs.

SNP	POSITION	ALLELES MINOR/MAJOR	OBSERVED HETEROZYGOSITY	P-VALUE (Hardy-Weinberg Equilibrium)	MINOR ALLELE FREQUENCY
rs6518591	chr22:18304021	G/A	0.262	0.4486	0.162
rs737866	chr22:18310109	G/A	0.327	0.9025	0.208
rs737865	chr22:18310121	C/T	0.328	0.9107	0.209
rs1544325	chr22:18311668	A/G	0.524	0.2598	0.492
rs8185002	chr22:18313048	G/T	0.33	0.9948	0.206
rs174675	chr22:18314051	T/C	0.417	0.802	0.302
rs5993882	chr22:18317533	G/T	0.299	0.4586	0.191
rs740603	chr22:18325177	G/A	0.488	0.731	0.398
rs4646312	chr22:18328337	C/T	0.424	0.7884	0.313
rs4633	chr22:18330235	C/T	0.504	0.7718	0.457
rs2239393	chr22:18330428	G/A	0.43	0.9021	0.317
rs4818	chr22:18331207	G/C	0.425	0.8359	0.313
rs4680	chr22:18331271	G/A	0.505	0.7154	0.457
rs4646316	chr22:18332132	T/C	0.313	0.2613	0.207
rs165774	chr22:18332561	A/G	0.378	0.6578	0.244
rs174696	chr22:18333176	C/T	0.439	0.4008	0.351
rs9306235	chr22:18335157	A/G	0.114	1	0.06
rs9332377	chr22:18335692	T/C	0.217	0.9743	0.122
rs165599	chr22:18336781	G/A	0.457	0.408	0.327
rs887199	chr22:18341955	A/G	0.319	0.9301	0.201
rs2518824	chr22:18342963	G/T	0.076	0.7802	0.038
rs887200	chr22:18343666	C/T	0.27	0.7455	0.164

SNPs with effects on *COMT* activity or expression are shown with **bolding** (Bray *et al.*, 2003; Lotta *et al.*, 1995; Meloto *et al.*, 2012; Nackley *et al.*, 2006; Tsao *et al.*, 2011).

5.4.3 *COMT* polymorphisms and pain: SNPs

In the patients several of the studied *COMT* SNPs (**Table 3; Fig. 13**) showed association ($p < 0.05$) with experimental pain (**Appendix 1: Tables 4-5**).

SNPs rs165774, rs174696 and rs8185002 were associated with intensity of pain after 48°C stimulation and rs737866 and rs737865 showed borderline associations ($0.05 < p < 0.1$) (**Appendix 1: Table 4**). Minor alleles of rs165774 (A), rs8185002 (G), rs737866 (G) and rs737865 (C) decreased pain intensity ($\beta = -0.300-0.464$), whereas rs174696C increased pain intensity ($\beta = 0.48$). Rs165774 showed the strongest association and remained significant also after correction for multiple comparisons ($p = 0.04$). Minor alleles of rs4646312 (C), rs2239393 (G) and rs4818 (G) showed a trend towards increased pain intensity after 43°C stimulation which lacked statistical significance ($p = 0.08-0.088$).

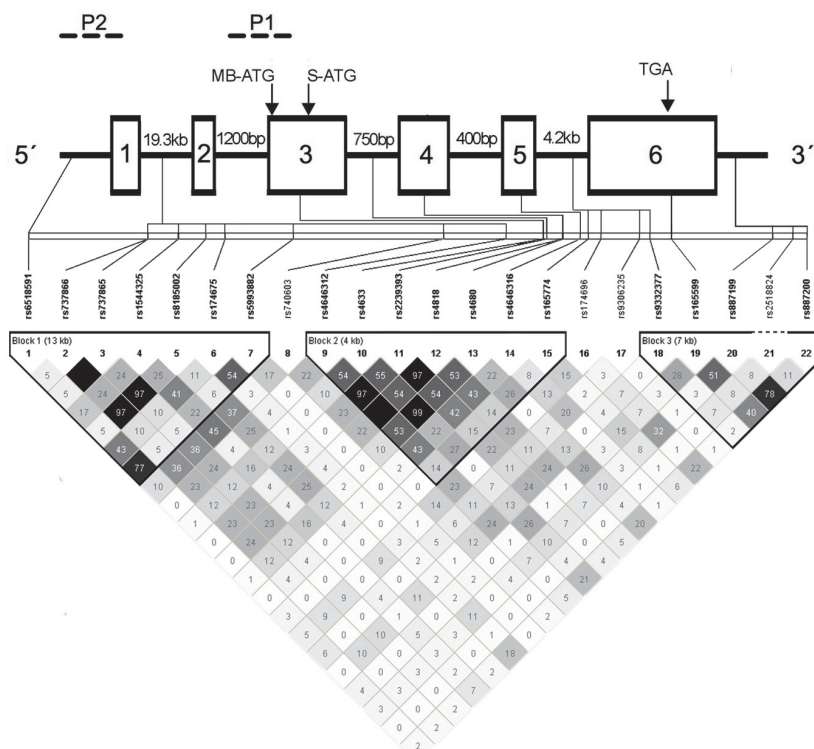


Fig. 13. Graphical representation of *COMT* SNPs, haplotypes, and linkage disequilibrium structure studied in patients (n=1000) undergoing breast cancer surgery. Numbers inside the square represent the linkage disequilibrium value between two SNPs and is $100 \times D'$. $|D'|$ varies between 0 (no disequilibrium) and 1 (maximum disequilibrium), and its value is colour-coded by shades of white to grey to black. Initiation (MB-*COMT* and S-*COMT*) and the stop (TGA) codons as well as rough location of promoter regions (P1 and P2) are also shown.

In cold pain intensity the strongest association was seen with rs887200, which was also associated with cold pain tolerance. Its phenotype effect was strongest and sustained the multiple comparison corrections (**Fig. 14; Appendix 1: Table 5**). The minor allele (C) was associated with decreased pain intensity and increased tolerance (recessive model). Also the minor allele of rs1544325 (A) was associated with increased pain intensity (dominant model) (45s, $p=0.005$, **Appendix 1: Table 5**; 60s, $p=0.004$, *data not shown*)(Kambur and Kalso, 2012, *unpublished data*). Rs1544325A also showed the most significant associations with cold withdrawal time, together with the minor allele of rs2239393 (G) ($p=0.007$, dominant and recessive models, respectively).

Several other SNPs of the first (rs737866 and rs737865) and the second (rs4646312C, rs4818G, rs4646316T and rs165774A) haploblocks as well as rs9306235, which is located between the 2nd and 3rd haploblocks showed associations with cold pain phenotypes. In general, minor alleles of the first haploblock SNPs (G and C, respectively) decreased pain intensity and increased time to withdrawal, whereas minor alleles of the other SNPs (rs4646312C, rs4818G, rs4646316T, and rs9306235A) had an opposite effect (**Appendix 1: Table 5**). SNP rs165774A, which was associated with decreased heat pain intensity, was associated with decreased cold pain intensity (90s, $p=0.03$, $\beta=-0.23$; *data not shown*). However, only association of rs887200 sustained multiple comparison corrections.

Clinical postoperative pain was associated with SNPs rs4646312, rs2239393 and rs4818, which all are located within the same (middle) haploblock ($p=0.006-0.007$)(Kambur and Kalso, 2012, *unpublished data*; **Appendix 1: Table 6**). SNP rs4680 was not significant. In all three SNPs the minor allele (C, G and G, respectively) carriers had higher postoperative pain scores than the patients being homozygous for the major allele ($\beta=0.25-0.30$). After corrections for multiple comparisons, association signals did not reach statistical significance ($p=0.08-0.1$)(*data not shown*). In phenotypes related to oxycodone consumption, there was an association with SNPs rs174696 and trends with rs740603, rs9332377 and rs887200 ($p=0.07-0.1$), which did not sustain multiple comparison corrections.

5.4.4 SNP rs887200

SNP rs887200 was for the first time shown to alter intensity to experimental pain. It was detected in the initial linear regression analysis, showing association with the intensity of cold pain at 30s. It was analysed further and it was associated with the intensity of cold pain consistently at all time

points (**Fig. 14A**) as well as tolerance to cold pain (**Fig. 14B**). A similar trend was also seen in the intensity of pain after noxious heat stimulation but failed to reach statistical significance (unpaired Student's *t* test, $p=0.1$; **Fig. 14C**). Also in the oxycodone phenotypes, there was a trend as minor allele carriers needed more oxycodone to achieve satisfactory analgesia and they also received more oxycodone in the PACU (Kambur and Kalso, 2012, *unpublished data*). Signals of oxycodone phenotypes, however, did not sustain multiple comparison corrections.

5.4.5 *COMT* polymorphisms and pain: haplotypes

For experimental pain phenotypes **heat pain** intensity showed the strongest association signal with haplotype TTACACA of block 2 (dominant model, uncorrected $p=0.003$, $\beta=-0.463$), which has a frequency of 24% (**Appendix 1: Table 7**). It also showed a trend toward decreased mean intensity of cold pain, but effect was not significant ($p=0.097$, $\beta=-0.212$). This haplotype was the only haplotype containing the minor allele (A) of the SNP rs165774, which was associated with heat pain in single SNP analyses. Within the block 1 haplotype AGCGGCT decreased intensity of heat pain (dominant model, uncorrected $p=0.04$, $\beta=-0.320$). It showed a trend toward decreased cold pain intensity at 30s and 45s (dominant model, uncorrected $p=0.061-0.08$, $\beta=-0.284 -0.317$) and prolonged withdrawal time (dominant model, uncorrected $p=0.054$, $\beta=3.898$).

In the **cold pain** phenotypes haplotype AATATCT of block 1 showed the strongest associations and increased pain intensity at all time points (dominant model, uncorrected $p=0.008-0.034$, $\beta=0.483-0.512$) and correspondingly shortened time to withdrawal (dominant model, uncorrected $p=0.005$, $\beta=-6.252$). After statistical corrections, however, these associations did not reach the level of statistical significance ($p=0.068-0.1$). Another haplotype of the same block, AATGTTT decreased pain intensity (dominant model, mean intensity, uncorrected $p=0.032$, $\beta=-1.425$; 45s $p=0.008$, $\beta=-2.028$). Among block 2 haplotypes CCGGGTG increased cold pain intensity at 30 s ($p=0.024$, $\beta=0.359$) and 45 s ($p=0.042$, $\beta=0.272$) as well as mean pain intensity ($p=0.031$, $\beta=0.25$), and decreased the withdrawal time ($p=0.044$, $\beta=-10.84$). Haplotype TCACGCG instead, decreased cold pain intensity (30 s, $p=0.003$, $\beta=-1.8$; 45 s, $p=0.02$, $\beta=-1.203$; mean, $p=0.011$, $\beta=-1.14$), and increased cold withdrawal time ($p=0.029$, $\beta=14.99$). It also showed a trend towards decreased intensity of heat pain (recessive $p=0.089$, $\beta=-0.913$). Association with cold pain intensity at 30s sustained after corrections for multiple comparisons ($p=0.033$).

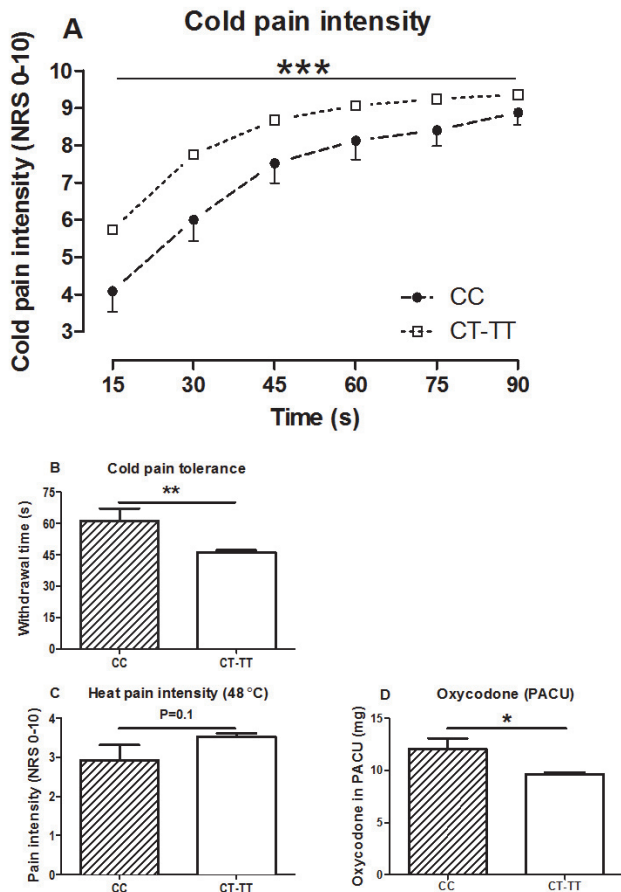


Fig. 14. A. Effect of SNP rs887200 on cold pain intensity. Asterisks indicate a statistically significant difference between the genotypes (2-way ANOVA for repeated measurements, effect of genotype, $p < 0.0001$). **B. Effect of SNP rs887200 on cold pain tolerance.** Asterisks indicate a statistically significant difference between the genotypes (unpaired Student's *t* test, $**p < 0.01$). **C. Effect of SNP rs887200 on intensity of pain after 5 s stimulation with noxious heat (48°C).** P-value indicates the difference between the genotypes (unpaired Student's *t* test). **D. Effect of SNP rs887200 on the amount of oxycodone (mg) administered in post anaesthesia care unit (PACU).** P-value indicates a statistically significant difference between the genotypes (unpaired Student's *t* test).

As rs887200 which showed the best single SNP association to cold pain, the haplotypes containing its minor allele were of special interest. Two haplotypes within block 3 had this allele. Of these, only the more common haplotype CGATC showed a trend towards lower cold pain intensity and prolonged withdrawal time. There were no associations between haplotypes and the phenotypes of postoperative pain or oxycodone consumption.

Among LPS, APS and HPS haplotypes LPS (CGG) was associated with increased pain intensity and shorter withdrawal times. HPS-haplotype (CCG) showed opposite effects under the recessive model which remained after corrections for multiple comparisons ($p=0.009$). LPS-haplotype also showed association with increased intensity of postoperative pain under dominant model, which sustained multiple comparison corrections ($p=0.02$) (**Appendix 1: Table 8**).

5.4.6 Interactions

In cold pain phenotypes there was an interaction between rs887200 and HPS (CCG)-haplotype and they together were associated with decreased intensity of cold pain (30s, $p=0.003$) and increased time to withdrawal (Kambur and Kalso, 2012, *unpublished results*). Similar interaction was seen in heat pain intensity. Rs887200 also showed interaction with APS (TCA)-haplotype also decreasing cold pain intensity (30s, $p=0.04$). There were no interactions between rs165774 in any of the experimental pain phenotypes.

6 DISCUSSION

6.1 Main findings

RODENTS. *Comt* knockout mice showed enhanced response to morphine in the hot plate test. In the tail flick test, which measures mainly spinal responses, *Comt* knockout mice lacked stress-induced analgesia, and their response to morphine was attenuated. Acute administration of both OR-486 and nitecapone increased mechanical and thermal nociception in mice, and these effects persisted after 5 days of repeated treatment. Both COMT inhibitors also increased the pronociceptive effects of carrageenan-induced inflammation. In *Comt* deficient mice, OR-486 failed to influence pain-related behaviour. In contrast to these findings, nitecapone reduced symptoms of neuropathic pain induced by the SNL. The development of mechanical and cold allodynia was partially prevented by treatment that started before surgery, and similar antiallodynic effect was achieved when the treatment started on the 2nd day after induction of neuropathy.

HUMANS. In breast surgery patients, the main finding was that the 3'UTR SNPs, rs887200 and rs165774 had the most significant effects on pain phenotypes. Effects of both SNPs on pain phenotypes were shown here for the first time. Both SNPs represent completely novel mechanisms of action, and in rs165774 the mechanism involves the formation of an alternatively spliced COMT variant (Meloto *et al.*, 2012). The magnitude of their effect exceeded the effects of other SNPs and associations sustained after all statistical corrections. The variants which have received virtually most of scientific attention so far, were insignificant. Several SNPs or haplotypes, however, were associated with experimental or clinical pain phenotypes (uncorrected $p < 0.05$).

Based on these results, a low or lacking COMT activity in rodents is associated with pronociception in thermal, mechanic, and inflammatory pain models, while the situation is reverse in the neuropathic pain models. These fairly unanimous findings, in a frame of pain models, allow a discussion of the mechanisms based on COMT substrates, their localization in

the pain tract and properties of COMT inhibitors. In humans, several SNPs were associated with pain phenotypes. For some SNPs there is some evidence regarding their molecular mechanism of action, but in most cases, their relation to COMT activity is not known. Therefore, in human patients it is rather relevant to discuss the relationship of *COMT* gene mutations than COMT activity, and pain.

6.2 Why does low COMT activity enhance pain?

The mechanisms underlying the effect of COMT inhibition and decreased COMT activity on nociception are unknown, however several hypotheses have been offered. COMT is expressed in several parts of nervous system that are involved in processing of nociception in the brain, as well as in spinal and peripheral structures. In the periphery, COMT is expressed in ganglia of the primary sensory neurons and in the spinal cord in superficial laminae of the dorsal horn (Hong *et al.*, 1998; Karhunen *et al.*, 1996). In the brain, COMT is expressed in several brain regions receiving nociceptive input from spinothalamic pathway, such as prefrontal cortex. A subpopulation of projecting neurons of another ascending nociceptive tract, the spinobulbar pathway, targets the regions in the brainstem, which contain catecholamine cell groups (A1-A2, A5-7, C1; Wall *et al.*, 2006). These areas integrate nociception with homeostatic, cardiorespiratory, and behavioural state processes, and modulate nociception at spinal and forebrain level. They also contain pre-autonomic neurons and regulate sympathetic activity in the periphery, offering another site of modulation where COMT could play a role. A1 neurons also project to the hypothalamus regulating the release of ACTH after trauma or noxious stimulation. ACTH is part of HPA-axis and it is released as a response to pain or trauma. It affects pain and stress responses, and is associated with chronic pain conditions such as fibromyalgia. Polymorphisms in *COMT* gene modulate endocrine stress responses of HPA convergently with polymorphisms in other genes of monoaminergic system (Jabbi *et al.*, 2007).

Initially, it was suggested that pronociceptive effects of low COMT activity are mediated via changes in the endogenous opioid system (see in details below, 6.4.2) (Zubieta *et al.*, 2003). As short-term administration of COMT inhibitors, while showing clear pronociceptive effects (**II**), did not modify opioid analgesia in mice, a lack of changes in opioid system is obvious (Kambur and Männistö, 2009, *unpublished results*). Therefore, pronociceptive effects of COMT inhibitors or

COMT polymorphisms cannot be explained by changes in synthesis of proteins in endogenous opioid system and also other complementary mechanisms have been suggested (Nackley *et al.*, 2007) (**I** and **II**). It seems that most of the studies, directly and indirectly, support the involvement of noradrenergic or adrenergic transmission in mediating the effects of low COMT activity on pain (Khasar *et al.*, 1999; Liang *et al.*, 2006; Nackley *et al.*, 2007; Meloto *et al.*, 2012; Tchivileva *et al.*, 2010).

β_2 and β_3 receptors. It has been shown that some of the effects of COMT are blocked by adrenergic β_2 or β_3 receptors antagonists (Nackley *et al.*, 2007). The density of adrenergic $\beta_{2/3}$ -receptors in the DRG is low, but is generally high in peripheral tissues and several parts of the brain (Nicholson *et al.*, 2005). The origin and nature of substances causing activation of β_2 or β_3 receptors are not known. Peripheral β_2 or β_3 receptors can be activated by noradrenaline released from sympathetic nerve endings, or by circulating catecholamines delivered to the site of action via the blood stream. It has been shown that in the DRG-neurons noradrenergic activation of β_3 -receptors stimulates the release of ATP, which can contribute to pain symptoms (Kanno *et al.*, 2010). On the other hand, a chronic stimulation of β_3 -receptors also increases the expression of mitochondrial uncoupling proteins (UCPs), which are found in primary sensory neurons that also express substance P (Horvath *et al.*, 2002), a pronociceptive peptide that lowers nociceptive thresholds (Horvath *et al.*, 2002a,b; Weyer *et al.*, 1999). UCPs represent an uncoupling pathway acting by proton sink mechanism, which allows mitochondria to avoid excessive production of ROS and to maintain a high rate of O_2 consumption (Guerrero-Castillo *et al.*, 2011).

COMT inhibitors can cause uncoupling of mitochondrial phosphorylation, supporting noradrenergic β_3 -mediated increase in UCP expression in sensory neurons as explanation for pronociceptive effects of COMT inhibitors (Borges, 2005,2003). A possible role of UCP in pronociceptive effects of COMT, however, has not been studied and requires further investigation. If pronociceptive effects are UCP-mediated, β -blockers could possibly also prevent other, non-nociceptive effects of mitochondrial uncoupling associated with COMT inhibitors.

DOPEGAL. It has been proposed that 3,4-dihydroxyphenyl-glycolaldehyde (DOPEGAL), a catecholamine metabolite formed by MAO, can produce potent mechanical hyperalgesia, and it may explain pronociceptive effects of catecholamines synthesized in primary peripheral nociceptors (Dina *et al.*, 2008). Under normal conditions, aldehyde reductase metabolizes DOPEGAL to 3,4-dihydroxyphenylglycol, which is further methylated by COMT to 3-methoxy-

4-hydroxyphenyl-glycol. Thus, a compromised COMT activity may lead to accumulation of DOPEGAL, offering a very straightforward explanation to the pronociceptive effect of COMT inhibitors. However, some of the original results on DOPEGAL have been withdrawn, and the role of DOPEGAL in pronociceptive effects of low or absent COMT activity remains unconfirmed (Dina *et al.*, 2009).

Whereas the adrenergic mechanisms have been shown to mediate pronociceptive effects of COMT inhibitors, their role in mediating pronociceptive effects of an inborn COMT deficiency has not been verified experimentally (Nackley *et al.*, 2007). In humans, β -receptor blockers can reduce chronic musculoskeletal pain symptoms in patients carrying a low COMT activity haplotype (Tchivileva *et al.*, 2010). Thus, adrenergic mechanisms seem to be involved also in pronociceptive effects of human *COMT* polymorphisms.

BRAIN PENETRATION and site of action of COMT inhibitors. In earlier studies, nitecapone has been considered a peripherally acting compound, which does not cross the blood-brain barrier (Männistö and Kaakkola, 1999; Nissinen *et al.*, 1988). However, a high dose of nitecapone (30 mg/kg; *i.p.*) inhibited also striatal COMT activity, indicating that nitecapone can cross the blood-brain barrier to some extent, and therefore may exhibit direct effects in the brain (III). The inhibition of COMT in the brain is modest, lasting only for a short period of time (III), and its implications remain unclear. However, its effect on dopamine levels may be quite limited as dopamine levels in the striatum are not increased even in *Comt* knockout mice (Huotari *et al.*, 2002). In the prefrontal cortex, however, the dopamine level is increased in the extracellular fluid when assessed with a no-net efflux method (Käenmäki *et al.*, 2010). This increase may have functional consequences, since in *Comt* knockout mice the elimination time of prefrontal dopamine is doubled as shown by voltametric studies (Yavich *et al.*, 2007). In line with *Comt* knockout studies, tolcapone, the COMT inhibitor which penetrates the blood-brain barrier, does not increase alone the rodent brain tissue or extracellular dopamine levels (Huotari *et al.*, 1999; Li *et al.*, 1998). However, after administration of *L*-dopa (Huotari *et al.*, 1999, 2002), clozapine, or K^+ -hyperpolarisation (Tunbridge *et al.*, 2004), tolcapone or complete COMT deficiency can increase dopamine levels. Thus, when COMT activity is seriously compromised, elimination of dopamine may be delayed, and there may be at least a shift towards an increased dopaminergic tone may occur in the brain. Therefore, it can be concluded that direct brain actions cannot be completely excluded from possible explanations of and may contribute to some extent to behavioural effects of nitecapone (II and III). Despite of that, COMT inhibition caused by

nitecapone in the periphery is significantly more pronounced and longer lasting, and thus may play a predominant role, rather than its effects in the brain. Nitecapone may also cross the blood-cerebrospinal fluid barrier. However, since intrathecal administration of nitecapone did not increase nociceptive sensitivity (II), spinal action cannot explain pronociceptive effects of COMT inhibitors in models of acute pain.

6.3 Effect of the pain type

The effects of low COMT activity and COMT inhibitors on nociception seem to depend on the pain type. In acute and inflammatory pain, the effects are pronociceptive and sensitivity to nociceptive stimuli is increased. In neuropathic pain, however, pronociceptive effects are lacking in experimental animal models (III) (Jacobsen *et al.*, 2010; Pertovaara *et al.*, 2001) as well as in human polymorphism studies (Armero *et al.*, 2005; Lötsch *et al.*, 2009b; Max *et al.*, 2006). In contrast COMT inhibitors have analgesic or antiallodynic effects (see in more detail below).

6.4 Low COMT activity and efficacy of opioids

6.4.1 Rodents

Comt knockout mice showed enhanced response to morphine in the hot plate test (I). Enhancement of opioid response in polymorphisms with low COMT activity has also been reported in human studies in healthy volunteers and cancer pain patients (see below).

We also found that in the tail flick test, which measures mainly spinal responses, a lack of COMT activity was related to loss of stress-induced analgesia and decreased morphine response. This indicates that COMT activity may be involved in the modulation of nociception and opioid responses not only in the brain, but also at spinal and/or peripheral levels. This can possibly occur via increased activation of $\beta_{2/3}$ -adrenergic receptors, as has been suggested in some other studies (Nackley *et al.*, 2007) and was discussed in detail above.

6.4.2 Mechanisms

It has been initially suggested that low COMT activity increases dopaminergic tone in certain brain areas, which in turn leads to a decrease in endogenous opioid peptides, and consequently to increased availability of opioid receptors (Zubieta *et al.*, 2003). Our studies supported this hypothesis indirectly, since opioid analgesia was not modified by short-term administration of COMT inhibitors (Kambur and Männistö, 2009, *unpublished results*), but only after inborn COMT deficiency (I). This also suggests that enhanced morphine responses are not due to changes in catecholamine dynamics caused by inhibition of COMT directly, but rather due to compensatory changes in the level of protein synthesis, such as an increase in opioid receptor synthesis. However, the impact of changes in catecholamine dynamics on opioid analgesia cannot be completely excluded, as the pharmacological inhibition of COMT is not complete, especially in the CNS, whereas in the knockout mice COMT activity is completely absent and the relative magnitude of the effect on COMT activity is greater.

OPIOID PEPTIDES. *In situ*-hybridization of opioid peptides in human *post-mortem* samples has not been unambiguous. In a more a recent study, the amount of proenkephalin mRNA was reduced only in the shell region of the nucleus accumbens (Nikoshkov *et al.*, 2008), but in another study there were no reduction in the amount of preproenkephalin mRNA, in any brain region studied. In contrast, their amount was increased in the caudate nucleus (Berthele *et al.*, 2005). In the thalamus, however, preproenkephalin was not detectable. Levels of Met-enkephalin peptide were within measurable range, and were decreased in the mediodorsal nucleus of the thalamus in Met-allele carriers (Kowarik *et al.*, 2012). β -endorphin level neither was detectable neither in the thalamus nor in any other brain region studied (cortex, basal ganglia). Together these studies suggest that the endogenous opioid system is regulated by *COMT* polymorphisms, but is not in line with the initial hypothesis of Zubieta *et al.* (2003), which explains changes in opioid receptor availability by alterations in opioid peptide levels.

OPIOID RECEPTORS. In human *post mortem* brain samples, genetic polymorphism (Val^{158/108}Met) which is associated with a low COMT activity (Met-allele) was associated with an increased amount of opioid receptors in several brain areas: the caudate nucleus, nucleus accumbens and mediodorsal nucleus of the thalamus (Berthele *et al.*, 2005). Increased level of μ -opioid receptors in Met-allele carriers was later confirmed in mediodorsal nucleus of the thalamus in healthy volunteers (Kowarik *et al.*, 2012). In the latter study the increase of μ -opioid receptors

in the thalamus was attributed to a decrease in Met-enkephalin level in the same area. Another opioid ligand, β -endorphin, was not detectable, and thus could not explain the observed changes. Indeed, the availability of μ -opioid receptors was increased in healthy volunteers with Val^{158/108}Met polymorphism and low COMT activity in the anterior thalamus and thalamic pulvinar in *in vivo* imaging ([¹¹C]carfentanil ligand-PET) study (Zubieta *et al.*, 2003). Thus, regardless of the mechanism, *COMT* polymorphisms have been shown to regulate μ -opioid receptor availability, and a low COMT activity increases availability of opioid receptors in several brain regions. Together these findings indicate that *COMT* polymorphisms modulate opioid responses.

6.4.3 *COMT* polymorphisms and postoperative opioid consumption

Opioids are the most effective drugs in treating pain and remain widely used. The main factors limiting and attenuating their use are variation in efficacy, adverse effects, addiction, development of tolerance, and opioid-induced hyperalgesia. Consequently, they attenuate outcomes of pain treatment and compliance. There is convincing evidence from *in vitro*, *ex vivo* and *in vivo* animal studies, studies in human *post mortem* tissues, *in vivo* human imaging, that show regulation of opioid system by *COMT*. *COMT* polymorphisms modulate not only the availability of opioid receptors, but also the effects of opioids, including their efficacy, adverse effects and opioid-induced hyperalgesia. *COMT* polymorphisms and other genetic predictors of opioid effects may be used to optimize and improve treatment of pain patients, once patient genetic information is available to healthcare professionals. However, there are several issues, which should be taken into account. Although the results of experimental studies have quite convincingly demonstrated changes in opioid system caused by *COMT*, the data showing effects of such changes on opioid treatment and outcomes in human patients are conflicting. Some of the clinical studies failed to show a significant effect (Huehne *et al.*, 2009; Lötsch *et al.*, 2009b), which may have several explanations.

1. Pain, opioid response, and requirements/consumption are complex multifactorial phenomena. Consequently, each of the contributing factors has a limited effect, and thus may explain only a fraction of the variation between individuals. It is not surprising that such effects can be seen more easily in experimental studies where the most confounding factors can be standardized and addressed. In a clinical setting, however, the effect ("signal") of the same magnitude may be hidden by variation ("noise") caused by

confounding factors, such as variation of pain symptoms, pathologies underlying the symptoms, stage of progression of the pathologies, clinical comorbidities, and other medications. In such cases, a low signal to noise-ratio in clinical studies and consequent lack of statistical significance of individual genetic factors do not diminish their relevance, but rather call for the other factors to be addressed – in clinical studies and in treatment of patients.

2. Since COMT is not regulating opioid responses alone, the effects of other factors have to be taken into account. Opioid response is being regulated by genes coding for opioid receptors, and those regulating metabolic inactivation and pharmacokinetic parameters of the drugs. Whereas the effects of genes belonging to the latter category may depend on the particular opioid used, the analgesic effect of opioids is generally mediated via μ -opioid receptors which are coded by *OPRM1*-gene. *OPRM1*-gene is also subject for genetic polymorphisms, and it has been shown that the effect of *COMT* SNPs on opioid response may depend on an *OPRM1*-variant (A118G) (Reyes-Gibby *et al.*, 2007). The effect of *COMT* SNP was most pronounced in carriers of AA variant, whereas in others it was less pronounced or not significant. Also another study pointed out the need to concomitantly address also other co-occurring genetic polymorphisms, which modulate the response and act as confounders (Lötsch *et al.*, 2009a).

3. Since COMT is proposed to regulate the opioidergic system via changes in dopaminergic tone in the CNS, also other factors regulating dopaminergic tone may modulate the effect of COMT on opioid responses. Polymorphisms in genes coding for dopamine receptors (*DRD1-4*), enzymes involved in biosynthesis and inactivation of dopamine (*DBH*, *MAOB*, *DAT*) and regulating other neurotransmitter systems (5-HT, NA) have been shown to modulate dopaminergic functions and phenotypes (Bertolino *et al.*, 2008; Bobb *et al.*, 2005; Bombin *et al.*, 2008; Borroni *et al.*, 2006; Xing *et al.*, 2007). Also other genes modulate the dopaminergic system. For example, angiotensin I converting enzyme (ACE) regulates dopaminergic tone in the CNS and its gene has been shown to have an interaction with *COMT* (Illi *et al.*, 2003; Jenkins *et al.*, 1996; Mertens *et al.*, 2010; Raghavendra *et al.*, 1998; Reardon *et al.*, 2000). Also *DBH* gene seems to be genetically linked with *COMT*, and/or may be regulated by the shared mechanisms (Xing *et al.*, 2007).

4. Finally, the effect of an individual *COMT* SNP on complex responses such as opioid responses or pain, is ultimately dependent on its molecular mechanism of action. The effect of SNP is mediated via its physico-chemical interactions with other components of transcriptional and translational machinery in its immediate and more proximal environment. Thus, the effect is dependent on the location of the SNP within the gene and its interaction with other factors, such as other SNPs, binding sites for transcription factors, and other regulatory elements (see below).

6.5 Mechanism of COMT inhibitors reducing the neuropathic pain

6.5.1 Experimental rodent model of neuropathic pain

At repeated doses once a day, nitecapone reduced symptoms of neuropathic pain induced by the SNL. Mechanical and cold allodynia were reduced when the treatment was started before or on the 2nd day after the induction of neuropathy. The antiallodynic effect of nitecapone was unexpected for two reasons: 1) it was opposite to pronociceptive effects seen in acute pain models, and 2) its duration of effect (over 24 hrs) exceeded manifold the duration of COMT inhibition (*ca.* 3 hrs) (III). Also other experimental studies on neuropathic pain have reported similar effects of COMT inhibitors (Jacobsen *et al.*, 2010; Pertovaara *et al.*, 2001). In a rat model of diabetes, nitecapone administered continuously in drinking water, decreased neuroplastic and nociceptive symptoms of neuropathy (Pertovaara *et al.*, 2001). In another study, OR-486 (30 mg/kg, *i.v.*) reduced spinal electrophysiological responses to nociceptive stimulation, and attenuated one of the neuroplastic changes underlying nociceptive sensitization in neuropathic pain, the spinal long-term potentiation (Jacobsen *et al.*, 2010; Zimmermann, 2001).

6.5.2 Possible mechanisms

The mechanisms underlying the antiallodynic effects of COMT inhibitors remain unknown. In diabetic neuropathy, nitecapone attenuated spontaneous activity of wide dynamic range neurons in the spinal dorsal horn offering a neurophysiological correlate to its antiallodynic effects (Pertovaara *et al.*, 2001). Since OR-486 also modulated C-fibre responses in the dorsal horn and spinal long-term potentiation (Jacobsen *et al.*, 2010), it seems obvious that COMT inhibitors directly or indirectly modulate spinal nociceptive transmission.

α_2 . The noradrenergic stimulation caused by local administration of noradrenaline or α_2 -agonists produces antinociception (Danzabrink and Gebhart, 1990; Reddy and Yaksh, 1980). However, the antihyperalgetic effect of nitecapone in diabetic neuropathy was dependent neither on α_2 -adrenoceptors nor μ opioid receptors (Pertovaara *et al.*, 2001).

α_1 , D_2 . Also other catecholamine receptors in spinal cord may be involved in nociception. Activation of α_1 -adrenoceptors in the dorsal horn promotes antinociception (Baba *et al.*, 2000). Additionally stimulation of spinal dopamine D_2 receptors is antinociceptive (Liu *et al.*, 1992; Munro, 2007; Pedersen *et al.*, 2005).

$\beta_{2/3}$. The adrenergic β_2 -receptors are also expressed in the DRG, in peripheral tissues, and several parts of the brain (Nicholson *et al.*, 2005). Initially, their role in antiallodynic effects in experimental neuropathy has been overlooked, as they were pronociceptive in models of acute pain (II) (Diatchenko *et al.*, 2005; Nackley *et al.*, 2007). Several recent studies, however, have shown that adrenergic β_2 -agonists alleviate neuropathic pain via activation of adrenergic β_2 -receptors (Choucair-Jaafar *et al.*, 2009; Yalcin *et al.*, 2010), and that adrenergic β_2 -agonism is necessary and sufficient for the antiallodynic effect of tricyclic antidepressants in animal models of neuropathic pain (Yalcin *et al.*, 2009). Interestingly, blockade of β_2 -adrenoreceptors blocked the effects of not only β_2 -agonists but also of tricyclic antidepressants. These findings were further supported by a small case study in neuropathic pain patients (Cok *et al.*, 2010). The mechanism of action of β_2 -agonists in neuropathic pain is not known. β_2 -agonists, however, decrease myeloperoxidase activity and lipid peroxidation, and increase the activity of superoxide dismutase and level of glutathione in inflammatory states (Elenkov *et al.*, 2000; Uzkeser *et al.*, 2012). They also modulate immune system responses for example by inhibiting tumor necrosis factor alpha (TNF- α) and interleukin 12 (IL-12) production. Thus, the effects of both, COMT inhibitors and β_2 -agonists in neuropathic pain patients should be re-evaluated. Also, based on their mechanism

of action, COMT inhibitors are expected to have synergistic effect with tricyclic antidepressants. This should be explored in further studies as, in perspective, this could be utilized in treatment of neuropathic pain. Whereas the effect of β_3 -agonism was not supported by the study of Yalcin *et al.* (2009), it could have an impact in some neuropathic pain states, such as diabetic neuropathy, as it seems to have disease modifying effects (Umekawa *et al.*, 1997; Kiso *et al.*, 1999; Weyer *et al.*, 1999).

ANTIOXIDATIVE EFFECTS. Antiallodynic effect of nitecapone in neuropathic pain model may be at least partially explained by its pharmacological actions other than the short-term inhibition of COMT. Nitecapone is a potent antioxidant and it scavenges reactive oxygen and nitric oxide radicals and prevents peroxidation of lipids at clinically relevant concentrations (Männistö and Kaakkola, 1999; Marcocci *et al.*, 1994a,b; Nissinen *et al.*, 1995; Suzuki *et al.*, 1992). Also, OR-486 may exhibit antioxidative properties to some extent. Oxidative stress plays an important role in neuropathic and other persistent pain conditions (Kim *et al.*, 2004b; Salvemini *et al.*, 1999; Tal, 1996; Wang *et al.*, 2004). In animal models of neuropathic pain, free radical scavengers have reduced phosphorylation of NMDA-receptors, central sensitization, allodynia and hyperalgesia (Gao *et al.*, 2005; Kim *et al.*, 2006c; Kim *et al.*, 2004b). Thus, antioxidative properties of nitecapone and OR-486 may contribute to their antihyperalgesic and antiallodynic effects. The duration of antioxidative action of nitecapone has not been directly studied in animals but there are some studies showing, indirectly, a prolonged action of nitecapone. For example, the protective effect of nitecapone in gastric lesions in rats lasted longer than 6 hrs, and the concomitant stimulatory effect on the release of prostaglandin E₂ was even 12 hrs or more (Aho and Lindén, 1992).

6.5.3 Humans

Unlike in some other types of pain, *COMT* polymorphisms have not increased incidence or intensity of the symptoms of neuropathic pain in any of the human studies, including in neuropathic pain patients (Armero *et al.*, 2005; Lötsch *et al.*, 2009b; Max *et al.*, 2006). The effect of nitecapone in our study (III) was comparable to that of drugs used in treatment of neuropathic pain in human patients, such as tricyclic antidepressants or gabapentin (30 mg/kg) in the same model (Joshi *et al.*, 2006; Leventhal *et al.*, 2007; Urban *et al.*, 2005). Since clinically used COMT

inhibitors are well tolerated, their possible antiallodynamic effects should be explored in future studies in rodents and humans to assess their potential in treatment of neuropathic pain.

6.6 *COMT* gene mutations and human pain

In our study (IV), the effects of *COMT* variation on pain were assessed using SNPs and haplotypes. In the human genome, SNPs and other bases are not transmitted alone, but rather in blocks. Thus, alleles of individual SNPs are usually accompanied by certain alleles of other co-transmitted SNPs which together form haplotypes. A prevalence of such sets of SNPs varies, and most common haplotypes cover the majority of population, whereas some are relatively rare or absent from certain populations. All SNPs that comprise a haplotype contribute to the DNA sequence of the gene, which determines its physico-chemical properties and interactions with enzymes and regulatory factors, including transcription factors and small RNAs. Moreover, the effects of SNPs are modulated by other SNPs and haplotypes, which form its functional environment. A co-occurrence of several SNPs within the single haplotype can also have effects that are not seen in individual SNPs. For example, several simultaneous SNPs can result in altered local stem-loop structures and more stable mRNA that slows down its processing, and consequently reduces the amount of translated protein (Nackley *et al.*, 2006). Such functional haplotypes modulate *COMT* activity and affect nociception more than individual SNPs, and thus provide a greater explanatory value and better face validity (Diatchenko *et al.*, 2005). Also in our study, an association of the haplotype with pain phenotypes was statistically significant, providing explanatory value equal to or greater than individual SNPs (IV).

6.6.1 Genetic associations (IV)

EXPERIMENTAL PAIN — HEAT and rs165774. In our study (IV) heat pain intensity (48°C) showed the strongest association with SNP rs165774. The subjects carrying the A-allele showed lower pain intensity than homozygous G-carriers. Also the haplotype containing this allele, TTACACA, showed association with heat pain. The level of statistical significance and magnitude of the effect (β) of haplotype and SNP analysis were virtually identical. As rs165774A was the only variant present in this haplotype exclusively, it would be plausible to assume that in both comparisons the signal is driven by rs165774A alone. However, as all rs165774A carriers

carried also the TTACACA-haplotype, we cannot formally exclude the impact of haplotype-specific effects. Rs165774A was also associated with decreased cold pain intensity (90s), but only associations with the heat pain sustained corrections for multiple comparisons. The effect of rs165774 on experimental pain has not been studied before. Rs165774 is located in the intronic region between exons 5 and 6, and is part of haploblock 2. Rs165774A leads to the expression of an alternatively spliced COMT isoform. This truncated isoform may be differentially expressed and more efficient in metabolizing noradrenaline, as suggested in a recent preliminary report associating it with temporomandibular dysfunction (Meloto *et al.*, 2012).

EXPERIMENTAL PAIN — COLD and rs887200. Regarding the cold pain phenotypes, the strongest association in our study was detected between a rare SNP, rs887200, and cold pain intensity. Homozygous minor allele (C) carriers were 22-23 % less sensitive to pain than the subjects with other genotypes. The magnitude of the effect of rs887200 in our study was greater than that of other *COMT* SNPs, including several SNPs which have shown associations in earlier studies. The patients having C/C genotype reported lower pain intensity ratings consistently across different time points. Rs887200C also showed trends toward decreased heat pain intensity and increased oxycodone requirements, which were not statistically significant, but were of considerable size and followed the predictions based on cold pain associations.

Rs887200 is located within a region adjacent to 3'UTR, which has recently been shown to be the main determinant of *COMT* activity and related behavioural phenotypes in animals (Segall *et al.*, 2010). In the study, which utilised eQTL analysis of inbred mouse strains, the variant was caused by a transposon. In humans, rs887200 has not been studied in pain phenotypes before, and its effects on *COMT* activity are currently unknown. Other SNPs in the *COMT* 3'UTR-region have been linked to neuropsychiatric phenotypes (Stein *et al.*, 2005; Shifman *et al.*, 2002) and altered *COMT* expression (Bray *et al.*, 2003). 3'UTR-variants do not directly alter the amino acid sequence of the protein, and therefore their mechanisms of action are less evident. Possible mechanisms include attenuation of interaction of the 3'UTR-area with negative modulators of expression such as microRNAs (Lewis *et al.*, 2003) with a consequent increase in expression. The *in silico* eQTL searches we performed did not provide evidence of this SNP affecting *COMT* expression levels significantly in the cell types available. This, however, is logical, as 3'UTR modulators are small regulatory molecules, which may be synthesised in neighbouring cells or only in particular conditions and expected to be lacking from the cell culture. Rs887200 is also

intragenic to a neighbouring gene, *ARVCF*, which codes for the armadillo repeat protein deleted in the velocardiofacial syndrome, but its impact seems rather unlikely.

Interestingly, rs887200 showed significant interaction with CCG (HPS)-haplotype, suggesting that they both may contribute to the decreased pain intensity. This interaction provides an explanation to the surprising results of HPS/HPS-haplotype and shows that rs887200 may act as a confounder masking other more moderate effects of LPS/APS/HPS-haplotypes.

SNPs and haplotypes of the 2nd haploblock. Clinical postoperative pain was associated with SNPs rs4646312, rs2239393, and rs4818 in the SNP and haplotype analyses, whereas rs4680 did not reach the level of statistical significance ($p=0.1$) (**Appendix 1: Tables 6 and 8**)(IV). In the SNP analysis, the carriers of the more rare alleles of these SNPs (rs4646312C, rs2239393G and rs4818G) reported higher postoperative pain scores at rest (uncorrected $p=0.006-0.007$) and during motion (uncorrected $p=0.009-0.01$) at the time when they needed the first dose of oxycodone, than the patients being homozygous for the major allele. Also in analysis of LPS/APS/HPS-haplotypes, which are defined by the SNPs rs4633, rs4818 and rs4680, CGG (LPS)-haplotype, containing minor alleles of rs4646312, rs2239393, and rs4818 was associated with increased postoperative pain ratings (rest pain, $p=0.008$, $\beta=0.251$; motion pain, $p=0.011$, $\beta=0.241$). The effect sustained multiple comparison corrections (rest pain, $p=0.02$).

One interpretation to this is that carriers of minor alleles tolerate more intense pain before requesting analgesic. However, in other phenotypes, the time of administration of the first oxycodone dose did not differ between the genotypes, which does not support this interpretation. SNPs rs4646312C and rs4818G showed a tendency towards higher pain ratings upon awakening (**Appendix 1: Table 6**). Rs4818G was also associated with increased cold pain intensity and decreased cold pain tolerance. Consistently, CGG (LPS)-haplotype increased the intensity of cold pain and decreased cold pain tolerance and CCG (HPS)-haplotype showed an opposite effect, which sustained corrections for multiple comparisons ($p=0.009$). In the earlier studies, however, LPS-haplotype and its marker SNP rs4818G have been associated with decreased postoperative and experimental pain intensity (Diatchenko *et al.*, 2006; George *et al.*, 2008b; Lee *et al.*, 2011). One study assessing rs4818, however, failed to confirm this (Kim *et al.*, 2006a). At first glance, both associations were equally surprising as they were opposite to the prediction of earlier pain studies in other pain phenotypes, which were supported by COMT activity experiments. The effects seen in our study, however, were most pronounced in the

recessive model. A recessive model compares homozygous carriers to the rest of the population, and presumes a recessive genotype effect, which is carried primarily by homozygotes (Lewis, 2002). HPS/HPS represents a relatively rare haplotype (n=18 in cold pain phenotypes), which was lacking, excluded, or extremely scarce in studies by Diatchenko *et al.* (2005, 2006) and other earlier studies. Thus, predictions regarding the HPS-haplotype were based on heterozygotes, omitting homozygote data and thus lacking sufficient predictive potential regarding HPS/HPS carriers. Nevertheless, among haplotypes introduced by Diatchenko *et al.* (2005, 2006), HPS haplotype has been predicted to be more sensitive to pain and to have lower COMT activity compared to other haplotypes. Thus it could be presumed, that these effects would be most pronounced in HPS/HPS carriers. However, such extreme variants can be accompanied by other mutations, which can compensate for insufficient COMT activity and, consequently, their phenotype effects (see below). We hypothesized that such compensatory variants would explain exceptional effects of LPS/LPS and HPS/HPS haplotypes on pain phenotypes.

On a general level, such compensatory mutations could act like rare variants, which have been shown to modulate the effect of common *COMT* variants, and act like confounding factors (Lötsch *et al.*, 2009a). In our study, the best candidates with potential to act as protective variants and confound the detrimental effect of the HPS allele in homozygous carriers, were rs887200 and possibly rs165774. Indeed, the analysis of epistatic interactions confirmed that there was an interaction between rs887200 and HPS-haplotype and in all cases the HPS/HPS haplotype was accompanied by the C/C-variant of rs887200, both contributing to the decreased pain intensity in the cold pain test. Rs887200 also showed interaction with APS (TCA)-haplotype, which decreased cold pain intensity. The magnitude of the effect was smaller ($\beta=-0.59$ vs. -0.97) apparently due to the fact that in the most of the patients APS-haplotype was accompanied by a single rs887200C-allele whereas HPS/HPS carriers had two of them.

As to LPS/LPS haplotype effects, they are to significant extent contributed by HPS/HPS and APS haplotype, which were included in the control group to which LPS/LPS haplotype carriers were compared. LPS/LPS carriers also mostly lacked the protective effect of rs887200C-allele, as 95% of LPS/LPS-carriers were rs887200T-homozygotes thus lacking even an individual C-allele. Additionally, Bray *et al.* (2003) showed that SNP rs737865 and rs165599 variants can modulate allelic expression of *COMT* mRNA in certain haplotypes, including rs4633C, via *cis*-acting mechanisms. Rs737865 and other SNPs and haplotypes of the 1st block may possibly modulate the effects of LPS/APS/HPS-haplotypes on pain phenotypes, since they have been associated

with cold pain phenotypes (uncorrected $p < 0.05$), and their effect on *COMT* mRNA expression has been shown in human tissues.

Taken together, it seems that the effects of functional *COMT* haplotypes of the 2nd haploblock on pain phenotypes may be modulated by 3' and 5'UTR SNPs showing rather complex interactions on molecular and/or phenotypic level. This is in line with earlier *in vitro* and *ex vivo* studies, which have shown genetic interactions between these variants (Bray *et al.*, 2003) and their importance in neuropsychiatric phenotypes (Shifman *et al.*, 2002; Hatzimanolis *et al.*, 2013). Impact of different *COMT* variants and their interactions remains to be verified and analysed in-depth in future studies. Linear regression analysis assumes that the effects of different genotypes are independent, and therefore does not allow reliable evaluation of such interactions. Thus, alternative analysis methods such as epistatic interaction analysis or multivariate regression analysis should be used in future studies to characterize such interactions. Moreover, statistical methods based on the assumption of independent effects, such as Bonferroni correction for multiple comparisons, are overconservative for assessing the effects of different *COMT* variants. Therefore, interactions between different variants and over conservativeness of Bonferroni corrections should be taken into account when interpreting earlier studies assessing multiple *COMT* variants.

6.6.2 Haplotypes and haploblocks (IV)

2nd HAPLOBLOCK. Among the detected haplotypes, the most interesting were those in the 2nd haploblock. TTACACA, the only haplotype containing a minor allele of rs165774 (A), has a frequency of 24% and was associated with decreased heat pain intensity (uncorrected $p = 0.003$, $\beta = -0.463$). In contrast, CCGGGTG, a haplotype containing a major allele (G), showed association with increased cold pain intensity after 30 and 45 s and decreased cold withdrawal time.

THE 3rd HAPLOBLOCK - experimental cold and rs887200 haplotypes. As rs887200 showed the best association with cold pain, the haplotypes containing its minor allele were of special interest. Within the 3rd haploblock two haplotypes had this allele. Neither CGATC, nor CGAGC reached the level of statistical significance. This can be explained by the fact that the main effect of rs887200 was seen in homozygous carriers which all represented CGATC-

CGAGC-diplotype. As homozygous carriers were lacking from both haplotypes, diplotype carriers were pooled together with other heterozygous carriers and the effect of diplotype was diluted. Finally, heterozygous carriers were compared against each other as CGATC-carriers were included in control group of CGAGC-haplotype and *vice versa*.

CLINICAL PAIN. In clinical pain, most significant associations were observed between the CGG-haplotype and increased intensity of postoperative pain (**IV**). CGG is present in CCGGGCG and CCGGGTG haplotypes which, however, showed only a trend lacking statistical significance. The lack of significance may mainly be due to the distribution of these variants into two haplotypes, thus contributing to increased postoperative pain scores in both of them. Consequently in the comparison of each haplotype, another haplotype containing CGG was included in the control group thus leading to lack of association signal. These results suggest that association signal may be primarily due to either individual SNPs or the CGG-haplotype, whereas possible modulatory effects of other SNPs in the 2nd haploblock remain to be studied by further diplotype and interaction analyses.

METHODOLOGICAL DISCUSSION. There are several issues that need to be taken into account when interpreting the results of Study IV. Firstly, approximately 25% of the breast cancer patients we studied had some previous chronic pain condition. LPS/APS/HPS haplotypes were associated with the prevalence of reported chronic pain. In LPS/LPS carriers the prevalence was 18.9%, while in HPS/HPS-carriers it was 35%. Thus, when we adjusted for earlier chronic pain conditions, we also corrected the effect of the haplotype. Secondly, it must be noted that follicular phase or post menopause were not addressed. It is known that *COMT* is downregulated by estrogen, and therefore its possible confounding effects cannot be excluded. Further studies are necessary to understand the interaction of such confounding factors with *COMT* genotypes and pain.

In clinical pain phenotypes there were less association signals and they were less significant compared to experimental pain, which might be due to several reasons. The data from clinical pain phenotypes was missing from some of the patients (n=20-70). For example, pain intensity was asked specifically when oxycodone was requested. However, it was not needed by some of the patients, and therefore pain intensity was not recorded. Also, the day-case surgery patients with likely milder symptoms were excluded from the analysis of oxycodone phenotypes. Thus, patients with presumably low pain intensity were excluded from postoperative pain intensity and

oxycodone data. This may represent a potential bias, since these patients were more prevalent in certain genotypes, including the rare variant rs887200-CC. These factors might affect the results of the haplotype analysis but also the analysis of individual SNPs.

In general, the data from postoperative phenotypes is more variable than experimental phenotype data. Although we adjusted for a number of confounders including the type of surgery, the extent of tissue damage, and duration of the operation, pre- and perioperative medication varied from patient to patient. Also, whilst the origin of experimental pain is clear, postoperative pain is likely to represent mixed pain, which contains not only inflammatory pain, but also a neuropathic component, as nerve fibers have been damaged to varying extent in virtually all of the patients. The impact of neuropathic component is particularly challenging to estimate, as in neuropathic pain low COMT activity is supposed to have opposite effects as compared with nociceptive pain. Finally, the impact of cognitive and emotional processes after a major surgery is greater, since the sense of security and controllability, typical to experimental pain tests, is lacking.

6.6.3 Phenotype-specific effects

A central question is why do the effects of different *COMT* SNPs appear, at least to some extent, be phenotype specific, if they act via changes in COMT activity via a common mechanism? This was observed in studies assessing rs4680 and haplotypes – within the same study rs4680 was associated with temporal summation of pain and haplotypes were associated with other pain phenotypes (Diatchenko *et al.*, 2006). Also in other studies rs4680 has been associated with pain responses if stimulation has been of higher intensity and of prolonged duration (Jensen *et al.*, 2009; Loggia *et al.*, 2011; Zubieta *et al.*, 2003). In our study, a different set of SNPs gave an association signal in cold and heat pain.

It must be kept in mind that in different pain phenotypes the extent of involvement of CNS-processes is different. If the stimulus is short, applied to a small area, and the intensity is relatively low, the involvement of CNS-processes is minimal. As intensity and duration of pain stimuli, and the area of stimulation increase, CNS-processes and endogenous pain modulation become more involved and contribute to the final pain experience to a greater extent. If the phenotype includes tolerance to pain (as in cold pressor task), the impact of CNS is even greater.

COMT has been shown to increase nociceptive sensitivity via peripheral mechanisms (II) but also in CNS where it modulates pain processing and expression of endogenous opioids and opioid receptors (Berthele *et al.*, 2005; Kowarik *et al.*, 2012; Loggia *et al.*, 2011; Mobascher *et al.*, 2010; Nikoshkov *et al.*, 2008; Zubieta *et al.*, 2003). The first effect has been shown to be mediated via adrenergic β -receptors and involves adrenergic or noradrenergic neurotransmission, and the latter effect has been attributed to the involvement of dopaminergic and endogenous opioid systems in prefrontal cortex and other brain areas processing nociception (Nackley *et al.*, 2007; Zubieta *et al.*, 2003). Genetic determinants of these two effects are partially overlapping. In the periphery, a major part of COMT activity is due to S-COMT, whereas in the brain, MB-COMT is the predominant isoform. Thus, SNPs specific to MB-COMT, such as rs737865 located in the first MB-COMT-intron, could modulate CNS-processing of pain while lacking peripheral effects (Bray *et al.*, 2003). SNPs located in the region coding for S-COMT, such as rs4818 and rs4633 (Diatchenko *et al.*, 2005, 2006; Tsao *et al.*, 2011) are also present in MB-COMT and thus could modulate both, the peripheral and CNS-effects. In the CNS, however, their effects may be modulated by MB-COMT-specific SNPs such as rs737865, which modulate the expression of rs4633-variants, and rs737865C relatively decreases expression of rs4633C-rs4680G-variant (Bray *et al.*, 2003).

In addition to these mechanisms, there may also be a longer transcript, which extends over conventional polyadenylation site at the 3'end, and covers rs165599 (Bray *et al.*, 2003) and a truncated variant caused by rs165773 (Meloto *et al.*, 2011). Both variants decrease expression of *COMT*. A variant containing rs165599 has been identified in the brain and may be more important in the modulation of CNS-processes, as it also has been associated with neuropsychiatric phenotypes. SNP rs165773 and the truncated variants may be more important in the periphery, as they are more efficient than non-truncated S- and MB-COMT in metabolizing noradrenaline, which is the main component of peripheral effects of low COMT activity. In the CNS, however, its effects may be opposite, since it reduces COMT expression decreasing COMT activity involved in dopaminergic CNS-processing. Thus, rs165773 and to some extent rs165599 show substrate specific effects.

SUBSTRATES. Differential effects on level of different COMT substrates could result in phenotype specific effects. If a SNP is modulating effects of noradrenaline or adrenaline, which are mediated via adrenergic $\beta_{2/3}$ receptors, its effects would be mainly seen in phenotypes in which peripheral catecholamines play a more important role, such as in pain sensitivity or

intensity after a brief stimulation. In contrast, the SNPs modulating dopaminergic transmission in CNS, such as rs4680, and consequently compensatory systems could have more pronounced effects in phenotypes in which CNS or targeted neural circuits are important. These include temporal pain summation after repetitive stimulation, pain intensity after prolonged stimulation, or opioid responses. It must be also noted that whereas the effects of haplotypes described by Diatchenko (2005, 2006), Nackley (2006) and Tsao (2011) have a strong peripheral component, the effects on CNS-processing have mostly been shown with rs4680 (Val^{108/158}Met) (Loggia *et al.*, 2011; Mobascher *et al.*, 2010; Zubieta *et al.*, 2003).

TISSUE and “STATE” SPECIFICITY. *COMT* variants may interact with systems regulating tissue- or “state” specific expression of *COMT*. In these cases, effects of such SNPs would be more pronounced in phenotypes that involve nociceptive processing in these tissues, or in corresponding pathophysiological states. Interaction of SNP with tissue and “state” specific mechanisms could provide another hypothetical explanation for the observed phenotype-specific effects of *COMT* SNPs.

COMBINATORIAL EFFECTS. From a molecular perspective, several genetic factors contribute to *COMT* expression, structure, or activity. SNP rs4680 (Val^{108/158}Met) modulates thermostability of *COMT* and consequently *COMT* activity, and the Met-variant has decreased stability (Lotta *et al.*, 1995). In Val-allele carriers, *COMT* activity is additionally modulated by rs4818 (Nackley *et al.*, 2006), which contributes to the expression of *COMT*. G-allele present in LPS-haplotype is associated with higher *COMT* expression and C-allele in HPS-haplotype with reduced *COMT* expression, at least in some tissues. Furthermore, in Val-carriers expression of *COMT* is modulated by rs737865 of the first MB-*COMT*-intron (Bray *et al.*, 2003). Rs737865C decreases relative expression of rs4633C-rs4680G-variant, as has been shown in the brain tissue. In Met-allele carriers, *COMT* activity is also modulated by rs4633. Rs4633T, which is present in APS haplotype increases expression of *COMT*, thus compensating for decreased stability of Met-variant at least in some tissues in a tissue-specific manner (Tsao *et al.*, 2011). It is also not clear if relative decrease in allelic expression of rs4633C-variant by rs737865C can increase the relative expression of rs4633T-variant. Part of Met-carriers, however, also carries rs165773A and an alternatively spliced *COMT* variant, which causes decreased expression but is more efficient in metabolizing noradrenaline (Meloto *et al.*, 2011). Finally, rs165599 located in the 3'UTR area can decrease expression of *COMT* at least in the brain (Bray *et al.*, 2003). This effect can be due to the alternatively spliced longer *COMT* isoform as rs165599 is located 3' from the conventional

polyadenylation site, and variants containing it have been detected in the brain (Bray *et al.*, 2003). We hypothesized that also rs887200C increases *COMT* expression, which is likely caused by an attenuated interaction with negative regulatory factors and prevention of negative modulation. However, other mechanisms cannot be totally excluded. For example, as Bray *et al.* (2003) showed, also other, longer *COMT* transcripts, may exist, and such alternatively spliced variants could offer an alternative explanation to the effects of rs887200C.

Thus, several polymorphisms modulate *COMT* expression and/or activity. The net effect on *COMT* activity in each subject may not be due to the effects of individual SNPs, but rather a result of a particular combination of these variants and their interactions. Different SNPs or their combinations may act via different/multiple mechanisms in additive, synergistic or antagonistic manner. Each individual also has two variants of *COMT*, which may differ in their structure and function, thus adding to the complexity of effects of *COMT* variants.

CONFOUNDING FACTORS and “observed” phenotype specific effects of *COMT*.

Finally, part of the observed phenotype specific effects of *COMT* variants may reflect methodological study- or phenotype-specific issues, rather than real phenotype-specificity. Studies comparing effects of different *COMT* variants are scarce and differ in their pain phenotypes as well as in the subjects studied. Thus, positive correlation of particular variants in one study and absence of effects in other study may be due to methodological differences. On the other hand, differences between phenotypes within the same study may arise from phenotype-specific factors, such as different inherent variation of the responses, scales of the used phenotypes, and size or magnitude of the overall effects.

6.6.4 Putative mechanisms of *COMT* variants

PUTATIVE MECHANISMS of ACTION of *COMT* SNPs. Whereas several SNPs within *COMT* gene have shown significant associations with clinical and experimental pain phenotypes, susceptibility to develop chronic pain condition or, with experimental or clinical pain intensity, the mechanism underlying these effects is not known. It seems likely that the effects are caused by changes in *COMT* function, such as decreased activity. Such change has been shown in SNP rs4680 (Val^{108/158}Met), which causes a change in the amino acid sequence of *COMT* from valine to methionine, increasing the thermolability of the protein and decreasing *COMT* activity *in vivo*.

The effects of vast majority of other SNPs within *COMT* gene on COMT activity and resulting nociceptive phenotype remain unknown. Practically all the other SNPs in *COMT* that have been studied in relation to pain are either synonymous, and do not change the amino acid sequence of the protein, or are located in introns. However, it cannot be excluded that the effect of the synonymous SNP on phenotype is in fact caused by another, coding or non-synonymous SNP that is tightly linked to it. However, this possibility seems rather theoretical.

Non-synonymous SNPs cause an exchange of amino acids, and nonsense SNPs can result in a premature stop codon, and thus their effect on the coded protein is evident. However, also synonymous and SNPs located within 5'- and 3'-UTRs, UTR-adjacent areas, and intronic and intergenic regions can be associated with different diseases. It has been estimated that only 8.7 % of SNPs associated with diseases are non-synonymous. In fact, synonymous and non-synonymous SNPs are as likely to be associated with the diseases as non-synonymous, and their effect sizes are similar (Chen *et al.*, 2010).

INTRONIC SNPs. In general, 52% of disease-associated SNPs are located in intronic regions. SNPs within the first intron are more likely to be associated with diseases than those in other introns. Especially SNPs located in the proximity of the 80th base from the beginning of the first intron seem to be more likely associated with diseases (0.85% probability vs. 0.5 %) (Chen *et al.*, 2010).

SNPs and mRNA. It has been shown that *COMT* haplotypes alter the secondary structure of mRNA modulating protein expression (Nackley *et al.*, 2006). SNPs, however, do not necessarily decrease expression of *COMT* gene or decrease COMT activity. Some of the SNPs, such as synonymous rs4633 (C¹⁶⁶T), located in 5'UTR of the RNA transcript, can increase the expression of COMT *in vitro*, which was explained by structural destabilization of mRNA, and consequent increase in translational efficiency (Tsao *et al.*, 2011). Since this mutation is usually present as part of a common haplotype with low or unstable COMT activity, it has been suggested to be a compensatory genetic change.

3'UTR. SNPs in the 3'UTR are as frequently associated with diseases as non-synonymous SNPs. SNPs in 3'UTR, and possibly in 5'UTR, may interfere with interaction between DNA and regulatory elements, such as microRNAs, and their role is underinvestigated. In our study (**IV**), SNP rs887200, which was associated with different pain phenotypes, is located in such an

untranslated region. The protective effect observed in homozygous carriers of rs887200C could be explained by an attenuated interaction with negative regulatory factors and a consequent prevention of negative modulation. In general, the effect of a SNP is due to its effect on physico-chemical properties of DNA, or resulting mRNA, and a consequent alteration of its interaction with other factors. Such factors include DNA- or RNA-processing enzymes, transcription factors, small RNAs and other *cis*- and *trans*-acting regulatory elements.

REGULATORY SITES. *COMT* gene contains several regulatory sites. Many of them are located within P1 and P2 promoter regions that contain binding sites for different transcription factors, such as AP-2, Ets-1, Sp1, and NF-D within the P2 coding for a longer transcript, and binding sites for factors HNF-4, Sp1, AP-2 and NF-IL6 in P1 coding for a shorter transcript. P1 also contains a binding site for C/EBPa that regulates tissue-specific expression of *COMT* (Tenhunen, 1996). Also, 3'UTR and possibly 5'UTR contain binding sites for regulatory factors. If a SNP is located within or in the vicinity of a binding site of a regulatory factor, it can modulate its function. Consequently, its own effect can depend on the presence or absence of a regulatory factor. For example, if a SNP would prevent the binding of a regulatory factor, its effect could be seen when the factor is present, resulting in a change in transcription, whereas when the factor is absent and the regulatory site is functionally silent the SNP would show no effect.

For example, a mutation in the estrogen-regulatory site providing negative regulation of *COMT* expression, could prevent such negative regulation, and its effect on protein expression and consequent clinical or physiological phenotypes would be greater during the phases of menstrual cycle when estrogen level is increased. *COMT* contains a regulatory site for another sex hormone, progesterone, as well. Regulation of *COMT* is also dependent on factors controlling tissue-specificity, such as C/EBPa (Tenhunen, 1996). Both tissue- and hormone specific regulation of *COMT* are clinically interesting, since they can help to narrow down and specify the group of patients or pain states in which certain SNPs would be important. For example, a SNP affecting estrogen-mediated regulation of *COMT* could be important in females, and its effects could be more important during the phases with high estrogen level and in tissues with a higher estrogen receptor density (for example vulvodynia, premenstrual syndrome or hormone-dependent cancer). In males, however, the same SNP would not have similar predictive value.

MicroRNA. *COMT* contains several microRNA binding sites (**Table 9**), and these microRNAs regulate gene functions. Their role has been shown in endothelial aging and inflammation (Qin *et al.*, 2012), regulation of cell stress and proliferation processes (Staszal *et al.*, 2011), cell death, epigenetic regulation, diabetic and other neuropathies, as well as in cancer and Huntington's disease.

GENE-GENE and OTHER INTERACTIONS. *COMT* is also regulated by other genes and by several physiological states, such as trauma, stroke, and inflammation. Both types of interactions are dependent on regulatory sites within the *COMT* gene. Such regulatory sites may contain different SNPs, which contribute to the genetic effect. Genes that interact with *COMT* include those coding for ACE (mediated via P21964, P12821), lipopolysaccharide-induced TNF factor (LITAF, mediated via P21964, Q99732), thyroid hormone receptor interactor (TRIP13), mediated by two different mechanisms (P21964, Q15645 and BioGRID:107707, BioGRID:114730) and 5'-3' exoribonuclease 2 (XRN2) mediated by two mechanisms, (P21964, Q9H0D6 and BioGRID:107707, BioGRID:116483). Others include a PCNA-associated factor (KIAA0101), regulator of G-protein signalling 2 (RGS2), ubiquitin C (UBC), valosin containing protein (VCP), and vitamin K epoxide reductase complex, subunit 1 (VKORC1) (Emanuele *et al.*, 2011; Illi *et al.*, 2003; Kim *et al.*, 2011; Lehner and Sanderson, 2004; Rual *et al.*, 2005; Schaafhausen *et al.*, 2011; Stelzl *et al.*, 2005; Vinayagam *et al.*, 2011; Yu *et al.*, 2013). Especially interactions with ACE and LITAF genes are interesting. ACE and *COMT* have potential interactions with the vascular system and pain states such as migraine in which it plays an important role. ACE is also involved in processing of vasoactive and other peptides and endothelial function. Interestingly, the use of ACE-inhibitors has been linked to CRPS and inflammatory pain (Fein *et al.*, 2009; de Mos *et al.*, 2009). On the other hand, interaction of *COMT* with ACE and the cardiovascular system may explain some aspects of interactions of blood pressure and pain and β -receptor mediated pronociceptive effects of low *COMT* activity. LITAF and TNF- α could link *COMT* to the immune system, nociceptive chemokines, 5-lipoxygenase and cyclooxygenase-2 pathways, and inflammatory pain.

7 CONCLUSIONS

Low COMT activity was shown to be pronociceptive in experimental animal models of acute and inflammatory pain, whereas COMT inhibitors were antinociceptive in a neuropathic pain model. In humans, pain phenotypes were modulated by several SNPs.

1. COMT deficiency in *Comt* knockout mice was associated with an enhanced response to morphine in the hot plate test, which is in line with the human studies showing increased availability of opioid receptors and enhanced morphine response in polymorphisms with low COMT activity. *Comt* knockout mice also showed decreased stress-induced analgesia in the tail flick test, suggesting weaker endogenous modulation of nociception on spinal/peripheral level. In the tail flick test, also opioid-induced antinociception was decreased, suggesting spinal and/or peripheral pronociceptive or antianalgetic effects of COMT deficiency.

2. Pronociceptive effects of several types of COMT inhibitors were confirmed in normal mice, and shown to be mediated primarily via mechanisms acting outside the CNS in COMT-dependent manner. COMT inhibitors increased both thermal and mechanical nociception, and increased inflammatory pain resulting from the pronociceptive effect of carrageenan in an additive rather than synergistic manner. Pronociceptive effects seemed to be mediated via peripheral mechanisms, as they were neither dependent on brain penetration of COMT inhibitors nor seen after intrathecal administration of nitecapone. Since a lack of *Comt* gene abolished further pronociceptive effects of OR-486, the effects are likely COMT-mediated. This was indirectly supported by the pronociceptive effect of an atypical COMT inhibitor CGP 28014.

3. In an experimental SNL model of neuropathic pain, a COMT inhibitor nitecapone partially prevented or reversed neuropathic pain symptoms, cold and mechanical allodynia, and hyperalgesia. Our results show that in contrast to the pronociceptive effects observed in acute and inflammatory pain models, in neuropathic pain, the effects of nitecapone are antiallodynic, antihyperalgesic or antinociceptive.

4. Pain phenotypes were modulated by several *COMT* SNPs in our cohort of 1000 breast cancer surgery patients. The effects were the most pronounced in two novel SNPs, the effects of which were described for the first time. Effects of these mutations on COMT activity are not known, but they are likely to represent novel mechanism of action.

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APPENDIX 1: TABLES 4-9

Table 4. Associations of *COMT* single nucleotide polymorphisms with intensity of thermal pain in breast cancer surgery patients (n=1000).

Table 5. Association between *COMT* SNPs and experimental cold pain in patients undergoing breast cancer surgery (n=900).

Table 6. Association of *COMT* SNPs with postoperative pain and oxycodone consumption.

Table 7. Association between *COMT* haplotypes and experimental pain in patients undergoing breast cancer surgery (n=900).

Table 8. Associations of *COMT* haplotypes with intensity of acute postoperative pain and *i.v.* oxycodone consumption in breast cancer surgery patients (n=1000).

Table 9. Predicted microRNA binding sites in *COMT* gene.

Table 4. Associations of *COMT* single nucleotide polymorphisms with intensity of thermal pain in breast cancer surgery patients (n=1000). Pain intensity was measured after 5s of noxious (48°C) and innocuous (43°C) heat stimulation with a numerical rating scale (NRS 0-10). Results are presented as empirical p-values based on linear regression analysis using additive, dominant and recessive models. Significant p-values are underlined and borderline associations (0.1>p>0.05) are shown with **bolding**. Variables have been adjusted for age, body mass index, anxiety (STAI score) and presence of pre-operative chronic pain condition.

SNP	Alleles minor/major	Heat pain intensity, 48°C						Heat pain intensity, 43°C								
		Additive			Dominant			Additive			Dominant			Recessive		
		P	β	P	β	P	β	P	β	P	β	P	β	P	β	
rs6518591	G/A	0.815	0.033	0.835	0.035	0.867	0.070	0.586	-0.045	0.480	-0.068	0.858	0.045			
rs737866	G/A	0.092	-0.228	0.059	-0.300	0.824	-0.090	0.978	0.002	0.698	-0.036	0.297	0.239			
rs737865	C/T	0.088	-0.230	0.056	-0.303	0.835	-0.080	0.933	0.007	0.744	-0.030	0.291	0.241			
rs1544325	A/G	0.547	0.067	0.634	0.084	0.618	0.090	0.628	0.031	0.931	0.009	0.480	0.075			
rs8185002	G/T	0.071	-0.246	0.047	-0.315	0.772	-0.120	0.993	0.001	0.614	-0.046	0.185	0.315			
rs174675	T/C	0.351	0.109	0.410	0.126	0.502	0.180	0.603	-0.035	0.417	-0.072	0.820	0.035			
rs5993882	G/T	0.421	0.108	0.339	0.153	0.997	0.000	0.878	-0.012	0.807	-0.023	0.880	0.034			
rs740603	G/A	0.288	-0.119	0.330	-0.155	0.470	-0.150	0.728	0.023	0.675	0.039	0.918	0.013			
rs4646312	C/T	0.561	0.068	0.772	0.044	0.424	0.210	0.166	0.094	0.435	0.069	0.080	0.262			
rs4633	C/T	0.300	0.114	0.290	0.179	0.547	0.110	0.280	0.069	0.654	0.044	0.174	0.149			
rs2239393	G/A	0.813	-0.030	0.454	-0.127	0.500	0.180	0.455	0.055	0.931	-0.009	0.088	0.265			
rs4818	G/C	0.542	0.072	0.733	0.052	0.438	0.200	0.190	0.089	0.485	0.062	0.085	0.258			
rs4680	G/A	0.257	0.125	0.230	0.203	0.543	0.120	0.238	0.075	0.569	0.056	0.162	0.154			
rs4646316	T/C	0.549	0.079	0.597	0.084	0.657	0.160	0.324	0.075	0.318	0.092	0.651	0.092			
rs165774	A/G	0.015	-0.312	0.003	-0.464	0.816	0.080	0.348	-0.070	0.118	-0.140	0.323	0.198			
rs174696	C/T	0.057	0.213	0.234	0.184	0.034	0.480	0.508	0.043	0.584	0.049	0.590	0.071			
rs9306235	A/G	0.441	0.173	0.442	0.182	0.832	0.250	0.271	0.142	0.355	0.126	0.238	0.818			
rs9332377	T/C	0.977	0.005	0.932	-0.015	0.663	0.290	0.677	0.039	0.881	0.015	0.258	0.437			
rs165599	G/A	0.345	0.111	0.780	0.043	0.112	0.410	0.784	0.019	0.998	0.000	0.549	0.089			
rs887199	A/G	0.414	0.109	0.191	0.208	0.421	-0.310	0.986	0.001	0.716	0.034	0.414	-0.180			
rs2518824	G/T	0.203	0.368	0.203	0.368	NA	NA	0.759	0.052	0.759	0.052	NA	NA			
rs887200	C/T	0.410	0.120	0.151	0.239	0.170	-0.640	0.866	-0.014	0.993	-0.001	0.606	-0.139			

Table 5. Association between COMT SNPs and experimental cold pain in patients undergoing breast cancer surgery (n=900). Cold pain intensity (NRS 0-10) was assessed during immersion of the hand to cold (5°C) water bath every 15 s for 90 s or until the withdrawal. Time of withdrawal (total time) has been used as an indicator of tolerance to cold pain. Variables have been adjusted for age, body mass index, anxiety (STAI score) and presence of pre-operative chronic pain condition. Results are presented as empirical p-values based on linear regression analysis using additive, dominant and recessive models. Significant p-values are underlined and borderline associations (0.1>p>0.05) are shown with **bolding**.

SNP	Alleles minor/ major	Cold pain intensity, 15s						Cold pain intensity, 30s						Cold pain intensity, 45s						Cold pain intensity, mean						Cold withdrawal time (s)											
		Additive			Dominant			Recessive			Additive			Dominant			Recessive			Additive			Dominant			Recessive			Additive			Dominant			Recessive		
		P	β	P	β	P	β	P	β	P	β	P	β	P	β	P	β	P	β	P	β	P	β	P	β	P	β	P	β	P	β	P	β	P	β		
rs6518591	G/A	0.751	-0.056	0.834	0.043	0.124	-0.848	0.972	0.006	0.496	0.129	0.087	-0.864	0.881	-0.020	0.701	0.061	0.137	-0.630	0.856	-0.022	0.688	0.055	0.102	-0.600	0.800	-0.463	0.399	-1.788	0.141	8.320						
rs737866	G/A	0.372	-0.150	0.378	-0.174	0.678	-0.202	0.082	-0.267	0.070	-0.328	0.566	-0.256	0.050	-0.253	0.059	-0.287	0.309	-0.380	0.114	-0.177	0.107	-0.212	0.542	-0.198	0.044	3.462	0.055	3.890	0.274	5.457						
rs737865	C/T	0.316	-0.168	0.308	-0.201	0.695	-0.191	0.074	-0.275	0.061	-0.338	0.571	-0.252	0.048	-0.256	0.055	-0.292	0.316	-0.375	0.104	-0.182	0.095	-0.220	0.552	-0.193	0.039	3.563	0.046	4.038	0.280	5.398						
rs15444325	A/G	0.554	0.082	0.031	0.473	0.211	-0.284	0.092	0.214	0.016	0.484	0.775	0.060	0.031	0.229	0.095	0.478	0.536	0.109	0.095	0.154	0.011	0.374	0.916	0.016	0.138	-2.107	0.007	-6.106	0.723	0.827						
rs8185002	G/T	0.447	-0.129	0.402	-0.165	0.907	-0.059	0.112	-0.247	0.087	-0.310	0.728	-0.162	0.055	-0.250	0.066	-0.279	0.316	-0.391	0.134	-0.170	0.118	-0.206	0.650	-0.153	0.058	3.302	0.061	3.796	0.397	4.414						
rs174675	T/C	0.970	-0.005	0.479	0.135	0.194	-0.425	0.681	-0.055	0.726	0.061	0.128	-0.435	0.532	-0.070	0.838	0.030	0.081	-0.439	0.625	-0.048	0.666	0.055	0.066	-0.400	0.955	-0.085	0.309	-1.987	0.104	5.446						
rs593882	G/T	0.827	0.037	0.344	0.188	0.087	-0.843	0.599	0.081	0.194	0.237	0.092	-0.761	0.760	0.040	0.319	0.153	0.114	-0.599	0.766	0.034	0.296	0.138	0.085	-0.565	0.308	-1.772	0.088	-3.478	0.212	6.313						
rs740603	G/A	0.736	0.047	0.440	0.153	0.688	-0.108	0.930	-0.011	0.699	0.070	0.487	-0.172	0.366	-0.098	0.686	-0.062	0.236	-0.246	0.759	-0.029	0.935	0.011	0.485	-0.126	0.917	0.151	0.989	0.029	0.856	0.502						
rs4646312	C/T	0.036	0.307	0.142	0.279	0.030	0.716	0.049	0.265	0.281	0.188	0.011	0.775	0.149	0.163	0.430	0.116	0.059	0.479	0.064	0.181	0.286	0.135	0.020	0.511	0.297	-1.571	0.982	0.044	0.017	-8.114						
rs4633	C/T	0.156	0.193	0.393	0.179	0.135	0.354	0.451	0.094	0.888	0.027	0.250	0.249	0.685	0.043	0.938	-0.013	0.428	0.144	0.463	0.067	0.989	0.002	0.207	0.199	0.943	-0.099	0.457	1.597	0.335	-2.345						
rs2259393	G/A	0.026	0.354	0.135	0.317	0.018	0.802	0.056	0.278	0.297	0.202	0.016	0.747	0.127	0.186	0.358	0.150	0.073	0.466	0.049	0.209	0.242	0.166	0.020	0.527	0.305	-1.716	0.744	0.730	0.007	-9.693						
rs4818	G/C	0.033	0.312	0.126	0.290	0.032	0.709	0.048	0.265	0.275	0.190	0.011	0.771	0.154	0.161	0.437	0.114	0.064	0.473	0.064	0.181	0.282	0.136	0.021	0.506	0.305	-1.545	0.972	0.070	0.017	-8.068						
rs4680	G/A	0.127	0.207	0.341	0.198	0.115	0.373	0.458	0.093	0.875	0.030	0.266	0.242	0.663	0.046	0.989	-0.002	0.438	0.142	0.450	0.068	0.957	0.007	0.209	0.198	0.981	-0.033	0.447	1.627	0.364	-2.213						
rs4646316	T/C	0.078	0.288	0.222	0.243	0.040	0.903	0.022	0.343	0.059	0.344	0.045	0.809	0.033	0.268	0.054	0.295	0.141	0.504	0.040	0.224	0.071	0.240	0.123	0.453	0.156	-2.380	0.496	-1.394	0.021	-10.477						
rs165774	A/G	0.117	-0.251	0.170	-0.262	0.254	-0.491	0.185	-0.194	0.275	-0.191	0.263	-0.442	0.150	-0.177	0.115	-0.232	0.739	-0.110	0.118	-0.167	0.079	-0.224	0.793	-0.075	0.185	2.178	0.139	2.909	0.808	1.075						
rs174696	C/T	0.976	-0.004	0.752	-0.061	0.689	0.113	0.670	-0.055	0.749	-0.057	0.696	-0.101	0.870	-0.018	0.755	-0.046	0.901	0.027	0.814	-0.022	0.660	-0.057	0.866	0.032	0.927	0.131	0.688	0.797	0.688	-1.162						
rs9306235	A/G	0.034	0.609	0.026	0.656	0.809	-0.482	0.010	0.679	0.010	0.698	0.591	0.982	0.043	0.446	0.048	0.450	0.505	1.024	0.014	0.472	0.013	0.489	0.688	0.535	0.228	-3.553	0.240	-3.567	0.654	-9.193						
rs932377	T/C	0.370	0.184	0.398	0.187	0.628	0.414	0.599	0.099	0.384	0.111	0.938	0.061	0.421	0.127	0.599	0.090	0.184	0.874	0.457	0.102	0.570	0.084	0.362	0.520	0.358	-1.933	0.475	-1.625	0.280	-9.484						
rs165599	G/A	0.780	0.041	0.738	-0.064	0.245	0.370	0.424	-0.108	0.288	-0.187	0.980	0.008	0.309	-0.115	0.187	-0.195	0.985	-0.005	0.399	-0.083	0.230	-0.154	0.873	0.034	0.614	0.760	0.304	2.028	0.541	-2.003						
rs887199	A/G	0.590	-0.089	0.783	-0.054	0.386	-0.406	0.166	-0.210	0.401	-0.152	0.056	-0.817	0.085	-0.220	0.206	-0.192	0.064	-0.667	0.128	-0.168	0.283	-0.141	0.081	-0.544	0.163	2.372	0.342	1.921	0.093	8.049						
rs2518824	G/T	0.670	-0.155	0.670	-0.155	NA	NA	0.227	-0.402	0.227	-0.402	NA	NA	0.531	-0.175	0.531	-0.175	NA	NA	0.420	-0.195	0.420	-0.195	NA	NA	0.936	-0.300	0.936	-0.300	NA	NA						
rs887200	C/T	0.376	-0.159	0.994	0.002	0.004	-1.624	0.044	0.332	0.262	-0.212	0.001	-1.725	0.059	-0.261	0.218	-0.196	0.010	-1.136	0.062	-0.224	0.275	-0.150	0.003	-1.111	0.094	3.094	0.303	2.181	0.013	14.55						

Table 6. Association of COMT SNPs with postoperative pain and oxycodone consumption. Results are presented as empirical p-values based on linear regression analysis. Significant p-values are **underlined** and borderline associations ($0.1 > p > 0.05$) are shown with **bolding**. Variables have been adjusted for type of surgery, age, body mass index, anxiety (STAI score) and presence of pre-operative chronic pain condition.

SNP	Motion pain when 1st oxycodone						Pain at rest when 1st oxycodone						Motion pain intensity upon awakening						Time of administration of 1st oxycodone					
	Additive			Recessive			Additive			Dominant			Recessive			Additive			Dominant			Recessive		
	P	β	P	β	P	β	P	β	P	β	P	β	P	β	P	β	P	β	P	β	P	β	P	β
rs6518591	0.744	0.028	0.928	-0.009	0.224	0.319	0.668	0.037	0.979	-0.003	0.173	0.358	0.661	0.062	0.981	0.004	0.207	0.537	0.386	-1.581	0.456	-1.590	0.491	-3.799
rs737866	0.671	0.036	0.621	0.049	0.996	0.001	0.852	0.016	0.809	0.024	0.950	-0.016	0.468	0.504	0.106	0.650	0.180	0.637	0.814	0.690	0.805	0.698	1.949	
rs737865	0.675	0.035	0.625	0.048	0.998	0.001	0.857	0.015	0.813	0.023	0.948	-0.016	0.451	0.102	0.484	0.111	0.648	0.181	0.564	1.003	0.593	1.091	0.725	1.787
rs1544325	0.864	0.012	0.908	-0.013	0.689	0.045	0.810	0.016	0.853	-0.020	0.558	0.066	0.591	-0.060	0.673	-0.075	0.655	-0.081	0.426	1.135	0.630	1.096	0.418	1.895
rs8185002	0.981	-0.002	0.836	0.020	0.542	-0.156	0.839	-0.017	0.997	0.000	0.542	-0.158	0.607	0.070	0.534	0.099	0.947	-0.027	0.331	1.710	0.366	1.844	0.560	3.076
rs174675	0.850	-0.014	0.999	0.000	0.674	-0.067	0.913	-0.008	0.901	0.012	0.650	-0.072	0.965	0.005	0.647	0.070	0.492	-0.180	0.123	-2.299	0.147	-2.820	0.340	-3.174
rs5993882	0.873	0.013	0.908	0.011	0.860	0.041	0.889	0.012	0.945	0.007	0.817	0.054	0.827	0.029	0.953	0.009	0.631	0.184	0.280	-1.871	0.341	-1.951	0.428	-3.926
rs740603	0.708	0.026	0.746	0.032	0.779	0.037	0.670	0.030	0.797	0.025	0.640	0.062	0.337	0.107	0.369	0.143	0.530	0.133	0.831	-0.306	0.496	-1.393	0.615	1.379
rs4646312	0.039	0.148	0.009	0.248	0.900	0.020	0.025	0.161	0.006	0.258	0.753	0.051	0.084	0.201	0.120	0.237	0.233	0.306	0.808	0.365	0.614	0.993	0.752	-1.053
rs4633	0.156	0.095	0.158	0.145	0.383	0.101	0.096	0.112	0.106	0.167	0.287	0.124	0.449	0.083	0.898	0.022	0.246	0.218	0.943	-0.100	0.259	2.427	0.164	-3.353
rs229393	0.020	0.188	0.010	0.281	0.377	0.151	0.011	0.206	0.007	0.296	0.274	0.189	0.196	0.163	0.254	0.194	0.348	0.250	0.964	0.069	0.788	0.554	0.744	-1.058
rs4818	0.043	0.146	0.010	0.243	0.895	0.021	0.029	0.157	0.007	0.251	0.754	0.050	0.104	0.189	0.155	0.217	0.230	0.308	0.764	0.451	0.567	1.124	0.762	-1.010
rs4680	0.174	0.091	0.157	0.146	0.445	0.089	0.108	0.108	0.105	0.167	0.338	0.112	0.343	0.103	0.643	0.078	0.263	0.210	0.821	-0.319	0.362	1.976	0.156	-3.462
rs4646316	0.409	0.066	0.431	0.078	0.624	0.105	0.398	0.068	0.388	0.085	0.705	0.081	0.908	-0.015	0.923	0.015	0.607	-0.177	0.257	1.897	0.178	2.765	0.928	0.402
rs174696	0.128	-0.119	0.146	-0.138	0.402	-0.174	0.083	-0.136	0.098	-0.157	0.337	-0.199	0.540	-0.079	0.600	-0.080	0.638	-0.163	0.929	0.146	0.694	0.777	0.522	-2.824
rs9306235	0.633	-0.033	0.323	-0.094	0.623	0.069	0.720	-0.025	0.351	-0.089	0.511	0.094	0.472	-0.080	0.349	-0.144	0.938	-0.018	0.607	0.740	0.982	0.044	0.311	2.961
rs9332377	0.788	-0.037	0.983	-0.003	0.190	-0.944	0.839	-0.028	0.915	0.016	0.110	-1.154	0.361	-0.202	0.411	-0.191	0.481	-0.835	0.561	1.663	0.498	2.042	0.732	-5.270
rs165599	0.718	0.037	0.579	0.061	0.536	-0.259	0.582	0.056	0.465	0.080	0.608	-0.214	0.660	0.072	0.486	0.124	0.416	-0.537	0.586	-1.145	0.391	-1.961	0.319	8.526
rs887199	0.888	0.010	0.575	-0.053	0.215	0.196	0.716	0.026	0.726	-0.033	0.168	0.218	0.896	-0.015	0.585	-0.084	0.530	0.161	0.777	-0.431	0.586	-1.080	0.770	0.970
rs2518824	0.704	-0.031	0.558	-0.057	0.745	0.077	0.776	-0.024	0.585	-0.053	0.617	0.118	0.543	-0.081	0.421	-0.127	0.839	0.078	0.796	0.440	0.981	-0.050	0.429	3.837
rs887200	0.569	0.160	0.369	0.160	NA	NA	0.331	0.173	0.331	0.173	NA	NA	0.298	0.304	0.298	0.304	NA	NA	0.430	-2.965	0.430	-2.965	NA	NA
	0.883	-0.013	0.827	-0.022	0.894	0.038	0.903	-0.011	0.807	-0.025	0.776	0.080	0.888	-0.021	0.742	-0.055	0.634	0.224	0.616	-0.928	0.831	-0.451	0.312	-5.996

Table 7. Association between *COMT* haplotypes and experimental pain in patients undergoing breast cancer surgery (n=900). Cold pain intensity (NRS) was assessed during immersion of the hand to cold (2°C) water bath every 15s for 90s or until the withdrawal. Time of withdrawal (total time) has been used as an indicator of tolerance to cold pain. Variables have been adjusted for age, BMI, anxiety (STAI score) and presence of pre-operative chronic pain condition. Results are presented as empirical p-values based on linear regression analysis using additive, dominant and recessive models. Significant p-values are underlined, and borderline associations ($0.1 > p > 0.05$) are shown with **bolding**.

First SNP	Last SNP	Haplotype	Frequency	Cold pain intensity, 15s						Cold pain intensity, 30s						Cold pain intensity, 45s					
				Additive		Dominant		Recessive		Additive		Dominant		Recessive		Additive		Dominant		Recessive	
				P	β	P	β	P	β	P	β	P	β	P	β	P	β	P	β	P	β
rs6518591	rs5993882	AATAICT	0.490	0.526	0.088	0.022	0.502	0.182	-0.305	0.080	0.222	0.011	0.512	0.820	0.048	0.034	0.226	0.004	0.483	0.607	0.090
rs6518591	rs5993882	AGCGGCT	0.197	0.418	-0.138	0.372	-0.176	0.899	-0.064	0.104	-0.253	0.080	-0.317	0.724	-0.164	0.051	-0.254	0.061	-0.284	0.314	-0.393
rs6518591	rs5993882	GATGTTG	0.165	0.727	-0.062	0.862	0.036	0.124	-0.848	0.298	0.000	0.521	0.122	0.087	-0.864	0.802	-0.034	0.789	0.043	0.137	-0.630
rs6518591	rs5993882	AAATGTTT	0.112	0.801	-0.054	0.990	-0.003	0.261	-1.124	0.009	-0.247	0.361	-0.191	0.063	-1.704	0.182	-0.220	0.413	-0.144	0.008	-2.028
rs6518591	rs5993882	AATGTTTG	0.031	0.197	0.524	0.197	0.524	NA	NA	0.259	0.421	0.259	0.421	NA	NA	0.228	0.377	0.228	0.377	NA	NA
rs4646312	rs165774	TTACACG	0.281	0.809	-0.036	0.719	-0.068	0.294	0.033	0.796	0.035	0.715	0.064	0.948	-0.021	0.485	0.080	0.473	0.105	0.752	0.085
rs4646312	rs165774	TTACACA	0.241	0.130	-0.242	0.191	-0.251	0.254	-0.491	0.213	-0.183	0.320	-0.175	0.263	-0.442	0.079	-0.166	0.144	-0.216	0.739	-0.110
rs4646312	rs165774	CCGGGTG	0.182	0.065	0.321	0.146	0.294	0.070	0.948	0.024	0.359	0.054	0.357	0.070	0.868	0.142	0.069	0.283	0.150	0.579	0.000
rs4646312	rs165774	TCACGCG	0.140	0.435	-0.150	0.846	-0.042	0.034	-1.423	0.128	-0.268	0.442	-0.152	0.003	-1.800	0.194	-0.192	0.478	-0.118	0.020	-1.203
rs4646312	rs165774	CCGGCGG	0.128	0.427	0.166	0.630	0.107	0.119	1.473	0.993	0.002	0.770	-0.060	0.199	1.112	0.639	-0.075	0.416	-0.139	0.185	0.963
rs4646312	rs165774	TTACATG	0.019	0.967	0.021	0.967	0.021	NA	NA	0.716	0.169	0.216	0.169	0.619	NA	0.605	0.205	0.605	0.205	NA	NA
rs9332377	rs887200	CAGTT	0.668	0.818	0.034	0.690	-0.076	0.246	0.367	0.379	-0.118	0.252	-0.201	0.991	-0.003	0.308	-0.115	0.177	-0.199	0.981	0.006
rs9332377	rs887200	CGATC	0.141	0.504	-0.143	0.504	-0.143	NA	NA	0.092	-0.330	0.092	-0.330	NA	NA	0.120	-0.256	0.120	-0.256	NA	NA
rs9332377	rs887200	TGGTT	0.114	0.398	0.187	0.398	0.187	NA	NA	0.584	0.111	0.584	0.111	NA	NA	0.599	0.090	0.599	0.090	NA	NA
rs9332377	rs887200	CGATT	0.037	0.551	0.211	0.551	0.211	NA	NA	0.315	0.327	0.315	0.327	NA	NA	0.979	0.007	0.979	0.007	NA	NA
rs9332377	rs887200	CGAGC	0.024	0.491	-0.273	0.491	-0.273	NA	NA	0.176	-0.490	0.176	-0.490	NA	NA	0.194	-0.396	0.194	-0.396	NA	NA
rs9332377	rs887200	TGGGT	0.015	0.628	0.414	0.628	0.414	NA	NA	0.938	0.061	0.938	0.061	NA	NA	0.184	0.874	0.184	0.874	NA	NA
rs4633	rs4680	CGG (LPS)	0.311	0.037	0.307	0.138	0.282	0.033	0.708	0.064	0.249	0.319	0.174	0.015	0.742	0.186	0.149	0.488	0.101	0.075	0.455
rs4633	rs4680	TCA (APS)	0.541	0.136	-0.203	0.335	-0.201	0.133	-0.356	0.496	-0.085	0.905	-0.023	0.293	-0.228	0.733	-0.036	0.922	0.016	0.479	-0.129
rs4633	rs4680	CCG (HPS)	0.147	0.538	-0.117	0.987	-0.003	0.034	-1.425	0.150	-0.251	0.497	-0.132	0.003	-1.802	0.217	-0.180	0.523	-0.104	0.019	-1.205
rs6518591	rs5993882	AATAICT	0.490	0.093	0.155	0.008	0.387	0.990	0.002	0.130	-2.152	0.005	-6.252	0.698	0.911	0.622	0.055	0.659	0.078	0.722	0.065
rs6518591	rs5993882	AGCGGCT	0.197	0.124	-0.174	0.108	-0.211	0.646	-0.155	0.052	3.382	0.054	3.898	0.393	4.456	0.067	-0.250	0.044	-0.320	0.779	-0.115
rs6518591	rs5993882	GATGTTG	0.165	0.788	-0.032	0.762	0.042	0.102	-0.600	0.793	-0.481	0.391	-1.824	0.141	8.320	0.943	0.010	0.985	0.003	0.867	0.072
rs6518591	rs5993882	AAATGTTT	0.112	0.257	-0.162	0.471	-0.110	0.032	-1.425	0.251	2.526	0.373	2.088	0.147	14.92	0.839	0.035	0.555	0.110	0.109	-1.276
rs6518591	rs5993882	AATGTTTG	0.031	0.216	0.335	0.216	0.335	NA	NA	0.089	-7.089	0.089	-7.089	NA	NA	0.300	0.555	0.080	0.555	NA	NA
rs4646312	rs165774	TTACACG	0.281	0.536	0.061	0.491	0.037	0.856	0.042	0.412	-1.259	0.059	-1.045	0.351	-3.34	0.805	0.123	0.440	0.118	0.323	0.278
rs4646312	rs165774	TTACACA	0.241	0.138	-0.158	0.097	-0.212	0.793	-0.075	0.215	2.038	0.168	2.709	0.808	1.08	0.015	-0.314	0.003	-0.463	0.828	0.076
rs4646312	rs165774	CCGGGTG	0.182	0.031	0.250	0.062	0.252	0.092	0.587	0.299	-1.852	0.667	-0.895	0.044	-10.84	0.534	0.086	0.490	0.112	0.923	0.040
rs4646312	rs165774	TCACGCG	0.140	0.167	-0.177	0.464	-0.105	0.011	-1.140	0.912	2.433	0.493	1.516	0.029	14.99	0.486	0.108	0.183	0.231	0.089	-0.913
rs4646312	rs165774	CCGGCGG	0.128	0.987	-0.002	0.719	-0.053	0.145	0.918	0.212	-0.236	0.784	0.628	0.095	-16.17	0.928	0.015	0.886	-0.026	0.331	0.701
rs4646312	rs165774	TTACATG	0.019	0.959	-0.017	0.959	-0.017	NA	NA	0.245	-6.060	0.245	-6.060	NA	NA	0.925	-0.039	0.925	-0.039	NA	NA
rs9332377	rs887200	CAGTT	0.668	0.383	-0.085	0.211	-0.159	0.861	0.037	0.578	0.834	0.278	2.133	0.556	-1.917	0.324	0.116	0.760	0.047	0.102	0.416
rs9332377	rs887200	CGATC	0.141	0.126	-0.218	0.126	-0.218	NA	NA	0.073	3.933	0.073	3.933	NA	NA	0.728	0.060	0.728	0.060	NA	NA
rs9332377	rs887200	TGGTT	0.114	0.570	0.084	0.570	0.084	NA	NA	0.475	-1.625	0.475	-1.625	NA	NA	0.932	-0.015	0.932	-0.015	NA	NA
rs9332377	rs887200	CGATT	0.037	0.676	0.098	0.676	0.098	NA	NA	0.758	-1.121	0.758	-1.121	NA	NA	0.898	0.038	0.898	0.038	NA	NA
rs9332377	rs887200	CGAGC	0.024	0.193	-0.343	0.193	-0.343	NA	NA	0.680	1.676	0.680	1.676	NA	NA	0.236	0.376	0.236	0.376	NA	NA
rs9332377	rs887200	TGGGT	0.015	0.362	0.320	0.362	0.320	NA	NA	0.280	-9.484	0.280	-9.484	NA	NA	0.663	0.290	0.663	0.290	NA	NA
rs4633	rs4680	CGG (LPS)	0.311	0.079	0.172	0.318	0.127	0.026	0.493	0.376	-1.334	0.888	0.276	0.024	-7.670	0.564	0.068	0.736	0.052	0.476	0.187
rs4633	rs4680	TCA (APS)	0.541	0.495	-0.062	0.999	0.000	0.235	-0.188	0.953	-0.082	0.410	-1.758	0.403	2.033	0.262	-0.123	0.235	-0.201	0.547	-0.115
rs4633	rs4680	CCG (HPS)	0.147	0.187	-0.167	0.505	-0.094	0.011	-1.142	0.219	2.396	0.495	1.487	0.029	15.01	0.418	0.124	0.148	0.248	0.090	-0.912

Table 9. Predicted microRNA binding sites in *COMT* gene.

MicroRNA	Location	Length (bp)	Binding site
miR-217	22:19956991-19957012	22	GCCCATGAGTGAGGATGCAGTIG
miR-129-5p	22:19957397-19957418	22	CTGAGTGGCAGAAAAGCAAAAAG
miR-214	22:19957539-19957559	21	ACAGCAGGCAC7GAAAAGCAGT
miR-220c	22:19957665-19957685	21	GCACAGGGGCC7CACG7GACT
miR-220	22:19957781-19957802	22	CCACACCGTGG7CTGCG7GCGCT
miR-324-3p	22:19957874-19957893	20	CCTGCCCC7GGCGCCAGACC
miR-324-3p	22:19957992-19958011	20	CCTGCCCCAGCAGCCCCAG7C
miR-29a	22:19958046-19958069	24	CAGCACCATGTCA7G7AAAACAGCTA
miR-29b	22:19958046-19958070	25	CAGCACCATGTCA7G7AAAACAGCTAA
miR-29c	22:19958046-19958069	24	CAGCACCATGTCA7G7AAAACAGCTA
miR-767-5p	22:19958047-19958067	21	AGCACCATGTCA7G7AAAACAGC
miR-485-5p	22:19958249-19958742	542	AGAGGCTGGGCC7GGGCCCTGCAGAGGGAAAAGGGGATGCTGGACGCTGCAGGCCACTGAG TGGCACAGGACCCACG7GTAACTCAGGGCAGGGCAGCCAACTCCACAGGCTACATGGATGG AGATGTGGATAGAAAGCATCTGCCCTGGGTGGTGTGGCTGACCCAAAGGGCTGTGGCACCC AGGGCTGATCCTGACTTGTGTCCCGAAGGGCGTTGGAGGGTATGGTGGCCATCCCTA CTCTGGTCCCATTTGCTCAGGCCCTGATCCCA7GCTGGCTGGGACAGGGCTGTGGGCCC AGGGAGCACCC7CCCCTCTCTGACCTTGCCTGTCTTTCCGGGAATGGGCCACTGGGGCTG CTGGACTGGAGGCCAAA7GCTCTCGGGGAA7CGTGGCGGGAAGCAGAGCGTGCAGGCAG CGGAGACTAAACAAGAAGCCCTGGCCCA7AGGGCCAGAGGAA7CAGGTGGACCA7CAACCA7ATG AGAGAA7CGTACCAGG

bp, basepairs

APPENDIX 2: ORIGINAL PUBLICATIONS I-IV

- I Stress-induced analgesia and morphine responses are changed in catechol-*O*-methyltransferase-deficient male mice.
- II Inhibitors of catechol-*O*-methyltransferase sensitize mice to pain.
- III Nitecapone reduces development and symptoms of neuropathic pain after spinal nerve ligation in rats.
- IV Effect of *COMT*-gene variants on experimental and acute post-operative pain in humans.

