THE ROLE OF CATECHOL-O-METHYLTRANSFERASE (COMT) AND THE EFFECTS OF COMT INHIBITION IN BRAIN AND IN CARDIOVASCULAR AND RENAL PATHOPHYSIOLOGY

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Academic Dissertation
To be presented, with the permission of Medical Faculty of the University of Helsinki, in auditorium 3, Biomedicum Helsinki on the 7th of September, 2007, at 13:00

Helsinki 2007
To Anu

“If the difference between the two groups is so small that you’ll need a statistical test to prove its significance, then it might not be a very important difference.”

Julius Axelrod,
A Nobel Prize winner in Physiology or Medicine 1970
and the discoverer of COMT enzyme
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This thesis is based on the following original publications, referred to in the text by the Roman numerals I-IV, and some unpublished data:


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# Abbreviations

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<th>Description</th>
</tr>
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<tbody>
<tr>
<td>ACE</td>
<td>angiotensin converting enzyme</td>
</tr>
<tr>
<td>ADHD</td>
<td>attention deficit hyperactivity disorder</td>
</tr>
<tr>
<td>Ang</td>
<td>angiotensin</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AP-1</td>
<td>activator protein-1</td>
</tr>
<tr>
<td>BBB</td>
<td>blood-brain-barrier</td>
</tr>
<tr>
<td>b.i.d</td>
<td>&quot;bis in dei&quot;, twice a day</td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>mouse strain (control mice)</td>
</tr>
<tr>
<td>CD4</td>
<td>leukocyte membrane protein</td>
</tr>
<tr>
<td>CD8</td>
<td>leukocyte membrane protein</td>
</tr>
<tr>
<td>COMT</td>
<td>catechol-O-methyltransferase</td>
</tr>
<tr>
<td>COMT (-/-)</td>
<td>catechol-O-methyltransferase deficient mice</td>
</tr>
<tr>
<td>CpG</td>
<td>cytosine-phosphate-guanine sequence in the DNA, often associated with the start of the genes</td>
</tr>
<tr>
<td>DA</td>
<td>dopamine</td>
</tr>
<tr>
<td>DDC</td>
<td>dopa decarboxylase enzyme</td>
</tr>
<tr>
<td>DDCI</td>
<td>dopa decarboxylase enzyme inhibitor</td>
</tr>
<tr>
<td>DNAase I</td>
<td>deoxyribonuclease I</td>
</tr>
<tr>
<td>DA</td>
<td>dopamine</td>
</tr>
<tr>
<td>DAT</td>
<td>dopamine transporter</td>
</tr>
<tr>
<td>DOPAC</td>
<td>3,4-dihydroxyphenylacetic acid</td>
</tr>
<tr>
<td>dTGR</td>
<td>double-transgenic rat harbouring human renin and angiotensinogen genes</td>
</tr>
<tr>
<td>ECF</td>
<td>extra cellular fluid</td>
</tr>
<tr>
<td>ED1</td>
<td>monocyte and macrophage membrane protein</td>
</tr>
<tr>
<td>ED2</td>
<td>monocyte and macrophage membrane protein</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EK</td>
<td>entacapone</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial fibrillar acidic protein, immunohistochemical marker recognizing astroglial cells</td>
</tr>
<tr>
<td>GFR</td>
<td>glomerular filtration rate</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrogen chloride</td>
</tr>
<tr>
<td>H_{2}O_{2}</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>HVA</td>
<td>homovanillic acid</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>intracellular adhesion molecule-1</td>
</tr>
<tr>
<td>IL-1β</td>
<td>interleukin-1 beta, immunological mediator</td>
</tr>
<tr>
<td>K_{m}</td>
<td>substrate concentration that produces half-maximal reaction velocity</td>
</tr>
<tr>
<td>LAT-2</td>
<td>type 2 L-amino acid transporter</td>
</tr>
<tr>
<td>L-DOPA</td>
<td>levodopa, 3,4-dihydroxyphenyl alanine</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
</tbody>
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**Abbreviations**

<table>
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>MAO</td>
<td>monoamine oxidase</td>
</tr>
<tr>
<td>MAT</td>
<td>methionine adenosyltransferase</td>
</tr>
<tr>
<td>Met</td>
<td>methionine, amino acid</td>
</tr>
<tr>
<td>MPP⁺</td>
<td>1-methyl-4-phenylpyridinium</td>
</tr>
<tr>
<td>MB-COMT</td>
<td>membrane bound catechol-O-methyltransferase</td>
</tr>
<tr>
<td>3-MT</td>
<td>3-methoxytyramine</td>
</tr>
<tr>
<td>MTHFR</td>
<td>methylenetetrahydrofolate reductase</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NA</td>
<td>noradrenaline</td>
</tr>
<tr>
<td>NaCl</td>
<td>sodium chloride</td>
</tr>
<tr>
<td>Na⁺/H⁺-exchanger</td>
<td>sodium transporter protein</td>
</tr>
<tr>
<td>Na⁺/K⁺-ATPase</td>
<td>sodium transporter protein</td>
</tr>
<tr>
<td>NET</td>
<td>noradrenaline transporter</td>
</tr>
<tr>
<td>NFκB</td>
<td>nuclear factor kappa B, transcription factor</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>OCD</td>
<td>obsessive compulsive disorder</td>
</tr>
<tr>
<td>6-OHDA</td>
<td>6-hydroxydopamine</td>
</tr>
<tr>
<td>8-OH-DG</td>
<td>8-hydroxydeoxyguanosine</td>
</tr>
<tr>
<td>3-OMD</td>
<td>3-O-methyldopa</td>
</tr>
<tr>
<td>OX-42</td>
<td>antibody against membrane protein in activated microglia/macrophage</td>
</tr>
<tr>
<td>P-</td>
<td>plasma</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>PDE-I</td>
<td>alkaline phosphodiesterase I</td>
</tr>
<tr>
<td>PFC</td>
<td>prefrontal cortex</td>
</tr>
<tr>
<td>p22phox</td>
<td>a component of NADPH oxidase</td>
</tr>
<tr>
<td>PTC</td>
<td>proximal tubular cells</td>
</tr>
<tr>
<td>RAA</td>
<td>renin-aldosterone-angiotensin</td>
</tr>
<tr>
<td>RER</td>
<td>rough endoplasmic reticulum</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase-polymerase chain reaction</td>
</tr>
<tr>
<td>S-</td>
<td>serum</td>
</tr>
<tr>
<td>SAH</td>
<td>S-adenosyl-L-homocysteine</td>
</tr>
<tr>
<td>SAM</td>
<td>S-adenosyl-L-methionine</td>
</tr>
<tr>
<td>S-COMT</td>
<td>soluble catechol-O-methyltransferase</td>
</tr>
<tr>
<td>SD</td>
<td>Sprague-Dawley rat</td>
</tr>
<tr>
<td>SN</td>
<td>substantia nigra</td>
</tr>
<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor alpha, immunological mediator</td>
</tr>
<tr>
<td>U-</td>
<td>urine</td>
</tr>
<tr>
<td>Val</td>
<td>valine, amino acid</td>
</tr>
</tbody>
</table>
| Vₘₙₙₙₙₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓₓ𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱𝐱toJson literally
Abstract

1. ABSTRACT

Catechol-O-methyltransferase (COMT) metabolizes catecholamines such as dopamine (DA), noradrenaline (NA) and adrenaline, which are vital neurotransmitters and hormones that play important roles in the regulation of physiological processes. The aim of this project was to provide novel information on the physiological and especially pathophysiological roles of COMT enzyme as well as the effects of COMT inhibition in the brain and in the cardiovascular and renal system.

To assess the roles of COMT and COMT inhibition in pathophysiology, we used four different study designs. The possible beneficial effects of COMT inhibition were studied in double-transgenic rats (dTGRs) harbouring human angiotensinogen and renin genes. Due to angiotensin II (Ang II) overexpression, these animals exhibit severe hypertension, cardiovascular and renal end-organ damage and mortality of approximately 25-40% at the age of 7-weeks. The dTGRs and their Sprague-Dawley controls tissue samples were assessed with light microscopy, immunohistochemistry, reverse transcriptase-polymerase chain reaction (RT-PCR) and high-pressure liquid chromatography (HPLC) to evaluate the tissue damages and the possible protective effects pharmacological intervention with COMT inhibitors. In a second study, the consequence of genetic and pharmacological COMT blockade in blood pressure regulation during normal and high-sodium was elucidated using COMT-deficient mice. The blood pressure and the heart rate were measured using direct radiotelemetric blood pressure surveillance. In a third study, the effects of acute and subchronic COMT inhibition during combined levodopa (L-DOPA) + dopa decarboxylase inhibitor treatment in homocysteine formation was evaluated. Finally, we assessed the COMT enzyme expression, activity and cellular localization during CNS inflammation-induced neurodegeneration using Western blotting, HPLC and various enzymatic assays. The effects of pharmacological COMT inhibition on neurodegeneration were also studied.

The COMT inhibitor entacapone protected against the Ang II-induced perivascular inflammation, renal damage and cardiovascular mortality in dTGRs. COMT inhibitors reduced the albuminuria by 85% and prevented the cardiovascular mortality completely. Entacapone treatment was shown to ameliorate oxidative stress and inflammation. Furthermore, we established that the genetic and pharmacological COMT enzyme blockade protects against the blood pressure-elevating effects of high sodium intake in mice. These effects were mediated via enhanced renal dopaminergic tone and suggest an important role of COMT enzyme, especially in salt-sensitive hypertension. Entacapone also ameliorated the L-DOPA-induced hyperhomocysteinemia in rats. This is important, since decreased homocysteine levels may decrease the risk of cardiovascular diseases in Parkinson’s disease (PD) patients using L-DOPA. The LPS-induced inflammation and subsequent delayed dopaminergic neurodegeneration were accompanied by up-regulation of COMT expression and activity in microglial cells as well as in perivascular cells. Interestingly, similar perivascular up-
regulation of COMT expression in inflamed renal tissue was previously noted in dTGRs. These results suggest that inflammation reactions may up-regulate COMT expression. Furthermore, this increased glial and perivascular COMT activity in the central nervous system (CNS) may decrease the bioavailability of L-DOPA and be related to the motor fluctuation noted during L-DOPA therapy in PD patients.
2. INTRODUCTION

Cellular methylation is a fundamental intracellular event, since it is involved in the synthesis of phospholipids, nucleic acids and in the metabolism of catecholamine neurotransmitters. Methylation reactions are also thought to modify protein function serving as on-off switches [Clarke, 1993; Philips et al., 1993; Parish and Rando, 1994] and regulating gene expression [Razin and Cedar, 1991; Tate and Bird, 1993; Bestor, 1998; Mill et al., 2006]. Regulatory methylation reactions are fairly stable but reversible reactions.

Catechol-O-methyltransferase (COMT) enzyme is one of the enzymes responsible for methylation reactions and is found ubiquitously in mammals [Guldberg and Marsden, 1975]. COMT is considered to be a constitutively active enzyme [Männistö and Kaakkola, 1999]. The main physiological role of COMT is to inactivate catecholamines such as dopamine (DA) via O-methylation. COMT also metabolizes other catechols such as levodopa (L-DOPA) and catechol estrogens. Catecholamines are metabolized mainly via two enzymes: O-methylated by COMT and oxidized via monoamine oxidase (MAO). The general consensus is that in almost all aminergic systems, the oxidation of catecholamines is the predominant metabolic pathway of the two. The lesser importance of COMT is also supported by the fact that the COMT gene appears not to be fundamental for life. Studies with COMT gene-deficient mice have shown that the mice appear to be apparently healthy and fertile [Gogos et al., 1998], whereas MAO deficiency in mice is associated with severe neurodevelopmental abnormalities [Cases et al., 1995; Upton et al., 1999; Bou-Flores et al., 2000; Yang et al., 2001; Holschneider et al., 2002].

Even though the O-methylation of catechols via COMT was first described almost five decades ago [Axelrod et al., 1958], only in recent years has COMT enzyme drawn increasing attention of different research groups as a possible etiological factor of pathophysiological processes in humans. Several studies have suggested that the importance of COMT increases during pathological situations. Some studies have also established links between the altered COMT activity and the etiology of diseases such as various cancers and schizophrenia. In schizophrenia, both high cortical as well as low subcortical COMT activity have been suggested to be a predisposing etiological factors [De Lisi et al., 2002; Bray et al., 2003; Glatt et al., 2003; Palmatier et al., 2004; Keltner, 2005; Abdolmaleky et al., 2006; Gogos and Gerber, 2006; Gothelf et al., 2007; McIntosh et al., 2007], whereas the low-activity allele of the COMT gene has been associated with increased carcinogenesis [Lavigne et al., 1997; Thompson et al., 1998; Palmatier et al., 1999; Matsui et al., 2000; Yim et al., 2001; Comings et al., 2003; Sazci et al., 2004; Inoue et al., 2005; Tanaka et al., 2006, 2007].

COMT may also play a role in the etiology of cardiovascular diseases. One of the well-known risk factors for high blood pressure is excessive sodium intake. DA is an important regulator of renal sodium homeostasis and natriuresis, both of which regulate the systemic blood pressure [José et al., 1998a; Aperia, 2000; Carey, 2001a]. The role of COMT in the renal dopaminergic
system and renal functioning, however, is still more or less obscure. In addition, most methylation reactions, such as O-methylation via COMT, use S-adenosyl methionine (SAM) as a methyl donor. As a result the SAM is degraded via an intermediate to homocysteine, which is a known individual risk factor for cardiovascular diseases, such as coronary artery disease [McCully, 1969; Clarke et al., 1991; Boushey et al., 1995; Hankey and Eikelboom, 1999; Lentz, 2005], promoting endothelial dysfunction and vascular smooth muscle cell proliferation [Castro et al., 2006]. Interestingly, homocysteine was also suggested to promote neurotoxicity and play a role in the etiology of neurodegenerative diseases [Reutens and Sachdev, 2002; Müller et al., 2004a]. The effects of COMT inhibitors on homocysteine formation, however, had not been reported.

Although increasing numbers of studies reporting correlation between the etiology of diseases and COMT activity have been published, the pathophysiological mechanisms remain mostly unknown and only scattered knowledge of COMT enzyme biochemistry during pathological situations is available. The present study was designed to increase our level of understanding of the physiological and pathophysiological roles of COMT enzyme and COMT inhibition in the brain and in the cardiovascular and renal systems using experimental animal models. The following chapters review our current knowledge of the COMT enzyme as a biologically active molecule and form a basis for the understanding of the physiological and pathophysiological roles played by COMT in the body.
3. REVIEW OF THE LITERATURE

3.1 Catechol-O-methyltransferase (COMT; EC 2.1.1.6)

The catecholamines DA, noradrenaline (NA) and adrenaline are vital neurotransmitters/hormones that play a key role in the regulation of physiological processes and the development of neurological, psychiatric, endocrine and cardiovascular diseases. The two enzymes primarily responsible for catecholamine metabolism are COMT and MAO. All catecholamines have a common precursor molecule, L-DOPA, which is also metabolized via COMT (see Fig. 3). The COMT enzyme inactivates catechol group-containing substrates via O-methylation. There are two different COMT isoenzymes: membrane-bound (MB-COMT) and soluble (S-COMT). The O-methylation of catecholamines (see Figure 1) and other catechols by COMT was first described in 1958 by Axelrod and coworkers [Axelrod et al., 1958; Axelrod and Tomchick, 1958].

![Catechol structure](image)

**Figure 1.** The O-methylation of dopamine (DA) via COMT. The COMT enzyme catalyzes the methylation of the hydroxyl group (-OH) in the 3-position to form 3-methoxytyramine (3-MT).

Following research introduced the first COMT inhibitors like pyrogallol [reviewed by Guldberg and Marsden, 1975]. The second-generation COMT inhibitors were taken into clinical use in the 1990s as adjunct drug therapy in the treatment of advanced PD [Männistö and Kaakkola, 1989, 1990, 1999]. The COMT gene and both protein isoforms were later characterized following the cloning of their polypeptide cDNAs [Salminen et al., 1990; Bertocci et al., 1991; Lundström et al., 1991]. The crystalline structure of rat S-COMT was finally revealed in 1991 [Vidgren et al., 1991, 1994]. The amino acid sequences of rat and human S-COMT exhibited 81% homology, and most importantly the active site was highly conserved [Vidgren and Ovaska, 1997].
3.1.1 Structure and enzymatic reaction

The COMT enzyme has a single domain structure in which the central $\beta$-sheet is surrounded by eight $\alpha$-helices. The active site of the COMT enzyme is located in the shallow groove of the outer surface of the protein. It contains the SAM-binding domain and the actual catalytic site. The three-dimensional structure also revealed the correct binding sequence of the Mg$^{2+}$, SAM and substrate. The binding site of SAM was located deeper than the Mg$^{2+}$-binding site. Thus, it is now known that the SAM binds first, then the Mg$^{2+}$ and finally the substrate. Importantly from the scientific perspective, the studies also revealed that all the residues crucial for binding of the substrates to the active site were identical in rat and human DNA. Only two amino acids were different in the active site [Vidgren et al., 1991, 1994, 1999; Vidgren and Ovaska 1997; Vidgren, 1998; reviewed in Männistö and Kaakkola, 1999], which suggests that there is a highly similar substrate spectrum and O-methylation reaction in rodents and humans.

In the O-methylation reaction, the COMT enzyme transfers the methyl group to one of the hydroxyl groups in the catechol substrate. Both COMT isoforms, MB-COMT as well as S-COMT, favour 3-O-methylation over 4-O-methylation due to the more suitable positioning of the side chain in the substrate in comparison to the hydrophobic protein residues of the catalytic site of the enzyme [Männistö and Kaakkola, 1999]. The COMT enzyme catalyzes the transfer of a methyl group from the universal methyl donor SAM in the presence of Mg$^{2+}$, although some other divalent metal ions can also function as catalysts [Guldberg and Marsden, 1975] (Fig. 2). Binding of the Mg$^{2+}$ to COMT enables conversion of the hydroxyl groups of the catechol to more easily ionizable forms. The presence of Mg$^{2+}$ ions is obligatory for the O-methylation reaction to occur. The COMT enzyme is able to O-methylate only one of the two catechol hydroxyls. As previously highlighted, the methyl donor in the COMT-catalyzed O-methylation reactions is SAM, which is formed from the essential amino acid methionine. After intermediate phases, S-adenosyl homocysteine (SAH) and homocysteine, the SAM can be transformed back to methionine completing the so-called methionine cycle (See below Fig. 2).

The methylation reaction via COMT can thus leads to increased homocysteine formation. Interestingly, homocysteine has been associated with numerous pathophysiological events as well as various diseases (See 3.2.2.1.1 and 3.3.2.1.1).
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Figure 2. The methionine cycle. The essential amino acid methionine is metabolized by methionine adenosyltransferase (MAT) to S-adenosyl methionine (SAM) which in turn functions as a methyl (-CH₃) donor in O-methylation reactions catalyzed by catechol-O-methyltransferase (COMT). In the reaction, SAM is transformed to S-adenosyl homocysteine (SAH). Adenosylhomocysteinase then metabolizes the SAM to homocysteine, separating the adenosine molecule from the SAH. In the presence of vitamin B₁₂ (B₁₂-vit) and methylated tetrahydrofolate (N⁵-methyl THF), homocysteine can be transformed back to methionine to complete the cycle. (ATP, adenosine triphosphate).

3.1.2 Substrates of the COMT enzyme

As explained, the COMT enzyme catalyzes an O-methylation reaction in which the methyl group is transferred to one of the catechol hydroxyl groups of the substrate (Fig.1). In biochemistry, adding a methyl group to a reactive molecule often satisfies its appetite for reactivity and thus methylation usually equals inactivation. This is also the case with the O-methylation of catechols, which are transformed to less reactive and more excretable forms. In the case of catecholamines, O-methylation abolishes the biological affinity of the catecholamines towards their aminergic receptors.

The main substrates of COMT are the endogenous catechols L-DOPA, DA, NA, adrenaline, as well as hydroxylated catechol estrogens, which are the nicotinamide adenine dinucleotide phosphate (NADPH)-dependent oxidation metabolites of estrogens. The exogenous xenobiotics and substances such as triphenols, benzerazide, carbidopa, dobutamine, isoprenaline, rimiterol, α-methyldopa, dietary polyphenols and all other catechol group-containing substances are also substrates for COMT [Axelrod and Tomchick, 1958; Guldberg and Marsden, 1975; Maruyama et al., 1996; Männistö and Kaakkola, 1999; Lautala et al., 1999; Zhu et al., 2000, 2001; Sipilä and Taskinen, 2004]. Interestingly, ascorbic acid (vitamin C), which does not have a benzene ring or a catechol structure, but rather a five-carbon (pentagon) ring with two hydroxyl groups at the 3- and 4-positions, is also O-methylated via COMT in vitro as well as in vivo [Blaschke and Hertting, 1971; Bowers-Komro et al., 1982]. Furthermore, millimolar concentrations of ascorbic acid inhibit COMT activity significantly in the spinal meninges in vitro [Kern and Bernards, 1997].
3.1.3 Distribution of COMT

3.1.3.1 Distribution of COMT at the tissue level

In mammals, the COMT enzyme is distributed ubiquitously in the body and has been located in most of the tissues investigated, e.g. brain, mesenteric organs, skin, lungs, adrenal glands, spleen, pancreas, uterus, ovaries, vasculature, erythrocytes, eye, as well as spinal meninges. The highest concentrations are found in the liver, kidneys and gastrointestinal tract [Guldberg and Marsden, 1975; Nissinen et al., 1988a; Schultz and Nissinen, 1989; Männistö and Kaakkola, 1999]. The S-COMT isoenzyme is usually the predominant isoform in the tissues, with the exception of brain parenchyma where MB-COMT is expressed more abundantly [Tenhunen and Ulmanen, 1993; Tenhunen et al., 1993, 1994].

3.1.3.2 Distribution of COMT at the cellular level

In the brain, COMT-immunoreactivity (-ir) is localized in the astrocytic cytoplasm [Karhunen et al., 1995], ependymal linings of the ventricles, astrocyte end feet around the capillary walls, capillary endothelium [Kaplan et al., 1979, 1981; Karhunen et al., 1994, 1995], postsynaptic dendritic spines [Kastner et al., 1994; Karhunen et al., 1995], and in developing microglial cells in neonatal rats [Shirakawa et al., 2004]. The presynaptic dopaminergic neurons exhibit no significant COMT activity [Rivett et al., 1983, Kaakkola et al., 1987; Karhunen et al., 1995]. Interestingly, higher COMT mRNA levels were noted in striatal and cortical neurons than in glial cells in a recent study [Matsumoto et al., 2003a]. However, this most likely only supports those previous findings showing that COMT mRNA levels correlate poorly with actual COMT protein levels [Tenhunen et al., 1994] and that COMT gene expression also appears to be regulated at the translational level [Lundström et al., 1995]. This hypothesis is further supported by a recent study showing differential changes in COMT mRNAs and corresponding protein expression in rat brain after treatment with the anti-depressant fluoxetine for 4 weeks [Fatemi and Folsom, 2007].

The COMT enzyme is also expressed in a wide variety of peripheral cells [Männistö and Kaakkola, 1999]. As in the aminergic neurons in the central nervous system (CNS), the peripheral sympathetic neurons apparently lack COMT activity [Guldberg and Marsden, 1975; Rang and Dale’s Pharmacology, 6th ed. 2007], although some controversy exists on this issue [Guldberg and Marsden, 1975]. In the renal tissue, the highest levels of COMT expression and activity are located in the epithelial proximal tubular cells (PTCs) where the DA is synthesized. Renal COMT expression was also found in the cells of the thick ascending part of the loop of Henle, in collecting ducts, urethral cells, renal capillary endothelium and in mesangial cells [Meister et al., 1993; Karhunen et al., 1994; Weisz et al., 1998; Zacharia et al., 2002; Dubey et al., 2003].
Interestingly, it has been suggested that pathological situations might alter the distribution of COMT at the cellular level. There is a preliminary finding of a small acute increase in COMT-ir and activity in activated microglial cells after local injection of glial toxin [Reenilä et al., 1997].

### 3.1.3.3 Distribution of COMT at the subcellular level

COMT is an intracellular enzyme that is found in all subcellular compartments [Ulmanen et al., 1997; Weisz et al., 1998; Männistö and Kaakkola, 1999]. No COMT activity has ever been reported in the outer plasma membrane, thus substrates have to be transported into the cells prior to methylation [Guldberg and Marsden, 1975; Männistö et al., 1992b; Ulmanen et al., 1997]. As explained earlier, COMT enzyme exists in two different isoforms, MB-COMT and S-COMT. The two isoenzymes are located in different subcellular compartments. This dissimilar subcellular localization of COMT isoenzymes is associated with the anchor region of MB-COMT [Jeffrey and Roth, 1984; Roth, 1992] that attaches MB-COMT to the rough endoplasmic reticulum (RER). Due to the lack of isoenzymes-specific antibodies, understanding of this differing subcellular distribution of MB-COMT and S-COMT has been obtained with centrifugation and following Western blotting studies.

S-COMT activity resides primarily in the nonsedimenting cytoplasmic fractions, whereas MB-COMT activity was found in the microsomal fraction [Jeffrey and Roth, 1984; Roth, 1992]. The MB-COMT is located in the RER and nucleus, whereas S-COMT is localized normally only in the cytosolic fraction [Tilgmann et al., 1992]. However, studies with cells expressing recombinant S-COMT also showed about 5% expression in the nucleus in kidney, liver, and brain homogenates [Ulmanen et al., 1997; Weisz et al., 1998].

Some situations appear to alter the subcellular localization of COMT. Interestingly, estrogens induce nuclear S-COMT in hamster kidney cells and increased nuclear S-COMT was also noted in human breast cancer tissue [Ulmanen et al., 1997; Weisz et al., 1998; 2000]. It was speculated that part of the up-regulated cytosolic S-COMT may be transported into the nucleus to protect the DNA from possible oxidative damage induced by hydroxylated catechol estrogens, the oxidative metabolites of estrogens [Zhu and Conney, 1998; Weisz et al., 1998, 2000; Hirose et al., 2007].

### 3.1.4 COMT isoenzymes

#### 3.1.4.1 One gene, two isoenzymes

The COMT gene in humans is located in chromosome 22, band 11q2. One characteristic of COMT genetics is that there is only one gene from which both longer (MB-COMT) and shorter (S-COMT) mRNA sequences are transcribed. The longer mRNA sequence of MB-COMT also contains the shorter mRNA
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sequence of S-COMT [Salminen et al., 1990; Lundström et al., 1991; Grossman et al., 1992; Winqvist et al., 1992; Tenhunen and Ulmanen, 1993; Tenhunen et al., 1993]. In humans, both transcripts are expressed in most tissues, with the exception of the brain, in which only the longer transcript was found in 16 regions studied [Hong et al., 1998; Matsumoto et al., 2003a]. Both rat and human S-COMT genes contain 221 amino acids, and their molecular masses are 24.8 kDa and 24.4 kDa, respectively. The amino acid sequences have 81% correspondences. The MB-COMT isoenzymes contain an additional 43 (rat) and 50 (human) amino acids. The corresponding molecular masses of the MB-COMT isoenzyme are 29.6 kDa and 30.0 kDa, respectively.

Expression of the COMT gene is controlled by two different promoters located within the COMT gene sequence [Salminen et al., 1990; Lundström et al., 1991]. The P1 promoter is located between the MB-COMT- and S-COMT-starting ATG codons and is thus capable of coding only for the S-COMT polypeptide. The regulation of P1 differs among tissues. The distal P2 promoter, however, was suggested to function constitutively and is expressed in all tissues. P2 can code for both isoforms by using the leaky scanning mechanism of translation initiation [Tenhunen and Ulmanen 1993; Tenhunen et al., 1993, 1994; Tenhunen, 1996].

The computer-based analysis of the promoter area of COMT has revealed the binding sites and hormone-response elements for estrogens and glucocorticoids, as well as multiple binding sites for transcription factor nuclear factor kappa B (NFkB) [Xie et al., 1999; Salama et al., 2006; Wentz et al., 2006]. Methylation of the phosphodiester-linked cytosine and guanine (CpG) islands within the promoter regions inhibits the binding of transcription factors, thereby inhibiting gene transcription [Iguchi-Ariga and Schaffner 1989; Klangby et al. 1998; Tate and Bird 1993]. Interestingly, there are also recent findings that the methylation status of the COMT gene promoter area could be altered in some diseases. The CpG islands in the promoter area of MB-COMT are methylated in endometrial cancer [Sasaki et al., 2003], whereas in schizophrenic and bipolar subjects, the promoter areas of MB-COMT are hypomethylated [Abdolmaleky et al., 2006].

3.1.4.2 MB-COMT and S-COMT

COMT is an intracellular enzyme that exists in membrane-bound and soluble forms. However, no logical explanation or physiological need for two COMT isoenzymes has ever been established. Both isoenzymes are biologically active and take part in cellular O-methylation. No difference in substrate spectrum has ever been reported. The expression of the isoenzymes varies among the tissues. The expression ratios of the two COMT isoforms in different tissues were obtained in Western blotting experiments. As explained earlier, S-COMT is the predominant isoenzyme in most tissues by a factor of 3 or higher, with the exception of the human brain in which the ratio between MB-COMT and S-COMT is 7 to 3 [Tenhunen et al., 1993, 1994].
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Although, there is substantially less MB-COMT in the majority of the tissues and organs, it has higher affinity (Km) for most of the substrates [Roth, 1992]. MB-COMT functions at lowμM concentrations in which the substrates normally exist in tissues, whereas S-COMT often requires 2-100 times higher substrate concentrations [Rivett et al., 1982; Rivett and Roth, 1982; Nissinen, 1984; Roth, 1992]. This kinetic difference favors O-methylation via MB-COMT and for this reason it has been suggested to be the physiologically more important of the two. S-COMT, on the other hand, has a much higher capacity (Vmax) which in some tissues is thousands-fold higher than the Vmax of MB-COMT. Due to the higher Vmax of S-COMT it was suggested to have increased importance during nonphysiological situations when higher O-methylation capacity is needed and in situations when substrate concentrations suddenly increased, such as during L-DOPA treatment [Roth, 1992; Huotari et al., 2002a].

Studies showing increased COMT expression and activity in cancerous tissue [Weisz et al., 1998, 2000; Tenhunen et al., 1999; Sasaki et al., 2003] and after neuronal injury [Rivett et al., 1983; Kaakkola et al., 1987; Reenilä et al., 1997] have provided evidence for the dissimilar behavior of these two isoenzymes in pathological situations. These studies have also suggested that the COMT enzyme may have increased importance during some pathological situations. However, the importance and the distinct roles of the isoenzymes in pathologic situations are still to be defined.

3.1.4.3 Methylation reaction kinetics of COMT isoenzymes

The COMT isoenzymes MB-COMT and S-COMT have major differences from the kinetic point of view. The two isoenzymes display physiologically significant differences in Km and Vmax which were studied in detail previously [Guldberg and Marsden, 1975; Rivett et al., 1982; Rivett and Roth, 1982; Malherbe et al., 1992; Roth, 1992; Lotta et al., 1995; Bonifati and Meco, 1998; Bonifacio et al., 2000; Sipilä and Taskinen, 2004]. The Km value of MB-COMT is significantly lower for most, if not all, substrates than that of S-COMT, meaning that MB-COMT is able to methylate catechol substrates with lower substrate concentrations compare to S-COMT [Malherbe et al., 1992; Lotta et al., 1995; Bonifacio et al., 2000; Sipilä and Taskinen, 2004]. The Km values of the isoenzymes vary widely among the substrates. In general, S-COMT has approximately 15-fold higher Km value for most substrates than MB-COMT [Malherbe et al., 1992; Lotta et al., 1995]. The Km value of S-COMT for L-DOPA, however, is only about 2.5 times higher [Lotta et al., 1995].

To define the physiological importance of the two isoenzymes it is important to note the physiological concentration at which the main substrates appear in the tissues. MB-COMT is the predominant isoenzyme at DA concentrations of <10 μM and at NA concentrations of < 300 μM, which are in the range of normal physiological concentrations of these catecholamines [Rivett et al., 1982; Rivett and Roth, 1982; Roth, 1992]. Thus, at normal physiological concentrations of DA and NA, MB-COMT is the more important isoenzyme of the two [Roth, 1992]. The Vmax of S-COMT, on the other hand, is much higher.
than that of MB-COMT. The MB-COMT $V_{\text{max}}$ for DA is 2-40 pmol/min per mg protein [Guldberg and Marsden, 1975; Roth, 1992], whereas the $V_{\text{max}}$ for the DA of S-COMT ranges from 50 pmol/min x mg protein in skeletal muscle to as high as 14,690 pmol/min in the liver [Guldberg and Marsden, 1975; Roth, 1992]. The physiological importance of this has been proposed to increase the importance of S-COMT in inactivation of xenobiotics and exogenous catechols such as L-DOPA. For example, during oral L-DOPA treatment in PD, the S-COMT is probably the predominant enzyme, due to its significantly higher $V_{\text{max}}$ and fairly small $K_m$ difference between the isoenzymes [Roth, 1992; Lotta et al., 1995; Bonifati and Meco, 1998).

3.1.5 COMT expression and activity

3.1.5.1 Regulation of COMT expression

The general consensus is that the COMT enzyme is neither easily induced nor suppressed [reviewed by Männistö and Kaakkola, 1999]. Prior to the thorough review article by Männistö and Kaakkola (1999), only a few studies had been published showing changes in COMT activity. However, the activity had only been shown to double at best [Guldberg and Marsden, 1975; Torda and Kvotenansky, 1983; Baldessarini, 1987; Lam, 1988; Li et al., 1991; Weisz et al., 1998]. Since then, many research groups have published results suggesting that COMT may in fact be more easily up-regulated or down-regulated than previously believed [Tenhunen et al., 1999; Xie et al., 1999; Zhao et al., 2001; Fujiwara et al., 2003; Jiang et al., 2003; Lee et al., 2004; Singh et al., 2005; Ogobure et al., 2006; Salama et al., 2006; Chen and Chen, 2007; Fatem and Folsom, 2007]. This regulation seems to occur at the promoter lever. Analysis of the COMT gene promoter area also suggested that it contained multiple half-sites of the estrogen-response element, a half-site for the glucocorticoid-response element, and multiple sites for NFκB [Xie et al., 1999; Salama et al., 2006; Wentz et al., 2006]. For example, estrogen down-regulates and progesterone up-regulates the COMT expression [Xie et al., 1999; Jiang et al., 2003; Salama et al., 2006]. Furthermore, COMT gene expression is under epigenetic regulation via gene promoter area methylation [Sasai et al., 2003; Abdelmalek et al., 2006; Mill et al., 2006]. Most importantly, these changes in gene and protein expression regulation seem to be associated with various pathological conditions.

The methylation status of gene promoter areas is an important mechanism affecting the expression of genes. This type of gene expression regulation is called epigenetic gene regulation. The term epigenetic refers to the study of changes in gene expression that do not involve changes in the genetic code. Two molecular switches – methylation and acetylation – have received particularly scrutiny [Bestor, 1998]. Since methylation usually equals inactivation of the substrate, methylation of the promoter area in DNA similarly decreases the transcription of the gene in question. Hypomethylation as well as acetylation in turn activates genes. One very interesting finding is that the methylation status of COMT isoenzymes promoter areas is altered during
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oncogenesis as well as in schizophrenia in humans [Sasaki et al., 2003; Abdolmaleky et al., 2006].

The MB-COMT promoter area (P2) is hypomethylated in patients with schizophrenia and bipolar disease in contrast to controls [Abdolmaleky et al., 2006]. On the other hand, the methylation status of the S-COMT promoter area (P1) did not correlate with schizophrenia [Murphy et al., 2005]. Interestingly, hypomethylation was also strongly associated with heavy alcohol use [Abdolmaleky et al., 2006], which is known to disrupt the methionine cycle and promote its toxic effects partly via epigenetic gene regulation [Loenen, 2006; Shukla and Aroor, 2006]. Alcohol abuse was also previously associated with schizophrenia and severity of bipolar disease [Regier et al., 1990; Cuffel, 1992; Goldstein et al., 2006]. There is also a curious finding that very high concentrations of ethanol inhibit recombinant MB-COMT activity but increase recombinant S-COMT activity in vitro [Reenilä et al., 1995].

The changes in COMT expression during carcinogenesis have also been reported. Tenhunen and co-workers (1999) studied the COMT isoenzyme protein expression in paired malignant and nonneoplastic human breasts. Both MB-COMT and S-COMT isoenzymes were increased in malignant tissue by 400% and 170%, respectively [Tenhunen et al., 1999]. A recent study by Sasaki and coworkers revealed that in endometrial cancer the MB-COMT promoter area was hypermethylated, suggesting down-regulated MB-COMT transcription [Sasaki et al., 2003]. The epigenetic regulation of COMT transcription [Mill et al., 2006] may thus have clinically important pathophysiological and physiological consequences.

3.1.5.2 Regulation of COMT activity

The most important factors affecting the tissue COMT activity are related to COMT expression and were considered in the previous chapter (see 3.1.5.1). The effects of tissue COMT isoenzyme ratios and tissue substrate concentration on COMT activity were also explained earlier (see 3.1.4.3). The genetic COMT polymorphism affects the general COMT activity and will be discussed in the following chapter (see 3.1.5.3). In the present chapter, the other factors known to alter COMT activity are reviewed.

There are also well-established gender-, age- and race-related differences in COMT activity [Guldberg and Marsden, 1975, Fahndrich et al., 1980; Philippu et al., 1981; Puzynski et al., 1983; Boudikova et al., 1990; Chen et al., 2004]. COMT activity in the liver of male subjects has been reported to be about 30% higher than that in females in humans [Boudikova et al., 1990]. COMT may play a more important overall role in catecholamine metabolism in males than in females. For example, COMT deficiency in mice increases DA concentrations in a sexually dimorphic manner. Male COMT (-/-) mice showed 3-4-fold higher DA levels in the prefrontal cortex (PFC), while female mice did not differ from their genetic wild-type littermates [Gogos et al., 1998]. These gender differences could be related to the suppressing effects of estrogens.
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towards COMT gene expression [Xie et al., 1999; Wentz et al., 2006; Salama et al., 2006]. During aging, the COMT activity in the liver increases by approximately 10-fold from birth to adulthood in humans [Guldberg and Marsden, 1975]. Continuous increase in COMT expression and activity from neonate to adulthood in humans was also established in the PFC [Tunbridge et al., 2007]. The functional COMT polymorphism (See 3.1.5.3) also affects the subjects COMT activity and forms the basis for the ethnic differences noted in COMT activity. In Caucasian subjects, the frequency of the low-activity Met allele is approximately twice as common as in East Asian populations and 2-10 times more prevalent than in Africans [Klemetsdal et al., 1994; McLeod et al., 1994, 1998; Palmatier et al., 1999; Ameyav et al., 2000]. Thus, Africans appear to have the highest and Caucasians the lowest general COMT activity of all the races.

3.1.5.3 Consequences of functional COMT polymorphism

There is a well-documented functional COMT G472A gene polymorphism (Val108/158/Met) in humans, that also affects the general COMT activity in the tissues [Scanlon et al., 1979; Boudikova et al., 1990; Lachmann et al., 1996; Weinshilboum et al., 1999; Shield et al., 2004; Chen et al., 2004]. The single nucleotide polymorphism that changes the amino acid at codon 158 in MB-COMT (108 in S-COMT) from valine (Val) to methionine (Met) is associated with lower tissue COMT activity in humans [Weinshilboum and Raymond, 1977; Scanlon et al., 1979; Boudikova et al., 1990; Lachmann et al., 1996; Chen et al., 2004]. Subjects homozygous for the Met allele have up to 4-times lower peripheral and 28% lower central COMT activity. It has belived that the polymorphism does not affect the mRNA or protein levels of the COMT isoenzymes [Scanlon et al., 1979; Boudikova et al., 1990; Lotta et al., 1995; Matsumoto et al., 2003b; Chen et al., 2004]. The difference in activity between these genotypes has been suggested to be associated with the thermolability of the COMT protein in Met allele subjects, which has been noted even at normal physiological temperatures [Scanlon et al., 1979; Boudikova et al., 1990; Lotta et al., 1995; Matsumoto et al., 2003b; Chen et al., 2004]. Studies with recombinant S-COMT revealed that the enzymatic activities of the two polymorphic genotypes do not differ [Lotta et al., 1995; Goodman et al., 2002]. However, a recent study also showed that the low-activity Met allele subjects had in fact 4-times less COMT protein in the liver tissue than Val allele subjects [Sullivan Doyle et al., 2004]. The lower tissue COMT protein concentration in Met allele subjects is also supported by immunohistochemical studies showing that the COMT immunoreactivity in Met/Met human tissue samples was approximately 54% lower for S-COMT [Chen et al., 2004]. The difference between MB-COMT immunoreactivity was shown to be smaller but also significant [Chen et al., 2004]. Thus, the lower activity of the Met allele subjects could be due to lower protein concentrations in tissues due to lesser transcription or to increased degradation and turnover rate of the protein. Previous studies have suggested that the mRNA expressions do not differ [Matsumoto et al., 2003b]. Thus, the thermolability seems to lead to shorter half-life and increased turnover rate. The proteolytic
systems of the body may contribute to the lower protein concentration by degrading the thermolabile unfolded protein.

There are also known differences in COMT polymorphism based on ethnic background. In Caucasian subjects, the frequency of the low-activity Met allele is approximately 50%, whereas in East Asians the frequency is 20-30% and with Africans even lower, e.g. 6% in Ghana. This is a broad generalization but the low-activity allele seems to be more common in ethnic groups originating closer to polar areas, whereas the high-activity Val allele is most common near the equator. [Klemetsdal et al., 1994; McLeod et al., 1994, 1998; Palmatier et al., 1999; Ameyav et al., 2000]. The ethnic differences in the COMT polymorphism are also interesting since the COMT (Val108/158/Met) polymorphism has recently been associated with several psychiatric diseases and various cancers, some of which also show some correlation in prevalence based on ethnic background [Karayiorgou et al., 1997; Mynett-Johnson et al., 1998; Inoue et al., 2005; Sazci et al., 2004; Mitrunen et al., 2001; Al-Hendy and Salama, 2006; Mikolajczyk et al., 2006; Glatt et al., 2003; Stein et al., 2006; Tanaka et al., 2006, 2007].

3.2 COMT in the CNS

The following chapters review the physiological and pathophysiological roles of COMT in the CNS. As explained earlier, the main function of the COMT enzyme is to inactivate the biologically active and vital catecholamines and other catechols in the body. To understand the physiological role of COMT we first must briefly overview the physiological role and metabolism of catecholamines in the CNS. This review focuses on the role of COMT in dopaminergic systems.

3.2.1 Physiological role of COMT in the CNS

The main endogenous substrates of COMT are the catecholamines (DA, NA and adrenaline) that take part in numerous vital physiological processes in the body. COMT enzyme plays a major role in the metabolism of these catecholamines (see Fig. 3).

In the CNS, DA functions as a neurotransmitter in dopaminergic pathways such as the motoric nigrostriatal, mesocortical, mesolimbic and tuberoinfundibular pathways. The dysfunction of these pathways may lead to diseases such as PD and schizophrenia. The dysfunctions of the major noradrenergic pathways are, in contrast, related to mood disorders.
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3.2.1.1 COMT and dopaminergic neurotransmission

When functioning as a neurotransmitter in the brain parenchyma, the major removal pathway of DA from the synaptic cleft is the neuronal reuptake (uptake1) mechanism via dopamine transporter (DAT) and to some extent also via other monoamine transporters such as noradrenaline transporter (NET). Following neuronal reuptake, the neurotransmitters are mostly recycled and transported through vesicular monoamine transporter-2 (VMAT2) into the
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intracellular vesicles. Only a small portion of neurotransmitters are metabolized via intraneuronal enzymes such as MAO [Kopin, 1985; Cass and Gerhardt, 1995; Morón et al., 2002; Cooper et al., 2003; Eisenhofer et al., 2004]. This vesicular storage system, however, is a dynamic equilibrium rather than a static system, as it is often misleadingly described. This misconception is that the vesicular stores of catecholamines exist in a static state until a stimulus evokes release into the extracellular space [Eisenhofer et al., 2004]. In fact, the vesicular stores of catecholamines exist in a highly dynamic equilibrium with catecholamines in the surrounding cytoplasm. The avid and rapid active transport of catecholamines from the cytoplasm into the vesicles, mediated by VMATs, counterbalances the passive outward leakage of the catecholamines from the vesicles. Although only a small fraction of the catecholamines in the cytoplasm escapes the vesicular sequestration, that fraction represents a major source of catecholamine metabolites [Eisenhofer et al., 2004]. The small portion of neurotransmitters catabolized after neuronal reuptake are primarily oxidized via MAO and the extraneuronal O-methylation pathway via COMT is usually the secondary metabolism route [Eisenhofer et al., 2004]. Thus, COMT has a more important role in the brain areas where the neuronal reuptake is not as predominant e.g. in PFC.

COMT was estimated to account for approximately 15% of the DA metabolism in the brain [Karoum et al., 1994]. Studies with COMT gene-deleted mice, which were introduced by Gogos and co-workers in 1998, have provided additional information on the role of COMT in catecholamine metabolism in the CNS. Studies with this mouse strain have confirmed that COMT is not crucially important in DA metabolism in the brain during normal conditions, but the importance is increased if the DA levels are for some reason increased [Gogos et al., 1998; Huotari et al., 2002a]. The PFC differs from the other brain areas from the perspective of catecholamine metabolism. In the PFC, COMT plays a more important role in DA metabolism. Instead of the 15%, O-methylation accounts for approximately 60% of the DA metabolism in the PFC [Karoum et al., 1994]. The predominant role of COMT in PFC DA metabolism may be related to the lesser expression of DAT in the PFC and subsequent decreased capacity of uptake1 in the PFC [Cass and Gerhardt, 1995; Sesack et al., 1998; Morón et al., 2002]. Since the DAT does not remove DA from the synaptic cleft as much in the PFC, the DA may diffuse further in brain parenchyma into areas where the glial uptake (uptake2) mechanism could remove it from the extracellular space. This hypothesis is also supported by a study showing that the inhibition of DAT by GBR 12909 did not influence the DA levels significantly in the cortical tissue [Huotari et al., 2002b].

COMT activity was suggested to increase acutely in activated microglial cells [Reenilä et al., 1997]. The upregulation of COMT in the glial cells, for any reason, might thus affect the DA neurotransmission especially in PFC.

3.2.1.2 COMT and the blood-brain-barrier (BBB)

In addition to playing an important role in inactivation of endogenous catecholamine neurotransmitters in the CNS, the COMT enzyme also
functions as an enzymological barrier in the cells forming the BBB [Lai and Spector, 1978]. In the BBB, COMT protects the neurons and the brain parenchyma from possibly harmful and biologically active peripheral catechol compounds and maintains, together with other metabolizing enzymes, the vital homeostatic difference between the main circulation and the brain parenchyma [Kaplan et al., 1979, 1981; Bradbury, 1985].

Some of the catechols like catecholamines do not penetrate through BBB because of their biochemical and physiological characteristics such as polarity and size. L-DOPA, on the other hand, even though being water soluble like catecholamines penetrates through BBB by using active amino acid transporters. For this reason, the L-DOPA is used as a prodrug to enhance DA synthesis in PD patients. Although the existence of this enzymological barrier is vital, from the perspective of L-DOPA medication it is also somewhat problematic due to low bioavailability of L-DOPA. Consequently, any changes in this enzymatic barrier activity might obviously have effects on the efficacy of L-DOPA treatment and could thus have even clinical relevance from the view point of L-DOPA treatment and PD.

3.2.2 Pathophysiological role of COMT in the CNS

The following chapters review the role and changes in COMT enzyme activity from the perspective of the CNS and neuropsychiatric pathophysiology. The focus is on the pathophysiology of dopaminergic systems.

3.2.2.1 Neuropathophysiology and COMT

Altered COMT activity has been noted in a few neurological diseases. COMT activity is decreased in the spinal cord of the Huntington’s disease patients [McGeer et al., 1993]. In contrast, slightly increased COMT activities were reported in Amyotrophic lateral sclerosis [Ekblom et al., 1993]. Of all the neurological diseases, PD is most often linked to the COMT enzyme due to the wide use of COMT inhibitors as adjunct medication in the symptomatic treatment of PD [Kaakkola, 2000; Gordin et al., 2003]. To the best of my knowledge, possible changes in COMT activity have never been studied in Parkinsonian patient post mortem brain samples. Some reports on the relationship between the COMT polymorphism and PD in Japanese population has been published [Kunugi et al., 1997; Yoritaka et al., 1997]. However, the general consensus is that no association between the COMT polymorphism and the etiology of PD exists [Hoda et al., 1996; Syvänen et al., 1997; Xie et al., 1997].

The inhibition of COMT activity has also been suggested to have neurodegenerative effects [Offen et al., 2001; Ogburn et al., 2006]. The pharmacological function of COMT inhibitors in PD treatment is to prevent O-methylation of L-DOPA to 3-OMD prior to uptake of L-DOPA to dopaminergic neurons. COMT inhibition prolongs the on-time and increases the
bioavailability of L-DOPA in PD patients [Kaakkola, 2000; Gordin et al., 2003]. Adjunct COMT inhibition also stabilizes the plasma L-DOPA levels in PD patients during L-DOPA medication [Müller et al., 2006]. Although L-DOPA is a most effective medication in the symptomatic treatment of PD, it may also have harmful and even cytotoxic effects in the CNS. It has been speculated that L-DOPA may increase the formation of reactive oxygen species (ROS) and increase oxidative damage in dopaminergic neurons may hasten dopaminergic neurodegeneration in PD patients. In addition to L-DOPA, catecholamines such as DA may also contribute to neurodegeneration via auto-oxidation [Stokes et al., 1999; Werner et al., 2001; Asanuma et al., 2004; Ogawa et al., 2005; Miyazaki et al., 2006]. According to this theory, O-methylation via COMT decreases the catechol auto-oxidation. COMT inhibition may thus increase oxidative cellular damage and promote neurodegeneration [Offen et al., 2001; Ogburn et al., 2006]. Most of the studies supporting the neurotoxicity of L-DOPA were performed in vitro, whereas only a few, if any, in vivo animal studies or clinical trials with PD patients have supported the hypothesis of the neurotoxicity of L-DOPA [reviewed in Müller et al., 2004b]. The situation is similar with other catecholamines. Most studies supporting the auto-oxidation theory are in vitro reports, whereas in vivo animal studies with the COMT-deficient mice and cynomologous monkeys have not reported increased oxidative stress, cytotoxicity or any other detrimental effects in the CNS following pharmacological or genetic blockade of COMT [Lyras et al., 2002; Forsberg et al., 2004]. This difference between the in vitro and in vivo studies could be explained by the simple fact that the DA auto-oxidation would hypothetically occur in the cells where the DA resides, that is, in dopaminergic cells where no significant COMT activity/expression has ever been noted [Rivett et al., 1983; Kaakkola et al., 1987; Karhunen et al., 1995]. The cellular distribution of COMT could thus easily explain the reported differences between the in vivo and in vitro studies and the fact why pharmacological COMT inhibition does not increase dopaminergic neurotoxicity in vivo [Offen et al., 2001; Lyras et al., 2002; Forsberg et al., 2004; Ogburn et al., 2006].

3.2.2.1 Homocysteine and neuropathophysiology

The O-methylation of catechols such as L-DOPA by COMT leads to formation of homocysteine [Zhu, 2002] (see Fig. 2). Increased homocysteinemia was noted during L-DOPA treatment in animals as well as in humans [Zürcher et al., 1993; Miller et al., 1997; Kuhn et al., 1998a; Müller et al., 1999, 2001, 2002; Obeid and Herrmann, 2006]. Homocysteine has been suggested to induce neurotoxicity and promote neurodegeneration. It was even suggested as an etiological factor in neuropsychiatric disorders such as stroke, dementia, alcoholism, depression, peripheral neuropathy, Alzheimer’s disease, and PD [Reutens and Sachdev, 2002; Bleich et al., 2003; Rogers et al., 2003; Müller et al., 2004a]. Homocysteine may increase the vulnerability of neuronal cells to excitotoxic and oxidative injury, increase beta-amyloid neurotoxicity and provoke excitation of cerebellar neurons via N-methyl-D-aspartic acid (NMDA) receptors. While normal activation of NMDA receptors can lead to both excitation and inhibition, homocysteine provoked only excitation of cerebellar
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neurons [Kim et al., 1987; Lee et al., 1988; Zeise et al., 1988; Lipton et al., 1997; Clarke et al., 1998; Gottfries et al., 1998; Outinen et al., 1998; Miller, 1999; Kruman et al., 2000; Ho et al., 2001; Loscalzo, 2002; Maler et al., 2003; Mattson and Shea, 2003]. From the perspective of PD and L-DOPA treatment, this is interesting, since much of homocysteine formation during L-DOPA treatment is probably related to COMT activity [Müller et al., 2001]. However, at the time of the planning of this thesis in 2002, the effects of COMT inhibition on homocysteine formation during L-DOPA treatment had not been studied.

3.2.2.2 Psychopathophysiology and COMT

The COMT enzyme and functional COMT polymorphism have been studied in numerous psychiatric diseases like schizophrenia, bipolar disorder, eating disorders, obsessive compulsive disorder (OCD), attention-deficit hyperactivity disorder (ADHD), alcoholism, and depression [Strous et al., 1997a; Ohmori et al., 1998; Kotler et al., 1999; Frisch et al., 2001; Shifman et al., 2002; Gabrovsek et al., 2004; Williams et al., 2005; Ohnishi et al., 2006; Frieling et al., 2006; Mikolajczyk et al., 2006; McIntosh et al., 2007].

A fairly convincing association between the high-activity Val allele of COMT and anorexia nervosa has been reported by different research groups [Frisch et al., 2001; Gabrovsek et al., 2004; Michaelovsky et al., 2005; Mikolajczyk et al., 2006]. One recent study, however, presented opposite results [Frieling et al., 2006]. The data for an association between COMT polymorphism and OCD or ADHD have been somewhat contradictory [Azzam and Mathews, 2003; Denys et al., 2006; Michaelovsky et al., 2005; Cheuk and Wong, 2006; Gothelf et al., 2007; Pooley et al., 2007]. However, this could relate to gender differences, as was suggested in a meta-analysis reporting that the Met allele genotype may associate with OCD in male, but not in females [Pooley et al., 2007]. Some evidence of association of low-activity Met allele with early onset major depressive disorder has been reported [Massat et al., 2005], whereas the Val allele has been associated with the bipolar disease and especially with manic behavior in some studies [Shifman et al., 2004; Abdolmaleky et al., 2006; Dickerson et al., 2006; Farmer et al., 2007].

The evidence for a genetic contribution to schizophrenia is strong [Kety et al., 1968; Kendler, 1983; Gottesman, 1991]. The genes related to synthesis (TH, DDC), transport (DAT) and degradation (COMT, MAO) of DA have been examined because of the evidence supporting the role of dopaminergic systems in the etiology of schizophrenia. In contrast to COMT, none of these other genes has reliably emerged as a risk factor for schizophrenia [Macciardi et al., 1994; Sobell et al., 1995; Daniels et al., 1995; Coron et al., 1996; Petit and Frebourg, 1996; Kunugi et al., 1998; Spurlock et al., 1998; Kojima et al., 1999; Faraone et al., 1999; Speight et al., 2000]. Furthermore, genes mapping the 22q11-q13 areas have been of interest, based on the association of psychotic symptoms in almost 30% of individuals with the rare velocardiofacial syndrome, which is cause by DNA deletions in the 22q11 region in 80-85% of the patients [Jurewicz et al., 2001]. Several studies link this same region to schizophrenia as well as to bipolar disease [Lachman et al., 1997; Takahashi
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et al., 2003; Potash et al., 2003; Palmatier et al., 2004; De Lisi et al., 2004; Gogos and Gerber, 2006; Gothelf et al., 2005, 2007; McIntosh et al., 2007]. As stated earlier, the COMT gene is located in that exact area (see 3.1.4.1).

The association studies between this functional polymorphism and schizophrenia have generated conflicting results, which is not unusual for genetic analysis of complex genetic diseases. Many factors can contribute to variability in association study findings among which gene-environment interactions [Caspi and Moffitt, 2006], endophenotype [Gottesman and Gould, 2003] and the low statistical power of individual research due to small sample size can lead to increased rate of both false-positive as well as false-negative results [Altshuler et al., 2000]. The contradicting results may also be explained with so-called bidirectional dopamine hypothesis, which suggests that both high and low dopamine levels in the brain may associate with psychotic symptoms [Keltner, 2005]. Numerous surveys have failed to show any correlation between COMT and schizophrenia [Strous et al., 1997b; Chen et al., 1999; Norton et al., 2002; Gallinat et al., 2003; Joo et al., 2005], whereas some have suggested a correlation with the low-activity Met allele genotype and schizophrenia [Kotler et al., 1999; Sazci et al., 2004; Gothelf et al., 2005]. The low-activity Met/Met genotype has been speculated to cause hyperdopaminergia in mesolimbic pathway, which may lead to impulsive, e.g. aggressive and suicidal, behaviour [Wei and Hemmings, 1999; Nolan et al., 2000; Strous et al., 2003]. In turn, cortical hypodopaminergia has been suggested to associate with schizophrenia due to decreased cognitive performance and increased negative symptoms. According to this theory, subjects with Val/Val alleles should have increased prevalence of schizophrenia than subjects with Met/Met alleles. This hypothesis has strong scientific support especially among high-risk sub-groups. Studies investigating these sub-groups such as families with multiple schizophrenics or Ashkenazi Jews as well as the largest population-based association study by Shifman and co-workers (2002) have reported a highly significant association between schizophrenia and Val-allele genotype [Shifman et al., 2002; Glatt et al., 2003; Kremer et al., 2003; Horowitz et al., 2005; Gothelf et al., 2007; McIntosh et al., 2007]. Furthermore, a recent meta-analysis suggestes Val allele as a small but reliable risk factor for schizophrenia in the populations of European ancestry from the analysis of the family-based association studies [Glatt et al., 2003].

Interestingly, a very recent study has reported a plausible evidence of strong association between the epigenetic regulation of COMT gene P2 promoter area and schizophrenia [Abdolmaleky et al., 2006]. Although the functional COMT polymorphism affects also the CNS COMT activity, the effect is a much milder than in peripheral tissues (See 3.1.5.3). Since COMT is responsible for about 60% of DA metabolism in the PFC [Karoum et al., 1994], the net reduction of DA metabolism in PFC due to low-activity COMT polymorphism is probably only about 15-20%. Direct regulation of transcription like epigenetic regulation could be speculated to have much stronger effects on subjects COMT activity even in the CNS. As explained earlier, the methylation status of the CpG islands in the gene promoter area affects directly the mRNA transcription of the gene. Hypermethylation of the promoter area equals
silencing of the gene. The promoter area of MB-COMT was shown to be hypomethylated in subjects with diagnosed schizophrenia or bipolar disease [Abdolmaleky et al., 2006]. The P2 promoter area had been previously suggested to be a strong candidate in another study investigating the association between COMT polymorphism and schizophrenia [Palmatier et al., 2004]. Thus, further research on epigenetic regulation of COMT gene might provide a novel link between the COMT enzyme and schizophrenia in the future. However, schizophrenics are a heterogeneous group of patients with varying symptoms and drug responses thus it is likely that also wide variety of etiological factors and mechanisms are involved in the development of this disease. Therefore, the COMT enzyme may probably explain, at best, only a small portion of the cases.

The association between alcoholism and COMT polymorphism was reported by Tiihonen and co-workers (1999). They showed a clear indication for the association between the late-onset alcoholism (type 1) and the low-activity Met allele of COMT in a Finnish population. When 123 alcoholics were compared with 246 race- and gender-matched controls, the odds ratio for alcoholism of subjects with COMT (Met/Met) was 2.51 over those with COMT (Val/Val). The frequency of the low-activity allele was also significantly higher in the alcoholics than in 3140 Finnish blood donors representing the general population [Tiihonen et al., 1999]. The same group also investigated the association between COMT polymorphism and early-onset (type 2) alcoholism and found no significant correlation [Hallikainen et al., 2000]. This association could relate to a fact that low activity Met allele may also correlate with depression as well as some other anxiety related disorders [Massat et al., 2005; Pooley et al., 2007].

3.3 COMT in peripheral tissues

3.3.1 Physiological role of COMT in peripheral tissues

The catecholamines DA, NA and adrenaline also function in the peripheral tissues. NA acts as a chemical transmitter in sympathetic postganglionic endings. The adrenal medulla synthesizes and secretes catecholamines into the circulation as part of a diffuse sympathetic discharge during activation of the sympathetic nervous system. In addition to these two well-known peripheral catecholaminergic systems – the sympathetic nervous and adrenomedullary hormonal systems – a third type of peripheral system exists involving DA as an autocrine or paracrine mediator in the mesenteric organs (e.g. gastrointestinal tract, spleen and pancreas) and most importantly in the kidney [Goldstein et al., 1995; Eisenhofer et al., 1995; 1997; 2004]. In the renal tissue, DA functions as a local paracrine mediator and plays a major role in regulation of natriuresis. Since natriuresis, on the other hand, has an important role in blood pressure regulation, one could speculate that changes in COMT activity may affect the blood pressure. For further understanding of this role of COMT in renal physiology, we first must overview the normal physiology of the kidney. The following chapters focus on renal physiology, long-term blood pressure regulation, the dopaminergic system and COMT.
3.3.1.1 Renal physiology, DA, and COMT

The primary sources of sodium in humans are from consumed nutrients and fluids containing sodium chloride (NaCl). Most of the sodium is lost from the body via the kidneys, whereas extrarenal sodium loss is usually minimal [Dahl, 1958; Baldwin et al., 1960; Rodriguez and Humphreys, 1995]. However, in the kidneys, only about 1% of the filtered sodium is excreted in the urine and 99% is reabsorbed by renal tubular cells. About 80% of this reabsorption takes place in proximal tubules [Weiner, 1990].

In the steady state, sodium excretion equals sodium intake; in other words kidney sodium excretion is enhanced or suppressed depending on the sodium intake. This is important, since water follows the sodium. If this balance is disturbed, either a sodium accumulation or deficit occurs, leading to changes in extracellular fluid volume (ECF). For example, during long-term sodium accumulation, the plasma volume is increased, leading to increased arterial pressure. A rise in arterial pressure in turn increases the glomerular filtration rate (GFR), increasing sodium filtration into the renal tubules. If sodium reabsorption in the tubules is not also enhanced, more sodium and water will be excreted, leading to decreased plasma volume and a subsequent decline in blood pressure. This phenomenon is called pressure-natriuresis and is believed to play a crucial role in long-term blood pressure regulation by regulating the ECF volume [Guyton et al., 1972; Guyton, 1991; Cowley, 1992; Hall and Granger, 1994].

However, as explained earlier, 99% of the filtrated sodium is normally reabsorbed in the tubules via sodium transport proteins such as Na⁺/K⁺-ATPase and Na⁺-H⁺-exchanger [Weiner et al., 1990]. Thus, neurohumoral systems, such as the dopaminergic system and the renin-angiotensin-aldosterone (RAA) system, that affect the activity of these sodium transport proteins play a crucial role in tuning of this pressure natriuresis by modifying the renal sodium reabsorption. Defects in these systems can affect the general blood pressure by shifting the pressure-natriuresis curve in relation to blood pressure (see Fig. 4). In other words, these neurohumoral systems can shift the sodium equilibrium and reset the balance to which the body aspires. The role of the RAA system in sodium homeostasis and in blood pressure regulation has been extensively studied, whereas the role of renal dopaminergic system, and especially the role of COMT, is still somewhat obscure.

One of the first reviews concerning the role of peripheral DA in the pathophysiology of hypertension speculated that DA might play predominant role during situation when sodium intake is high, whereas in situations of sodium deficit the DA takes a backseat to NA and other sodium retaining factors [Kuchel and Kuchel, 1991]. This hypothesis was supported by the result showing that activation of α-adrenergic receptors stimulated the tubular Na⁺-K⁺-ATPase activity at low sodium concentrations, but not at saturating concentrations [Baines et al., 1985]. Further support for this hypothesis was attained later by data showing that DA inturn inhibited renal Na⁺-K⁺-ATPase activity at saturating sodium concentrations, but not at low sodium concentrations.
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concentrations [Ibarra et al., 1993]. Furthermore, Baum and Quigley (1998) later reported that in isolated perfused rabbit kidney, the DA antagonized the stimulation in transport produced by NA, but had only little effect in the absence of NA tonus [Baum and Quigley, 1998].

These results thus support the preliminary hypothesis that these renal natriuretic and anti-natriuretic factors operate in dynamic equilibrium and are linked, possibly in the form of cascade [Kuchel and Kuchel, 1991]. These major factors affecting the renal sodium excretion as well as blood pressure regulation have been reviewed in detail previously by Mervaala (1995) and are listed below (see Fig. 4).

![Figure 4](image-url)

**Figure 4.** a) During normal balanced situations in healthy subjects, sodium excretion correlates directly with sodium intake. b) The neurohumoral systems and the direction of their enhanced function on the pressure-natriuresis curve in the kidney. Continuous curve represents the normal situation. Dashed pressure-natriuresis curve represents the hypothetical situation when the pressure-natriuresis curve has shifted right, e.g. in hypertensive subjects. Letter A represents factors such as anti-hypertensive medication, dopamine (DA), natriuretic peptides (ANPs) [Jin et al., 1988; Sagnella and MacGregor, 1994], nitric oxide (NO) [Romero et al., 1992; Raji, 1993], prostaglandins (some) [Capdevila et al., 1992; Campese, 1994; Quilley and McGiff, 1994], kallikrein-kinin system [Ferri et al., 1994], calcium (Ca) [Porsti, 1991; Wuorela, 1993; Hatton and McGarron, 1994] and potassium (K) [Mervaala et al., 1992; Adrogue and Madia, 2007]. Letter B represents factors such as the RAA system, endothelins [Tolins and Shultz, 1994], sympathetic nervous system [De Champlain et al., 1969; Dietz et al., 1980; Oparil et al., 1989; Campese, 1994; DiBona, 2000] and insulin [Sechi et al., 1994; Morris and Connell, 1994]. Figures are modified from the original data of Guyton et al. (1972).

Since renal DA is a hormone essential for regulating renal natriuresis, the enzymes important in DA synthesis and metabolism will next be discussed.

Circulating DA was previously believed to originate from sympathetic nervous system spill-over, due to inefficient conversion of DA via β-hydroxylase. This
hypothesis was previously used to explain the observation that urinary excretion of DA and its metabolites exceeds that of NA and its metabolites [Kopin, 1985]. However, it is no longer tenable because the effectiveness of the β-hydroxylase in the sympathetic nerves is now known to be about 90% [Eisenhofer et al., 1996]. A substantial portion of the peripheral DA therefore originates from sources independent of the autonomic nervous system. These peripheral dopaminergic systems include the renal dopaminergic system [Goldstein et al., 1995, 2003; Eisenhofer et al., 1995; 1997; 2004]. In fact, the other peripheral dopaminergic systems, e.g. in the mesenteric organs, constitute a major site of DA synthesis, accounting for about 45% of all the DA produced in the body [Eisenhofer et al., 1995; 1997].

The renal DA has since been shown to be produced in situ from the circulating precursor L-DOPA via decarboxylation by dopa decarboxylase enzyme (DDC). Renal DA originates from the circulating plasma L-DOPA which in turn originates from spill-over of sympathetic nerve endings and the adrenomedullary system as well as other peripheral DA systems [Seri et al., 1990; Soares da Silva and Fernandes, 1990; Eisenhofer et al., 1995; 1997]. The kidneys filter the circulating L-DOPA [Ball and Lee, 1977; Ball et al., 1978; Zimllichman et al., 1988; Grossman et al., 1991; Wolfowitz et al., 1993] and type 2 L-amino acid transporters (LAT-2) on the lumen side of the PTCs have been suggested to be mainly responsible for L-DOPA uptake into these cells. The LAT-2 have also been suggested to constitute the rate-limiting step of the renal tubular DA synthesis in vitro [Pinho et al., 2004]. However, it is also known that the L-DOPA enters the PCTs via Na+-dependent mechanism [Pestana et al., 2001; Pinho et al., 2004]. Interestingly, β2-receptor stimulation has been suggested to down-regulate the L-DOPA uptake in the tubular cells and decrease DA synthesis [Carranza et al., 2000]. The conversion of L-DOPA to DA in the kidneys accounts for virtually all the free DA in the urine. Urinary DA therefore reflects the local renal-dopaminergic natriuretic system, not filtration of circulating DA [Eisenhofer et al., 2004].

Two enzymes play a major role in L-DOPA metabolism in the kidney, DDC and COMT. Most of the renal L-DOPA is decarboxylated via DDC to DA. There is well-established evidence that inhibition of DDC attenuates the natriuresis, blunts the excretory responses of DA to volume challenge and decreases the urinary excretion of DA [Sowers et al., 1984; Ball and Lee 1977; Williams et al., 1986; Yoshimura et al., 1987]. Furthermore, salt loading increases DDC activity in the proximal tubules [Hayashi et al., 1991]. COMT in turn accounts for approximately 10% of the peripheral L-DOPA metabolism and O-methylation of the L-DOPA to 3-O-methylldopa (3-OMD) [Nutt and Feldman, 1984]. The metabolism of renal DA also occurs via two different pathways, methylation and oxidation. COMT is responsible for O-methylation of DA to 3-MT and MAO in turn catalyzes the oxidation of DA to DOPAC [Fernandes and Soares da Silva, 1994]. The major urinary metabolite of this metabolism is the homovanillic acid (HVA), which is the end-product of both pathways [Wang et al., 2001]. Inhibition of COMT increases natriuresis and urinary excretion of DA [Eklöf et al., 1997; Hansell et al., 1998; Odlin et al., 1999; Wang et al., 2001; Vieira-Coelho et al., 2001]. This natriuretic effects os even more pronounced than after administration of L-DOPA [Eklöf et al., 2004].
1997]. MAO inhibition, on the other hand, does not increase natriuresis or DA excretion [Wang et al., 2001; Odlind et al., 2001a; 2001b]. Thus, metabolism of renal DA via O-methylation appears to be the physiologically more important metabolic pathway and MAO may be more of a housekeeping enzyme [Aperia et al., 2000] (see Fig. 3).

3.3.1.2 Effects of renal dopaminergic and RAA systems in kidney physiology

The renal dopaminergic and RAA systems are important regulators of sodium balance and blood pressure. The significance of the RAA system in blood pressure regulation has been well established. There is also accumulating evidence suggesting that renal DA production and/or DA receptor function may be defective in arterial hypertension, as well as in renal parenchymal diseases [José et al., 1998a; Aperia, 2000; Carey, 2001a]. The following chapter reviews the biochemical effects of the dopaminergic and RAA systems in the kidney. Briefly, the RAA system inhibits natriuresis via angiotensin type I (AT1) receptors and the dopaminergic system increases natriuresis via dopamine type I (D1)-like receptors (D1 and D5) in the PTCs. On the other hand, intracellular Na+ regulates both DA and Ang II receptor availability and their cellular responses in the plasma membrane of the renal PTCs [Efendiev et al., 2003]. The opposing effects and interaction of the renal dopaminergic and RAA systems are next discussed.

The regulatory effects of DA on blood pressure and natriuresis are mediated through the renal DA1-5 receptors. The DA receptors have been classified via their physiological function into two groups: D1-like receptors (D1 and D5) and D2-like receptors (D2, D3, and D4) [José et al., 1998a; Aperia, 2000; Carey, 2001a]. Increased dopaminergic activity and stimulation of DA1-like receptors promotes natriuresis and diuresis through inhibition of sodium transport proteins such as Na+/K+-ATPase and Na+-H+-exchanger in the tubular cells [Baines et al., 1992; Chen and Lokhandwala, 1993; José et al., 1998a; Aperia, 2000; Carey, 2001a; Pedemonte et al., 2005], shifting the pressure-natriuresis curve to the left. Approximately 50% of the basal renal Na+ excretion has been estimated to be mediated by the paracrine action of renal DA on PTC D1-like receptors in rats and in dogs [Pelayo et al., 1983; Siragy et al., 1989]. Stimulation of the RAA system and AT1 receptors in turn opposes these effects by increasing sodium reabsorption via stimulation of sodium transport protein activities. Angiotensin evokes antinatriuretic effects by attaching to the AT1 receptors found in the PTCs, efferent and afferent arterioles and glomerular mesangium. In the PTCs, the AT1 receptor activation increases Na+ transport across the cell from the tubular lumen into the renal interstitial spaces and peritubular capillaries by stimulating Na+-H+-exchanger and Na+/K+-ATPase activities [Matsusaka and Ichikawa, 1997]. This shifts the pressure-natriuresis curve to the pathological direction, i.e. to the right. AT1 receptor activation also constricts the renal arterioles and contracts the glomerular mesangium, resulting in decreased intrarenal blood flow and GFR [Carey and Siragy, 2003], whereas DA increases the renal blood flow and
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GFR [Aperia, 2000]. During conditions of sodium loading, D_2-like receptors act synergistically with the D_1-like receptors to increase renal sodium excretion [Eklöf, 1997; José et al., 1998b]. The main D_2-like receptor expressed in renal proximal tubules in rats and humans is the D_3 receptor [O’Connell et al., 1998; Muhlbauer et al., 2000]. The disruption of the D_3 receptor gene has been shown to produce renin-dependent hypertension and decreased ability to excrete sodium after acute saline loading in homozygous mutant mice [Asico et al. 1998]. Moreover, D_1 and D_3 receptors may interact and positively regulate each other’s expressions [Levavi-Sivan et al., 1998; Zeng et al., 2006b] and the transregulation of D_1 and D_3 receptors may be defective in hypertension [Zeng et al., 2004]. AT_2 receptors in turn inhibit the PTC Na^+K^+-ATPase activity via NO/cGMP-dependent pathway increasing sodium excretion [Hakam and Hussain, 2006] and oppose vasoconstrictive effects of the AT_1 receptors [Carey, 2001b]. Furthermore, AT_2 receptor null mice show exaggerated antinatriuretic responses to systemic Ang II infusion and are unable to generate a normal pressure-natriuresis response [Siragy et al., 1999; Gross et al., 2000]. The AT_2 receptor tonically inhibits the angiotensin-converting enzyme (ACE) in AT2 null mutant mice [Hunley et al., 2000] and also inhibits renin biosynthesis and Ang II formation [Siragy et al., 2005].

There is also a direct interaction between these two different hormonal systems. DA has been suggested to down-regulate the AT_1 receptors in the PTCs in rats and in humans [Cheng et al., 1996; Wang et al., 2003], whereas Ang II inhibits DA uptake in the rat kidneys in vitro [Choi et al., 2006]. DA-dependent natriuresis via D_1-like receptors is dependent on AT_2 receptors, as was shown in a study in which Fenoldopam, a selective D_1-like receptor agonist, induced natriuresis in rats that was attenuated by PD-123319, a specific AT_2 receptor antagonist [Salomone et al., 2007]. Transregulation of AT_1 and D_3 receptors have been established in renal PTCs in rats [Zeng et al., 2005]. Activation of the D_3 receptor also decreases AT_1 receptor expression in rat renal PTCs [Zeng et al., 2006a]. Furthermore, disruption in D_1 and AT_1 [Zeng et al., 2003], as well as D_1 and D_2-like [Ladines et al., 2001] receptor interaction occurs in spontaneously hypertensive rats. Thus, although the primary effects of these two systems, dopaminergic and RAA, are in opposition, the normal functioning of either system is also dependent on that of the other.

3.3.1.3 Detoxification role of COMT

In a manner similar to that in the BBB, the COMT enzyme also functions as an enzymological barrier in the peripheral tissues. Although anatomical structures such as the basement membrane are responsible of the cross-separation and maintenance of homeostasis between different bodily compartments, the existence of the enzymological barrier is another important way to maintain this, since some liposoluble or small compounds and molecules using active transport systems can easily penetrate most anatomical structures. COMT functions as a phase II enzyme and is one of the enzymes forming the enzymological barrier against the free translocation of biologically active
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molecules and substances such as catechols in the body [Guldber and Marsden, 1975; Männisto and Kaakkola, 1999]. The localization of COMT shows that the enzyme is most active in bodily structures located between different compartments of the body separated by the circulation. As explained, COMT activity is high between the lumen of the gut and the gut circulation (gut wall) and between the enterohepatic circulation and main circulation (liver). Furthermore, the prevention of free translocation of catechols in the body also requires high levels of COMT activity between the main circulation and vital organs such as the brain (BBB and endothelium in general). In these structures, COMT functions as part of the enzymological barrier, protecting tissues and organs from nonspecific effects of biologically active and possibly harmful circulating catechol group-containing compounds.

Most of the detoxification of catechols via COMT occurs in organs related to first-pass metabolism such as the liver and gut wall, both of which show high COMT activities. COMT metabolizes various exogenous pharmaceuticals and dietary substances containing catechol structures such as L-DOPA, dietary polyphenols etc. (see. 3.1.1.4). The COMT enzyme is also found in other bodily structures and organs through which exogenous catechols could enter the body, e.g. the skin and lungs.

Another important detoxication role of COMT is related to estrogen metabolism. The hydroxylated catechol estrogens are the metabolites of cytochrome P450 enzyme NADPH-dependent oxidation of estrogens (estradiol and estrone) in the liver. The catechol estrogens have been associated with carcinogenesis [Cavalieri and Rogan, 1998] and these effects were suggested to be mediated via increased oxidative DNA damage. COMT plays a major role in metabolism and inactivation of the reactive hydroxylated catechol estrogens, 2-hydroxy- and 4-hydroxyestrogens, converting them to tumor suppressing 2-methoxy- and 4-methoxyestrogens [Männistö and Kaakkola, 1999; Zhu, 2002]. Induction of the nuclear S-COMT in pathologic situations could be the cells´ own protective response to increased nuclear catechol estrogen metabolite levels by inhibiting possible oxidative DNA damage [Zhu and Conney, 1998; Weisz et al., 1998, 2000; Hirose et al., 2007]

3.3.2 Pathophysiological role of COMT in peripheral tissues

3.3.2.1 Pathophysiological role of COMT in the cardiovascular system

As stated earlier, the COMT enzyme plays an important role in the metabolism of catecholamines (DA, NA and adrenaline), which are the vital neurotransmitters/hormones occupying a key role in the regulation of physiological processes in the cardiovascular system.

At the time of our study, no association between hypertension and the COMT enzyme had been reported in humans. Since then, however, the possible
association between hypertension and cardiovascular diseases with functional COMT polymorphism has drawn the interest of several research groups. The Val/Val genotype of the COMT enzyme was associated with higher systolic blood pressure among 2966 Scandinavian individuals without self-reported diabetes or current use of antihypertensive drugs. The Val/Val genotype was also more likely to be found among individuals with current antihypertensive medication [Hagen et al., 2007]. The researchers speculated that the effects may have derived from the increased metabolism of DA. Surprisingly and in apparent contrast with the blood pressure results in the same study by Hagen and co-workers, patients with Val/Val genotype tended to have lower prevalences of self-reported heart disease than those with the Met/Val or Met/Met genotypes [Hagen et al., 2007]. Similar results were also recently reported in a Japanese population [Kamide et al., 2007]. In this study the Val allele was also associated with hypertension in Val/Val subjects. The results of studies investigating the correlation between functional COMT polymorphism and acute coronary events have been contradictory [Eriksson et al., 2004; Happonen et al., 2006].

3.2.2.1 Homocysteine and cardiovascular pathophysiology

The O-methylation of catechols via COMT also increases the formation of homocysteine (See Fig. 2), which is known individual risk factor for cardiovascular diseases. Homocysteine is known to increase endothelial dysfunction and vascular smooth muscle cell proliferation, decrease NO bioavailability in the endothelium and increase oxidative stress in vascular wall [McCully, 1969; Clarke et al., 1991; Boushey et al., 1995; Hankey and Eikelboom, 1999; De Bree et al., 2002; Aguilar et al., 2004; Lentz, 2005; Castro et al., 2006; Voutilainen et al., 2007]. In addition, homocysteine increases platelet aggregation and interacts with the coagulation cascade and fibrinolysis, thus modifying the normal endothelium to a more prothrombotic phenotype [Perna et al., 2003].

However, although the hyperhomocysteinemia has been long known to correlate with increased cardiovascular disease risk the direct causality is yet to be proven, since no generally accepted pathophysiological mechanism has ever be reported. This absence of specific mechanism has led to a speculation that homocysteine might be an epiphenomenon, simply reflecting the presence of some other proatherogenic factor actually responsible increased risk for cardiovascular diseases. Nevertheless, hyperhomocysteine has a well proven positive correlation with the increased risk of cardiovascular diseases (see above).

Thus, due to its role in catecholamine metabolism and homocysteine formation, the COMT enzyme may also play a role in the pathophysiological processes of cardiovascular diseases.
3.3.2.2 Role of COMT in carcinogenesis

Since altered COMT activity and expression as well as detoxification of toxic metabolites and substances via O-methylation have been associated with carcinogenesis, the following chapter overviews the research of COMT in the pathophysiology of cancers.

There have been numerous studies linking the COMT enzyme and especially the functional polymorphism of the enzyme to the etiopathology of various cancers. The general hypothesis is that high COMT activity protects from the oxidative DNA damage of catechol estrogens whose metabolites have been suggested to promote carcinogenesis. The functional Val(108/158)Met polymorphism in the COMT gene affecting the activity of the enzyme has been linked to oncogenesis in various types of cancers. The low-activity Met allele may be associated with breast, prostate, renal cell cancer and uterine leiomyomas [Lavigne et al., 1997; Thompson et al., 1998; Palmatier et al., 1999; Matsu et al., 2000; Yim et al., 2001; Comings et al., 2003; Sazci et al., 2004; Inoue et al., 2005; Tanaka et al., 2006, 2007]. Some studies have also suggested that in breast cancer, the low-activity Met allele may correlate with metastasis and aggressivity of the cancer [Matsu et al., 2000]. A similar tendency towards further advanced cancer was noted in a prostate cancer patient [Tanaka et al., 2006]. Interestingly, there is also a difference in COMT isoenzyme expression in breast cancer tissue. Although both S-COMT and MB-COMT concentrations are increased, the relative amount of MB-COMT is higher compared to the non-malignant control breast tissue. The absolute amount of MB-COMT increased by 400%, whereas S-COMT increased by only 170% [Tenhunen et al., 1999].

The majority of population-based studies supporting the association between COMT polymorphism and breast cancer have been done with Asian subjects, whereas in Caucasian populations, the association has been found mostly in some subgroups, such as subjects with high body mass index (BMI) or long-term contraceptive/hormone therapy use, both of which are known individual risk factors for breast cancer [Mitrunen et al., 2001; Lavigne et al., 1997; Thompson et al., 1998]. Many of the studies found no association with the low-activity Met allele and breast cancer. Most of these studies were conducted in Caucasian populations in which the Met allele is naturally more common [Gaudet et al., 2006; Modugno et al., 2005; Ahsan et al., 2004; Saintot et al., 2003; Millikan et al., 1998]. In sporadic prostate cancer, the COMT Met allele is a risk factor, whereas the Val allele may be protective [Tanaka et al., 2006; Nock et al., 2006]. Catechol estrogens also play an important role in estrogen-induced prostate cancer [Cavalieri et al., 2002]. There are also studies showing the abnormal methylation status of COMT gene promoter areas in endometrial tumors [Sasaki et al., 2003] and COMT polymorphism is also associated with uterine leiomyomas in different ethnic groups [Al-Hendy and Salama, 2006]. One study with Caucasian women failed to demonstrate a similar correlation, although the trend was in a similar direction [Denschlag et al., 2006].
3.3.3 Effects of COMT inhibition in peripheral dopaminergic systems

Although COMT is expressed abundantly in the kidney [Meister et al., 1993; Karhunen et al., 1994; Weisz et al., 1998; Männistö and Kaakkola, 1999], the importance of COMT compared with another catechol-metabolizing enzyme MAO in the regulation of kidney functions and modulation of renal dopaminergic tone, however, has been somewhat of a dispute in the past. Some studies indicated COMT as the more important enzyme of the two in renal catecholamine metabolism and natriuresis [Eklöf et al., 1997; Odlind et al., 2001a, Wang et al., 2001], whereas some earlier studies had suggested that MAO could be more important of the two enzymes in intrarenal catecholamine metabolism [Vieira-Coelho et al., 1993; Fernandes and Soares-da-Silva, 1994]. The generally accepted consensus today seems to be that MAO is more of a housekeeping enzyme in the kidney, whereas COMT has more important physiological role in the renal catecholamine metabolism and regulation of sodium homeostasis [reviewed in Aperia, 2000]. Thus, changes in COMT activity should also affect DA-dependent natriuresis and sodium balance. COMT inhibitors increase the renal DA-dependent natriuresis and DA concentration, as measured with a microdialysis technique [Wang et al., 2001]. The natriuretic effects of COMT inhibitors are probably mediated through increased renal DA contents but COMT inhibitors have also been proposed to have direct affinity to D1-like receptors [Vieira-Coelho et al., 2001]. However, no indisputable evidence for this has been provided, since even the study by Vieira-Coelho and co-workers only used D1 receptor antagonists to support their hypothesis. The blockade of D1 receptors, however, also blocks the effects of DA on D1 receptors; thus, no unchallenged evidence was provided. In anesthetized rats, acute COMT inhibition by entacapone produces a profound DA receptor-dependent natriuresis [Eklöf et al., 1997; Odlind et al., 1999, 2001a; Wang et al., 2001]. Entacapone also produced an over 5-fold increase in sodium excretion and this effect was reduced by 70% when administered together with SCH 23390, a known D1 receptor antagonist [Hansell et al., 1998]. Interestingly, another substance that inhibits O-methylation only in vivo, CGP 28014, produced a significant increase in DA and DOPAC excretion in the same study without increasing sodium excretion. In the brain, CGP 28014 effectively inhibits O-methylation via COMT without being an effective COMT enzyme inhibitor in vitro [Männistö et al., 1992a; Törnwall and Männistö 1993; Kaakkola et al., 1994]. It has been suggested that CGP 28014 may inhibit the so-called uptake2 (nonneuronal uptake) process, leaving an excess amount of DA in the extracellular space, thus preventing O-methylation by keeping the substrate in a different compartment than COMT [Hansell et al., 1998]. This hypothesis would also explain the renal finding of Hansell and co-workers (1998). However, these studies suggest that urinary DA concentration may not function as the most reliable indicator of renal dopaminergic activity in every situation. This was also supported by a microdialysis study investigating the effects COMT and MAO inhibition in renal DA and 5-hydroxytryptamine metabolism in rats. In this study the DA concentration in the renal interstitial fluid was increased by 150%, although the urinary DA increased by only 40%
after administration of COMT inhibitor. Since the D_1,5 receptors appear in both the basolateral and apical surfaces of the PTCs, some of the natriuretic effects may also be mediated via DA receptors in the apical membrane [Wang et al., 2001].

Interestingly, Odlind and coworkers studied natriuresis during acute sodium infusion on anesthetized COMT-deficient mice and found that the mice were surprisingly unable to excrete as much sodium into the urine as their genetic wild-type littermates [Odlind et al., 2002], a result that was not in line with previous studies into the effects of COMT inhibition and natriuresis. Previous reports have also provided compelling evidence that renal production of DA in response to high sodium intake may be blunted in patients with salt-sensitive hypertension [Pestana et al., 2001]. Salt-sensitive African-American subjects also show a deficiency in the renal dopaminergic-dependent natriuretic response to acute sodium load [Damasceno et al., 1999], a concept that may be explained by ethnic COMT polymorphism differences.

3.4 COMT inhibitors

3.4.1 History of COMT inhibitors

The first compound inhibiting the COMT enzyme, pyrogallol (1,2,3-trihydroxybenzene), was discovered in the late 1950s [Axelrod and Laroche, 1959]. The discovery and studies of other first-generation COMT inhibitors such as gallates, tropolone, U-0521 and ascorbic acid during the following decades was thoroughly reviewed in Guldberg and Marsden (1975). The second-generation COMT inhibitors, entacapone and tolcapone, were introduced in the late 1980s [Bäckström et al., 1989; Borgulya et al., 1989; Männistö and Kaakkola 1989, 1990] and have been reviewed in Männistö and Kaakkola (1999).

This section of the review focuses on the characteristics of the two COMT inhibitors in clinical use today, entacapone and tolcapone, as well as on nitecapone, which is also a second-generation COMT inhibitor we have used in our studies. Of these three, tolcapone is most effective in the CNS because it penetrates through BBB efficiently, whereas nitecapone is most effective in peripheral COMT inhibition and does not penetrate through the BBB [Forsberg, 2005].

3.4.2 Characteristics of COMT inhibitors

3.4.2.1 Pharmacodynamics

COMT inhibitors function by binding to the enzyme directly and preventing binding of the substrate to the active site of the COMT enzyme. The second-generation COMT inhibitors – entacapone (OR-611), nitecapone (OR-462) and tolcapone (Ro 40-7592) – are the so-called competitive or tight-binding
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type of inhibitors and share the same feature: they all have the nitrocatechol
structure, an electronegative substituent (-NO₂), at the 5-position of the
catechol ring [Vidgren et al., 1999; Learmonth et al., 2002, 2004]. In addition,
there is also a hydroxypyridine inhibitor of O-methylation, CGP 28014.
Interestingly, this compound is inactive in vitro but a potent COMT inhibitor in
vivo. The in vivo properties of CGP 28014 resemble most closely those of
tolcapone [Waldmeier et al., 1990].

![Molecular Structures](image)

Figure 5. Molecular structures of tight-binding nitrocatechol inhibitors of COMT: entacapone,
tolcapone and nitecapone.

The second-generation nitrocatechols bind tightly but reversibly to the same
active site of the COMT enzyme as the endogenous catechol substrates
[Lotta et al., 1995]. However, the electronegative –NO₂ group makes them
poor substrates. The side chain of these compounds ultimately defines the
degree of O-methylation. Approximately 3% of tolcapone molecules are O-
methylated during the binding reaction, whereas entacapone does not appear
to be methylated at all [Bäckström et al., 1989; Borgulya et al., 1989].
Although the investigations being conducted in different laboratories are not
fully comparable, the half maximal inhibitpry concentrations (IC₅₀) estimated
for nitecapone, entacapone and tolcapone in the rat liver are 310 nM, 160 nM
and 40 nM, respectively [Schultz and Nissinen, 1989; Zürcher et al., 1990;
Nissinen et al., 1992; reviewed in Kaakkola et al., 1994].

Here the characteristics of ascorbic acid should also be commented. Ascorbic
acid is a substrate for COMT and about 5% of excreted ascorbic acid is in the
methylated form [Blaschke and Hertting, 1971; Bowers-Komro et al., 1982].
However, ascorbic acid also functions as a COMT inhibitor and millimolar
concentrations of ascorbic acid can reduce COMT activity significantly [Kern
and Bernards, 1997].

3.4.2.2 Pharmacokinetics

Although all the second-generation nitrocatechols are potent inhibitors of the
COMT enzyme, the pharmacokinetic properties of entacapone, nitecapone
and tolcapone differ somewhat. Unfortunately, only a few of the kinetic studies
have compared the inhibitors and for this reason these values should be
compared with caution.
The oral bioavailability of tolcapone is about 50% at dosages of 20-40mg/kg, whereas the oral bioavailability of entacapone at 5.7 mg/kg varies between 10-35%. All nitrocatechols have small volume of distribution and are extensively bound to plasma proteins [Keränen et al., 1994; Männistö and Kaakkola, 1999; Heikkinen et al., 2001]. In addition to lower oral bioavailability, entacapone is also eliminated more rapidly than tolcapone in rats [Funaki et al., 1994; Savolainen et al., 2000].

In a study comparing the kinetic properties of entacapone and tolcapone in rats, the COMT activity in the brain was more effectively inhibited with the tolcapone after continuing oral administration of these nitrocatechols at 10 mg/kg b.i.d. for 7 days [Forsberg et al., 2003]. Thirty minutes after the tolcapone administration, the striatal COMT activity left was only about 35%, whereas 30 min after the entacapone administration, approximately 55% of the striatal COMT activity remained. Six hours after the administration the entacapone effect had been abolished completely, whereas tolcapone still inhibited almost half of the striatal COMT activity. In the kidney, the effects were similar, although stronger than those noted in the brain parenchyma. Approximately 90-95% of the renal COMT activity was inhibited with both compounds 30 min after the oral administration. However, after 6 h, entacapone inhibited only 60% of the COMT activity whereas the tolcapone still inhibited about 90% of the activity; after 8 h the values were 30% and 80%, respectively. Nitecapone is considered to be more peripherally active than entacapone and much more so than tolcapone. Nitecapone at 10 mg/kg p.o. inhibits duodenal COMT activity by over 90% 1 h after administration in rats. Eight hours after administration, the COMT activity was still inhibited by approximately 20%. No effects on striatal COMT activity were noted [Nissinen et al., 1988b]. In the kidney, oral administration of nitecapone at 30 mg/kg inhibited COMT activity by over 90% 1 h after administration [Vieira-Coelho et al., 2001].

Second generation COMT inhibitors are poor substrates for COMT. Entacapone and nitecapone are not O-methylated in humans, whereas approximately 3% of the tolcapone has been reported to methylate via COMT. All COMT inhibitors are abundantly metabolized in the liver. These nitrocatechol inhibitors are mainly metabolized via glucuronidation and excreted via biliary tract to feces or via circulation and kidney filtration to urine. Only less than 1% of the molecules are excreted unchanged in the urine. [Taskinen et al., 1991; Wikberg et al., 1993; Da Prada et al., 1994].

3.4.2.3 Other properties of COMT inhibitors

Interestingly, previous studies have revealed that in addition to COMT inhibition, entacapone and nitecapone, also have anti-inflammatory properties, scavenge ROS, act as iron chelators and inhibit lipid peroxidation [Suzuki et al., 1992; Marcocci et al., 1994; Haramaki et al., 1995; Orama et al., 1997; Lal et al., 2000]. As explained earlier, O-methylation of catechols via COMT inactivates the reactive hydroxyl group of the catechols, making them less...
reactive and toxic. The catechols are especially reactive due to their unique characteristic of two adjacent hydroxyl groups. O-methylation by COMT has been speculated to have protective effects by preventing the auto-oxidation of these two adjacent hydroxyl groups, which elicits the production of hydrogen peroxide (H$_2$O$_2$) and ROS such as the superoxide anion (O$_2^-$) and hydroxyl radical (OH$^-$) [Kopin, 1985; Miller et al., 1996; Kuhn et al., 1998b]. This theory suggests that COMT inhibition may increase ROS formation and oxidative stress with resulting cytotoxic effects. The O-methylated metabolites 3-OMD, 3-MT and HVA may also possess antioxidant properties [Miller et al., 1996; Nappi and Vass, 1998]. On the other hand, inhibition of O-methylation should increase 3,4-dihydroxymandelic acid concentrations (see Fig. 3), which is a metabolite of NA with powerful antioxidative potential [Ley et al., 2002]. There are numerous in vitro studies showing that auto-oxidation of catechols may have cytotoxic effects. The role of COMT in this in vivo, however, is still unclear. It is important to underline that all of the studies supporting the COMT enzymes significant role in this theory were performed in vitro [Stokes et al., 1999; Werner et al., 2001; Offen et al., 2001; Ogawa et al., 2005; Ogburn et al., 2006; Miyazaki et al., 2006], whereas in vivo studies with COMT-deficient mice or cynomolgous monkeys have reported no increased cytotoxicity, auto-oxidation or increased ROS formation following pharmacological or genetic blockade of COMT [Lyras et al., 2002; Forsberg et al., 2004]. In addition there is also a study showing that treatment with COMT inhibitor could reverse renal abnormalities in diabetic rats by decreasing oxidative stress and inflammation in the kidney [Lal et al., 2000].

Nitrocatechol COMT inhibitors may also have direct affinity for D$_1$ receptors [Vieira-Coelho et al., 2001]. Entacapone, nitecapone and tolcapone all produce increased natriuresis although urinary DA excretion was not increased after administration of entacapone or tolcapone. Interestingly, nitecapone treatment resulted in the most effective natriuresis and also increased urinary DA excretion. The natriuretic effect of all these compounds was abolished after administration of the D$_1$ antagonist SCH 23390 [Vieira-Coelho et al., 2001]. However, since the SCH 23390 also abolishes the effects of renal DA itself, this hypothesis of the direct D$_1$ affinity for COMT inhibitors is still merely a hypothesis.

### 3.4.3 Clinical use of COMT inhibitors

L-DOPA medication is the golden standard in the treatment of PD. Oral L-DOPA treatment was first introduced in the 1950s and is still the most effective treatment for motor dysfunctions such as the akinesia and hypokinesia seen in Parkinsonian patients. However, there are well-established problems related to L-DOPA treatment, e.g. the wearing-off phenomenon related to decreased plasma L-DOPA concentrations and the on-off phenomenon, whose etiology is still unknown [Jankovic, 2005; Waters, 2005]. Wearing-off is related to the inefficient tissue concentration of L-DOPA resulting from the metabolism of L-DOPA. The on-off phenomenon describes the sudden end of the positive effect of L-DOPA, which does not appear to be related to tissue or plasma L-DOPA concentrations. High L-DOPA
Review of the literature

counts, on the other hand, may lead to side effects such as dyskinesias. Thus, a constant L-DOPA concentration would be most optimal. The clinical usage of COMT inhibitors in the treatment of PD is in fact related to this very purpose. Adjunct COMT inhibition lengthens the so-called on-time in Parkinsonian patients and increases the bioavailability of L-DOPA [Parkinson Study Group, 1997; Kaakkola, 2000; Gordin et al., 2003].

L-DOPA is formed from the amino acid tyrosine via hydroxylation by TH. L-DOPA in turn functions as a precursor for synthesis of DA in cells containing DDC, e.g. neuronal dopaminergic cells or renal PTCs (see Fig. 3). Oral exogenous L-DOPA medication is used to increase the DA concentration in the CNS of Parkinsonian patients [Kaakkola, 2000; Gordin et al., 2003]. However, the bioavailability of oral exogenous L-DOPA is poor, due to inefficient absorption and peripheral L-DOPA metabolism and only about 1% of the orally digested L-DOPA ever reaches the brain parenchyma unchanged [Männistö and Kaakkola, 1990]. To decrease the peripheral metabolism of L-DOPA, DDC inhibitors such as carbidopa or benserazide are usually combined with L-DOPA treatment. In addition to DDC, the L-DOPA is also metabolized by COMT. Approximately 70% of the L-DOPA is metabolized to DA via DDC and of the rest the major portion is metabolized via COMT to 3-OMD, some is oxidized via tyrosinase to DOPA quinone and transamination via tyrosine aminotransferase represents a minor pathway [Nutt and Fellman, 1984]. However, during L-DOPA+DDC inhibitor therapy, O-methylation represents the major pathway [Le Witt, 1989; Männistö and Kaakkola 1990]. Thus, addition of COMT inhibitor to PD medication increases the bioavailability and increases the half-life of L-DOPA, leading to fewer motor fluctuations and increase in on-time by producing more constant L-DOPA levels in L-DOPA + DDC inhibitor-treated PD patients [Kaakkola, 2000; Gordin et al., 2003].

Two COMT inhibitors, entacapone and tolcapone, are currently in clinical use. [Bäckström et al., 1989; Borgulya et al., 1989; Männistö and Kaakkola 1989, 1990]. Initially the tolcapone appeared to be the better of the two molecules, being able to penetrate more readily through the BBB and cell membranes. This characteristic of tolcapone was believed to offer an advantage, since in addition to peripheral methylation, also central methylation of L-DOPA via COMT was inhibited. However, this enhanced cell membrane permeability was fairly soon shown to be in fact a disadvantage, because tolcapone presented clinically significant liver toxicity in patients and was later withdrawn from clinical use. Currently, tolcapone has been allowed back into clinical use, but only under regular controls of patient liver transaminases. Entacapone, on the other hand, exerts no liver toxicity or any other lethal side effects. The capacity of COMT in the periphery is probably so high that only a minor fraction of the enzyme is ever needed [Männistö and Kaakkola, 1999]. As a result, the total COMT inhibition may be difficult to achieve but this may also be crucial for the safety of peripherally active COMT inhibitors such as entacapone [Männistö and Kaakkola, 1999]. However, this is only speculation since even total COMT deficiency is not lethal in mice lacking the COMT gene [Gogos et al., 1998], although to the best of my knowledge, no studies on aged COMT-deficient mice have ever been conducted.
Aims of the study

4. AIMS OF THE STUDY

The role of the COMT enzyme may increase during pathological situations. Numerous recent studies have suggested that functional COMT polymorphism affecting the COMT activity associates with the etiology of various diseases. Thus, increased COMT activity during pathological situations may be important. However, only scattered information of the pathophysiological role of COMT enzyme at the biochemical level has been published. This thesis was designed to increase our level of understanding about the physiological and pathophysiological roles of COMT enzyme and to study the possible correlations and associations between COMT and different pathophysiological events using experimental animal models and pharmacological as well as genetic COMT blockade. The role of COMT was studied in the brain and in the cardiovascular and renal systems.

The present study had the following aims:

To assess the effects of the COMT inhibitor entacapone in the prevention of Ang II-induced perivascular inflammation and renal damage in a hypertensive animal model with increased RAA system activity (I).

To investigate the role of pharmacological and genetic COMT inhibition in long-term blood pressure regulation in mice exposed to a high-sodium diet (II).

To explore whether COMT inhibition can decrease the formation of homocysteine in rats during L-DOPA-induced homocysteinemia (III).

To investigate the possible changes in COMT activity, expression and localization in the rat brain during lipopolysaccharide (LPS) induced inflammation and subsequent dopaminergic neurodegeneration (IV).
5. MATERIALS AND METHODS

5.1 Animals and chow (I-IV)

I) In the first study we used 4-week-old double-transgenic (dTGR) male rats and their age-matched normotensive Sprague–Dawley (SD) controls were used in the study. The dTGR harbour human angiotensinogen and renin genes and due to this over expressing angiotensin II as described elsewhere [Ganten et al., 1992; Bohlender et al., 1997]. The rats had free access to chow (Harlan, 2018 Global Rodent Breeding, NaCl 0.8% w/w) and drinking water.

II) We used 10-week-old COMT (-/-) mice and their wild-type counterparts. COMT (-/-) mice were generated as described by Gogos and co-workers [Gogos et al., 1998]. The mutated COMT allele was originally introduced into a mixed 129Sv/C57BL/6J genetic background and, by multiple generations backcrossing, the mutation was introduced into a more homogeneous C57BL/6J genetic background. Heterozygous male and female mice were bred to produce mice of all three genotypes. The mouse population was regularly enriched using C57BL/6J males or females bred with COMT heterozygotes. Mice were bred and genotyped in the University of Kuopio, Finland. Part of the mouse tail was clipped from 3- week-old pups for genotyping as described elsewhere [Gogos et al., 1998; Huotari et al., 2002a]. The lack of COMT protein in the liver and kidney was verified by western blotting. During the recovery period and during recording of baseline values, mice were kept on a normal sodium diet (Harlan, 2018 Global Rodent Breeding, NaCl 0.8% w/w). Thereafter, COMT (-/-) and wild-type mice (n = 7 and n = 8, respectively) received a high-sodium diet (normal sodium diet with added NaCl 6% w/w; Riedel de Haen, Seelze, Germany) for 3 weeks.

III) Male Wistar rats, weighing (200-250g, Han/Kuo, Institute of Biomedicine, University of Helsinki) were housed four per cage in a thermostatically controlled room at 23 ± 2 °C at a relative humidity of 40-70%. The room was artificially illuminated from 7 am to 7 pm. Tap water and standard laboratory food (Harlan, 2018 Global Rodent Breeding, NaCl 0.8% w/w) was available ad libitum except for the last 8 hours before the experiment when only water was given.

IV) Male Wistar male rats, (250-350g, Han/Kuo, Institute of Biomedicine, University of Helsinki), were kept 4 per cage under 12 hours light/dark cycle. Food (R36, Tamro, Helsinki, Finland) and water were given ad libitum.

All the experiments and procedures were approved by the Animal Ethics Committees of the Universities of Kuopio and/or Helsinki, Finland, and correspond to the principles of the American Physiological Society.
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5.1.1 The collection of tissue, urine, plasma and serum samples (I-IV)

Prior to tissue or blood sample collection, the animals were anesthetized with CO2/O2 (70/30%) (AGA, Riihimäki, Finland) and then sacrificed with either decapitation (I, III, IV) or with cervical dislocation (II). Tissue samples were promptly dissected, snap-frozen in either liquid nitrogen (I, II, IV), isopentane (–35ºC) (immunohistochemistry) (I) or in liquid nitrogen (whole brain samples for TH immunohistochemistry) (IV), and finally all stored at –80ºC until analyzed. Tissue samples for morphology (I, II) and COMT, OX-42, and GFAP immunohistochemistry (IV) were fixed in 4% buffered paraformaldehyde or 10% formaline at room temperature. The heart, kidneys, and liver were removed, washed with ice-cold saline, blotted dry, and weighed. The ratio of the heart weight to body weight was calculated as an index of cardiac hypertrophy (I). Blood samples were taken into chilled tubes with or without ethylenediaminetetraacetic acid (EDTA) (4.5 mM) as anticoagulant and centrifuged at +4ºC for 15 minutes; then plasma and serum were stored at -80ºC (I).

In some studies, the animals were housed individually in metabolic cages for two days at predefined occasions (in the beginning of the study and at the end) (I, II). In metabolic cages the animals had free access to tap water and chow. Food intake and consumption of water were recorded, and urines were collected over 24-h periods. Urine volumes were measured and samples stored at -80 ºC until the biochemical determinations were performed. For measurements of urine catecholamine concentrations 1 ml of hydrogen chloride (HCl) was placed in urine bottles beforehand to prevent the catecholamine degradation before analysis. For determination of other urine variables, the animals were housed in metabolic cages for additional day. The urine samples were collected in metabolic cages similarly as the urine samples for catecholamine determinations with the exception that no HCl was added to the urine bottles prior to the collections (I, II).

5.2 Chemicals (I-IV)

5.2.1 Drugs

COMT inhibitors were used in all four studies (I-IV). The COMT inhibitors administered in the individual studies were: entacapone (I, III IV), tolcapone (IV), and nitecapone (II) (all gifts from Orion Pharma, Espoo, Finland). The COMT inhibitors were dissolved in a few drops of Tween 20 and diluted in saline (NaCl 0.9%). The vehicle used in the studies was NaCl 0.9%. The L-DOPA and carbidopa, were dissolved in 5% Gum Arabic according to manufacturer’s instructions (Orion Pharma, Espoo, Finland) (III).
Materials and methods

The dosage and administration routes used were as follows:

I) The dTGR and normotensive SD control rats were divided into four groups: (1) dTGR control group (n=20); (2) dTGR + entacapone group (n=10); (3) SD control group (n=10); and (4) SD + entacapone group (n=6). Entacapone (30 mg/kg i.p.) (Orion, Espoo, Finland) was given every 12 h for 3 weeks. As a separate experiment, the acute effects of entacapone (30 mg/kg i.p.) on urinary sodium and catecholamine excretions were examined in 5-week-old dTGR (n = 6 in dTGR control and entacapone-treated dTGR groups).

II) After the 3-week experimental period, subgroups of wild-type mice (n = 4 in each group) were given either nitecapone 30 mg/kg i.p. b.i.d. (Orion Pharma, Espoo, Finland) or vehicle for 8 days. As an additional experiment, the acute effects of a single injection of nitecapone (30 mg/kg i.p.) on urinary excretion of catecholamines were examined in wild type mice receiving a normal sodium diet (n = 6 in both nitecapone-treated and control groups).

III) Acute study: Entacapone (0, 10 or 30 mg/kg, i.p.) and carbidopa (0, 2.5, 7.5 or 25 mg/kg, i.p.) were given 30 minutes before the L-DOPA (0, 10, 30 or 100 mg/kg, i.p.) treatment. Blood was collected 2 hours after the last treatment for dose-response studies and at 0.5, 1, 2, 4 or 8 hours after L-DOPA for the time-course study. Subchronic study: Rats were treated with levodopa (3 x 100 mg/kg/day p.o.) + carbidopa (3 x 25 mg/kg/day p.o.) with or without entacapone (3 x 30 mg/kg/day p.o.) for 5 days to mimic the clinical situation and blood was collected on day 5, two and 8 hours after the last L-DOPA doses.

IV) The first dose of entacapone (30 mg/kg, i.p.) or tolcapone (15 mg/kg, i.p.) was given 1 hour before the stereotaxic infusion of LPS. Thereafter rats were administered with entacapone (30 mg/kg, i.p.) or tolcapone (15 mg/kg, i.p.) b.i.d. for two weeks. The last injection was given 12 h before the dissection of the brain samples.

5.2.2 Reagents

LPS (from Escherichia coli, serotype O111:B4, Sigma Chemical Company) was dissolved in phosphate-buffered saline (PBS) (IV). The chloral hydrate (trichloroacetaldehyde monohydrate, 2,2,2-trichloro-1,1-ethanediol) (IV), Xylazine (II), and Ketamine (II) were used as an preoperative anesthetics. The buprenorfine (Temgesic) was used as a post-operative analgesic (II). A few drops of Tween 20 were used in the reconstitution of entacapone, nitecapone, and tolcapone into the saline vehicle (IV). Homocysteine was obtained from Sigma-Aldrich (St. Louis, MO, USA) (III). Tissue adhesive (3M) and Silk (6-0) were used to secure the telemetric catheter into the carotid artery (II).
5.3 COMT

5.3.1 COMT activity (I, II, IV)

The rats were decapitated and the brain and kidney samples were snap-frozen in liquid nitrogen and stored at -80°C before enzyme analysis (I, IV). The striatal and nigral tissue samples were dissected promptly, weighted and homogenized by sonication (IV). Thin renal cortex samples were sliced with a razor blade before homogenization [Reenilä, 1999] (I, II).

The COMT reaction utilizing DHBAc as a substrate [Schultz et al., 1989; Reenilä, 1999] was conducted as previously reported [Nissinen and Männistö, 1984; Reenilä et al., 1995]. DHBAc concentration used routinely in the COMT assay was many folds higher than the K_m for MB-COMT and half-saturating for S-COMT preparations obtained from rat brain [Nissinen, 1985]. Routinely, the enzyme preparation (100 μl) was incubated for 30 min at 37°C with SAM and DHBAc as a substrate in 250 μl of total volume. After incubation, the reaction was terminated with ice-cold perchloric acid and centrifuged. The supernatants were injected into an HPLC for analysis. Vanillic acid and isovanillic acid were analyzed. The tissue samples were filtered through 0.45 μm polyvinylidifluoride filter (Millipore, Japan) before HPLC analysis. Routinely, samples without enzyme and samples without substrate were run as blanks. Reaction with kidney tissues was made at the same protein level as brain homogenates. Due to the higher COMT enzyme activity in the kidney, the reaction products were diluted (1:10 - 1:20) with homogenizing buffer before HPLC analysis [Reenilä, 1999].

Samples were injected (Waters 717plus autosampler with cooler) into a HPLC system which consisted of an isocratic pump (Waters 510 or 515, Waters Association, Millford, MA, USA) and reversed phase column with precolumn. The reaction products were detected with ESA coulometric detector 5100 A (ESA Inc., Bedford, MA, USA). The response of detector was recorded and calculated with Millenium 32 Software. The mobile phase, 0.1 M Na_2HPO_4, pH 3.2, 0.15 mM EDTA and 15 % (vol/vol) methanol, was used at 1.0 ml/min flow rate as previously described [Reenilä, 1999].

5.3.2 COMT enzyme expression (Western blot) (II, IV)

Tissue samples were homogenized by using an ultrasonic cell disrupter. Ten μl of homogenized sample was taken and diluted with 40 μl of Tris/ EDTA 20 mM/1 mM. The proteins were separated on 10 % SDS-PAGE followed by electrophoretic transfer onto nitrocellulose membranes. After blocking, the filters were incubated with a guinea pig antiserum against rat recombinant S-COMT and MB-COMT proteins (1:1000 dilution) in PBS/0.1 % Tween 20 and 5 % (w/v) non-fat dry milk for 1.5 hours at room temperature, followed by sheep anti-guinea pig Ig (1:1000 dilution) (Boehringer-Mannheim, Mannheim, Germany), for 1 hour at room temperature. The visualization was done with ECL-method (Pierce, Rockford, IL) [Karhunen et al., 1994]. The bands were
scanned and intensities were measured with computer densitometry (Syngene, GeneGenius, Gel Documentation and Analysis System, Synoptics Ltd., Cambridge, UK).

5.3.3 COMT genotyping (Southern blot) (II)

The genotypes of the animals were determined by Southern blot analysis as described earlier [Huotari et al., 2002a]. Genomic DNA was extracted from mouse tail clippings and probed with a 1 kb fragment adjacent to the right arm of the targeting construct of the mouse COMT cDNA, corresponding to exons 2-4. Wild-type and recombinant restriction-specific fragments are 11.5 and 3.5 kb, respectively; heterozygous mice have both fragments. The wild type (WT; COMT +/+) and homozygote (COMT -/-) mice were used in the experiments (II).

5.4 Immunohistochemical studies (I, IV)

For OX-42, GFAP, and COMT immunohistochemistry (IV) the animals were anesthetized with chloral hydrate 350 mg/kg 2 and 14 day after bilateral LPS and vehicle infusion, perfused with 250 ml of 4% paraformaldehyde and post fixed for 2 hours. The specimens were incubated with non-immune swine serum for 20 min at room temperature, with the primary antibody diluted 1:200 for anti-COMT (produced by Dr. Carola Tilgmann, Orion Pharma, Finland), 1:100 for anti-OX-42 (Pharmingen, San Diego, CA USA) or 1:50 for anti-GFAP (a generous gift from Prof. I. Virtanen, University of Helsinki, Finland) overnight at 4°C, and with the secondary antibody diluted 1:200 in PBS. The mounted specimens were examined with Leitz Aristoplan fluorescence microscope with fluorescein or with rhodamine specific filters. The specificities of the GFAP antibody and COMT antiserums have been described elsewhere [Lundström et al., 1992; Karhunen et al., 1994; Lumme et al., 1996]. The monoclonal anti-GFAP has been produced at the Pathology department in University of Helsinki, Finland [Paetau et al., 1985]. COMT antibody used reacts with both COMT isoforms, MB-COMT and S-COMT, since there is no known isoform specific COMT antibody. OX-42 antibody reacts with the CR3 complement (C3bi) receptor found on most monocytes, granulocytes, dendritic cells, peripheral macrophages and microglia. It appears to recognize a common epitope shared by CD11b and CD11c [Robinson et al., 1986; Ford et al., 1995]. The COMT immunohistochemistry in dTGR and SD rats were conducted as described above (I).

For demonstration of tyrosine hydroxylase (TH) positive neurons in the SN area, the animals were anesthetized with chloral hydrate 350 mg/kg and the unilateral infusions of LPS or NaCl were made. Fourteen days after the infusion animals were decapitated. The brains were frozen in dry ice and cut in 10 μm coronal cryostat sections. The sections were fixed in cold acetone (+4 °C) for 5 minutes and processed for TH immunohistochemistry. Between each step, sections were rinsed two times for 5 min each in Trisma-buffered
saline (TBS). Sections were incubated in 10% normal goat serum, 1% BSA in 0.3% Triton-X in TBS for 1 hour at room temperature, and rabbit anti-TH polyclonal primary antibody (AB 152; 1:100; Chemicon) for two hours at 37 °C. Sections were then rinsed two times with TBS, incubated in biotinylated goat anti-rabbit secondary antibody (1:250; Vector Laboratories, Burlingame, CA) for 1 hour at room temperature, rinsed three times in TBS for 5 minutes each and incubated in avidin-biotin complex (Vectastain Elite, Vector Laboratories) for 30 min. Sections were then rinsed three times in TBS. Visualization of TH-immunoreactivity occurred with the use of AEC (3-amino-9-ethyl-carbazole, containing 30% H2O), which provided a bright red cytoplasmic label. The immunostained sections were viewed and photographed using Leica IM500 and Leica QWIN software (Leica Microsystems AG, Heerbrugg, Switzerland) (IV).

For kidney immunohistochemistry, the frozen samples were processed and semi quantitative scoring of leucocytes (ED1, ED2, CD4, CD8), intracellular adhesion molecule-1 (ICAM-1) and COMT (antibody – a generous gift from Dr Carola Tilgmann) were performed as described in detail previously [Reenilä et al., 1997; Mervaala et al., 2000; Müller et al., 2000; Finckenberg et al., 2003] (I). Kidney crysections (5 μm) were treated with acetone (10 min at -20ºC), chloroform (10 min at room temperature) and air-dried. Primary antibodies (mouse anti-rat ED1, ED2, CD4, CD8; Serotec, Norway), mouse anti-rat ICAM-1 (Serotec, Norway) were applied for 60 min at room temperature. The samples were then treated with secondary antibody (peroxidise-conjugated rabbit anti-mouse immunoglobulins; DAKO A7S, Glostrup, Denmark) for 30 min at room temperature. After enhancing the signal with the third antibody (HRP-F(ab´)2 goat anti-rabbit (Zymed, San Francisco, CA) for 30 min at room temperature, the reaction was made visible by an AEC (3-amino-9-ethyl carbazole) solution containing hydrogen peroxidase. Finally the samples were counterstained with hematoxyline and mounted. The expression and relative amount of of 8-OH-DG positive label per sample was determined with computerized densitometry (Leica IM500 and Leica QWIN software; Leica Microsystems AG, Heerbrugg, Switzerland) [Finckenberg et al., 2003]. The immunohistochemistry and semi quantitative scoring for nitrotyrosine and 8-hydroxydeoxyguanosine (8-OH-DG) were conducted with same method as TH immunohistochemistry (described above).

5.5 Other biochemical variables

5.5.1 Tissue protein concentrations (I, II, IV)

Tissue protein concentrations were measured with an Ultrospec III Spectrophotometer (Pharmacia LKB Biotechnology, Uppsala, Sweden) utilizing the dye binding method [Bradford, 1976] (I, II, IV).
5.5.2 Other enzymatic activities

5.5.2.1 TH activity (IV)

Tyrosine hydroxylase (TH) activity was used as a marker for dopaminergic neurons (IV). The enzyme reaction was based on a previous report [Naoi, 1988]. The enzyme preparation (20 μl) was incubated with 100 mM sodium acetate buffer, pH 6.0, 10 mM (NH₄)₂Fe(SO₄), 1 mM dl-6-methyl-5,6,7,8-tetrahydropteridine and 100 μM tyrosine in 250 μl of total volume for 10 min at 37ºC. After addition of 4 M perchloric acid (25 μl) and centrifugation, the reaction product L-DOPA was analyzed with the reversed-phase HPLC system utilizing fluorescence spectrometer (Model LS-5, Perkin Elmer Ltd., Buckinghamshire, UK) at 281 nm excitation and 314 nm emission wavelength [Mandai et al. 1992]. The mobile phase was 0.1 M H₃PO₄, pH 3.00, 20 mM citric acid, 0.15 mM Na₂EDTA, 1 mM octanesulphonic acid and 10 % (vol/vol) methanol with flow rate of 1.0 ml/min [Reenilä, 1999].

5.5.2.2 PDE-I activity (IV)

Alkaline phosphodiesterase I (PDE-I, EC 3.1.4.1.) activity was measured spectrophotometrically (Ultraspec III, Pharmacia LKB Biotechnology, Uppsala, Sweden) from samples prepared as previously described [Storrie and Madden, 1990]. It has been previously shown that macrophage activation with competent stimulus causes 2-7 fold increase in PDE-I activity [Edelson and Erbs, 1978]. Acute neuronal injury also caused significant increase in PDE-I activity together with activation of brain macrophages, microglia [Reenilä et al., 1997]. Both of these studies have used PDE-I as the macrophage/microglia activation marker. In rat brain PDE-I has been found to localize in epithelial cells and glial cells but not in neurons [Narita et al., 1994; Bollen et al., 2000]. Therefore PDE-I activity (formation of p-nitrophenol from p-nitrophenyl-thymidine-5´phosphate) was used in this study as a marker of microglial activation.

5.5.3 Tissue and urine catecholamines and their metabolites (I, II, IV)

High performance liquid chromatography connected with electrochemical detection (HPLC-EC) was used for quantification of tissue and urine DA, NA, L-DOPA, DOPAC and HVA as previously described [Männistö et al., 1992a; Reenilä, 1999].

The tissue and urine catecholamines were analyzed by using the isocratic ion-pair reversed-phase HPLC method with electrochemical detection. The urine samples were transferred to polyethylene vials containing 1 ml perchloric acid and immediately frozen to –80ºC until analyzed. The tissue samples were frozen as they were after dissection to –80ºC until analyzed. After centrifugation and collection of supernatant, the urinary catechols and their metabolites were measured electrochemically following alumina adsorption
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and ion-pair reverse-phase HPLC. The tissue samples were first homogenized with sonication and then centrifuged. The supernatant was collected and the catechols and their metabolites were assessed as from the samples (described above). An internal (3,4-dihydroxybenzylamine, 3,4-DHBA) and external standards were used during procedures [Reenilä, 1999].

5.5.4 Markers of the oxidative stress (I)

5.5.4.1 S-8-isoprostane (I)

At the age of 7 weeks, the rats were decapitated and blood samples were taken for serum 8-isoprostaglandin F₂α determinations. Serum 8-isoprostane concentrations were determined by ELISA (Cayman, Ann Arbor, Michigan, USA) according to the instructions of the manufacturer [Mervaala et al., 2001].

5.5.4.2 The p22phox mRNA (I)

The NADPH oxidase has been associated with cardiovascular pathophysiology via increased oxidative stress [Griendling, 2000a, 2000b]. The mRNA expression of the subunit of NADPH, p22phox, was determined to reflect the expression of NADPH oxidase. For determination of p22phox mRNA expression in the kidney by reverse transcriptase-polymerase chain reaction (RT-PCR), total RNA was first extracted with Trizol (GIBCO) reagent, and treated with DNAase I (Deoxyribonuclease I; Sigma Chemical Co., St. Louis, Missouri, USA). The RNA was reverse-transcribed with Enhanced Avian RT-PCR kit (Sigma Chemical Co.), and amplified using Accutaq DNA polymerase (35 cycles, 94°C 1 min, 50°C 30 s, 72°C 45 s, for p22phox, amplified product 203 bp in size; and 32 cycles, 94°C 1 min, 58°C 30 s, 728C 45 s, for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), amplified product 193 bp in size). The following RT-PCR primers for p22phox and GAPDH were used:

p22phox forward, AAAGAGGAAAAAGGGCTCCA;
p22phox reverse, CTGCCAGCAGGTAGATCACA;
GAPDH forward, TGGGGCAGCCCAGAACATCA;
GAPDH reverse, GCCGCTGCTTCACCACCTT.

The intensity of the resulting bands was measured by a computer-based imaging system (Genetools; Syngene, Cambridge, UK). The density of the p22phox band was expressed relative to the density obtained for GAPDH.

5.5.4.3 The 8-OH-dG immunohistochemistry (I)

The immunohistochemistry for 8-hydroxydeoxyguanosine (8-OH-DG) expression was conducted as previously described (see 5.4). The primary antibody for 8-OH-dG used was polyclonal goat anti-8-OH-dG (Chemicon International, Temceula, USA). The biotinylated anti-goat was used as
secondary antibody (Vector Laboratories, Burlingame, CA, USA). The expression and relative amount of 8-OH-dG positive label per sample was determined with computerized densitometry (Leica IM500 and Leica QWIN software; Leica Microsystems AG, Heerbrugg, Switzerland).

5.5.4.4 Nitrotyrosine immunohistochemistry (I)

The immunohistochemistry for nitrotyrosine expression was conducted as previously described (see 5.4). The primary antibody for nitrotyrsine used was polyclonal rabbit anti-nitrotyrosine (Upstate Biotechnology, Lake Placid, NY, USA). The biotinylated anti-rabbit was used as secondary antibody (Vector Laboratories, Burlingame, CA). The expression and relative amount of nitrotyrosine positive label per sample was determined with computerized densitometry (Leica IM500 and Leica QWIN software; Leica Microsystems AG, Heerbrugg, Switzerland).

5.5.4.5 Cysteine and glutathione kidney concentrations (I)

The concentration of thiol compounds glutathione and cysteine in the kidney was determined fluorometrically after labelling with monobromobimane and separating various thiols on an HPLC column as previously described [Levonen et al., 2000].

5.5.5 S-creatinine (I, II)

The serum creatinine was determined using routine laboratory techniques.

5.5.6 P-homocysteine (III)

Plasma homocysteine concentrations were measured by means of a fluorescence polarisation immunoassay, using the Imx instrument (Abbot Laboratories, Chicago, Ill, U.S.A.).

5.5.7 Creatine clearance (I, II)

Plasma and urine creatinine levels were determined by routine laboratory methods and creatinine clearance was calculated from these values.

5.5.8 Urine sodium excretion (I, II)

The urine sodium concentrations were determined by using routine laboratory techniques.
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5.5.9 U-albumin (I)

Urinary albumin (albuminuria) was measured by ELISA with rat albumin as a standard (Celltrend, GmbH, Lückenwalde, Germany).

5.6 Blood pressure (I, II)

5.6.1 Tail-cuff (I)

The systolic blood pressure and the heart rate (HR) of the pretrained rats were measured with a tail cuff analyzer (Apollo-2AB, Blood Pressure Analyzer, Model 179-2AB, IITC Life Science, Woodland Hills, CA, USA). Before measurement, each rat was warmed at 29°C for 30 minutes to attain detectable pulsations from tail artery. The heart rate was obtained during blood pressure measurement. Systolic blood pressure was determined by the arithmetic mean of the three consecutive results without disturbances in the signal.

5.6.2 Radiotelemetry (II)

Blood pressure and heart rate were recorded from the left carotid artery using TA11PA-C20 telemetric implants (Data Science) and DataQuest IV system (Data Science). The animals were anesthetized and positioned under an operating microscope. The left common carotid artery was dissected visible under a microscope. The sympathetic nerve following artery was separated from the surface of the artery. The sterile catheter tip was inserted into the left common carotid artery under microscope using the branching point of internal and external arteries as a landmark. The catheter was then fixed in position using 6-0 silk ligatures and tissue adhesive [Carlson and Wyss, 2000; Butz and Davisson, 2001]. All mice were allowed 10 days of recovery from surgery before any measurements were made. Baseline values were recorded continuously for 3 days. The data were sampled every 5 min for 10 s, continuously day and night, with a sampling rate of 1000 Hz.

5.7 Stereotaxic surgery (IV)

Rats were anesthetized with chloral hydrate (350 mg/kg i.p.) and positioned in a stereotaxic apparatus (David Kopf Instruments, USA). Unilateral infusion of LPS (10 μg) or vehicle solution was made paranigral into area above the right substantia nigra pars compacta (referred later on as lesion side) to the stereotaxic coordinates [Paxinos and Watson, 1986] 3.2 mm anterior, 2.1 mm lateral and 2.0 mm cranial from interaural line [modified from Iravani et al., 2005]. The infusion volume of 1 μl was delivered over the time period of 5 minutes, and after each injection, the needle was left in place for an additional
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5 minutes to avoid the reflux along the needle tract. The body temperature was maintained at approximately 37°C during and after operation by using a thermo blanket and heating lamp. Bilateral LPS and vehicle infusions were conducted in animals operated for immunohistochemistry.

5.8 Arterial responses in vitro (II)

The samples from abdominal aorta were carefully excised and cleaned of adherent connective tissue. Three millimeter (mm) sections of abdominal aortic were used in the assay. The endothelium-intact arterial rings were stretched on stainless steel hooks and mounted in an organ bath chamber in Krebs-Ringer buffer (pH 7.4) of the following composition (mM): NaCl (119.0), NaHCO3 (25.0), glucose (11.1), CaCl2·2H2O (1.6), KCl (4.7), KH2PO4 (1.2), MgSO4·7H2O (1.2), which was aerated with 95% O2 and 5% CO2. The rings were equilibrated for one hour at +37°C at a resting tension of 1.5 g. The force of contraction was measured with an isometric force-displacement transducer and registered with a polygraph (FTO3C transducer, Model 7C8 Polygraph; Grass Instrument Co., Quincy, MA, USA). The presence of intact endothelium in the vascular preparation was confirmed by observing the relaxation response to 1 μM acetylcholine (ACh) in rings precontracted by 1 μM noradrenaline (NA) or by 0.1 μM NA. Cumulative concentration contractile response curves were determined for NA and DA. Cumulative relaxation responses to ACh and sodium nitroprusside were examined after precontraction with 1 μM NA for mesenteric and aortic arteries, and 0.1 μM NA for the renal artery [Mervaala et al., 2001].

5.9 Data analysis and statistics (I-IV)

Data are presented as means ± SEM. The differences were considered significant when P < 0.05. Statistically significant differences between mean values were tested by ANOVA and Fisher’s least significant difference test (I), ANOVA and the Tukey’s multiple range test (II), Sigma Stat using a paired t-test (III), or by analysis of variance and Newman-Keuls test (IV). The data was analysed using SYSTAT statistical software (SYSTAT Inc., Evanston, Illinois, USA) (I, II).
6. RESULTS

6.1 The effects of COMT inhibition (I-IV)

The effects of pharmacological COMT inhibition were investigated in all four study designs. The dosages, study design and main effects are listed below (Table 1). Study IIIa represents the acute study and IIIb the subchronic study.

Table 1. The main effects of pharmacological COMT inhibition. Double-transgenic rat (dTGR), twice per day (b.i.d), intraperitoneal administration (i.p.), oral administration (p.o.).

<table>
<thead>
<tr>
<th>Study</th>
<th>Drug treatment</th>
<th>Animals (species)</th>
<th>Main drug effects</th>
</tr>
</thead>
</table>
| I     | Entacapone 30 mg/kg i.p. b.i.d. | dTGR (rat) | • 85% reduction in albuminuria  
• Total prevention of mortality |
| II    | Nitecapone 30 mg/kg i.p. b.i.d. | C57BL/6J (mice) + 6% NaCl diet/mice | • Normalized sodium-induced elevation in blood pressure |
| IIIa  | Entacapone (0, 10mg or 30 mg/kg, i.p.) | Wistar (rat) + L-DOPA (0, 10, 30 or 100 mg/kg i.p.) and carbidopa (0, 2.5, 7.5 or 25 mg/kg, i.p.) | • Prevented acute formation of homocysteine |
| IIIb  | Entacapone (3 x 30 mg/kg p.o. per day) | Wistar (rat) + L-DOPA (3 x 100 mg/kg per day p.o.) and carbidopa (3 x 25 mg/kg per day p.o.) | • Prevented sub-chronic formation of homocysteine |
| IV    | Entacapone (30 mg/kg i.p. b.i.d.) or Tolcapone (15 mg/kg i.p. b.i.d.) | Wistar (rat) + paranigral injection of LPS 10 μg | • Did not increase or ameliorate neurotoxicity |

The detailed results of the individual studies (I-IV) are presented in the following chapters.
6.1.1 Effects of entacapone on angiotensin II-induced renal injury (I)

Hyperactive RAA system following overproduction of Ang II is associated with renal perivascular inflammation and increased oxidative stress leading to increased mortality and extensive renal damage in dTGRs [Luft et al., 1999; Mervaala et al., 1999a, 2000; Müller et al., 2000]. These effects are mediated via blood pressure-dependent mechanisms as well as blood pressure-independent mechanisms such as NFkB and activator protein-1 (AP-1) pathways [Luft et al., 1999; Mervaala et al., 1999a, 2000; Müller et al., 2000]. In addition to inhibiting the O-methylation of catechols, COMT inhibitors possess anti-inflammatory and antioxidant properties [Lal et al., 2000; Lyras et al., 2002; Forsberg et al., 2004]. We studied the effect of the COMT inhibitor entacapone in the prevention of Ang II overexpression-induced renal damage in dTGRs.

6.1.1.1 Effects of entacapone on mortality and kidney function

The dTGRs developed severe hypertension and among untreated dTGRs, the cardiovascular mortality was 25% (five out of 20). The 24-h albuminuria was 300-fold greater than that of normotensive Sprague-Dawley (SD) rats (Fig. 6). The dTGRs showed increased serum creatinine levels and decreased creatinine clearance (Table 2).

Entacapone treatment (30 mg/kg i.p. twice per day for 3 weeks) prevented cardiovascular mortality completely and decreased 24-h albuminuria by approximately 85% (Fig. 6). The renoprotective effects were most likely not mediated via a pressure mechanism, since only a small (13 mmHg) decline in systolic blood pressure in entacapone-treated dTGRs was noted (I: Fig. 1a). The serum creatinine level was completely normalized (p<0.05), and creatinine clearance increased significantly (p<0.05) compared with untreated dTGR rats (Table 2).

Fig 6. Bar graph showing the effects of 3 weeks entacapone (EK) (30 mg/kg i.p. b.i.d.) treatment on 24-h albuminuria in double-transgenic rats (dTGRs). Sprague-Dawley rats (SD) were used as control animals. Mean ± SEM are given, n= 10 in each group; *p<0.01.
Results

Table 2. Serum creatinine and creatinine clearances. Double-transgenic rats (dTGRs) harbouring human angiotensinogen and renin genes, entacapone (EK), Sprague-Dawley rats (SD). Mean ± SEM are given, n = 10 in each group. *p<0.05 compared to SD, #p<0.05 compared with entacapone treated dTGRs.

<table>
<thead>
<tr>
<th>Variables</th>
<th>dTGR</th>
<th>dTGR + EK</th>
<th>SD</th>
<th>ANOVA p=value</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-creatinine (μmol/l)</td>
<td>63.4 ± 3.6 *#</td>
<td>43.9 ± 1.9</td>
<td>46.7 ± 0.5</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Creatinine clearance (ml/min per g kidney wet weight)</td>
<td>0.41 ± 0.03 *#</td>
<td>0.61 ± 0.04 *</td>
<td>0.81 ± 0.04</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

6.1.1.2 Morphological evaluation of the effects of entacapone in kidney and in heart

The quantitative morphological evaluation grading scale of the renal and heart tissue samples was described in the original article (I). Untreated dTGR kidneys exhibited severe glomerular and vascular damage with mesangial proliferation, glomerular sclerosis and necrosis, increased intimal and media thickness and deposition of matrix (Fig. 7a). Profound vascular damage with fibrinoid necrosis and vast myocardial infarcts were observed in the heart of dTGRs (I: Fig. 2c). The hypertensive dTGRs also developed extensive cardiac hypertrophy.

Entacapone significantly ameliorated Ang II-induced vascular and glomerular lesions in the kidneys (Fig. 7b and 7c). However, the entacapone treatment resulted in no significant protection against Ang II-induced myocardial damage or cardiac hypertrophy (I: Fig. 2d and 2f).

Figure 7. a) Representative photomicrograph from the kidneys of untreated double-transgenic rats (dTGRs). The comparable photograph of dTGRs treated with entacapone for 3 weeks (b). Kidney damage scores are shown in bar graph (c). Values are means ± SEM, n = 9-10 in each group. *P<0.05 compared with Sprague-Dawley rats (SD).
6.1.1.3 Effects of entacapone on markers of oxidative stress and inflammation

The oxidative stress in dTGRs was evaluated with four different methods, including renal nitrotyrosine expression, renal 8-OH-DG expression, serum 8-isoprostane concentration, and NADPH subunit p22phox mRNA expression. In addition the concentration and redox state of cysteine and glutathione in the kidney were assessed. The renal nitrotyrosine and 8-OH-DG expressions were markedly increased in dTGR compared with SD rats (Fig. 8a and 8b). The serum 8-isoprostane concentration was increased by 130% in dTGR compared with SD rats (Fig. 8c). The p22phox mRNA expression was increased by over 1000% in dTGR rats compared to SD rats (Fig. 8d).

![Fig. 8](image_url)

Fig. 8. Representative bar graphs of the markers for oxidative stress. Renal nitrotyrosine expression representing the NO interaction with superoxide anion O₂⁻ (a). Renal 8-OH-DG expression, a marker for oxidative DNA damage (b). Serum 8-isoprostane concentration, a marker for lipid peroxidation (c). Renal p22phox mRNA expression (d).
Mean ± SEM are given, *p<0.05, n = 9-10 in each group.
Results

Treatment with entacapone ameliorated all of these markers of oxidative stress in dTGRs. Entacapone significantly decreased the expressions of renal nitrotyrosine (Fig. 8a) and 8-OH-DG (Fig. 8b). Entacapone also completely normalized the serum 8-isoprostane concentrations (Fig. 8c). The p22phox mRNA expression was also markedly but not statistically significantly ameliorated by entacapone treatment (Fig 8d). Entacapone did not influence the concentration or redox state of cysteine or glutathione in the kidney (data not shown).

The significantly increased leukocyte infiltration (ED1, ED2, CD4, and CD8-immunopositive cells), and ICAM-1 overexpression in the renal perivascular space in dTGR compared with SD rats is presented below (Table 3). Entacapone significantly ameliorated Ang II-induced perivascular inflammation and ICAM-1 overexpression in dTGRs (Table 3). In SD rats, entacapone had no effect on the number of inflammatory cells or ICAM-1 expression in the kidney (data not shown).

<table>
<thead>
<tr>
<th>Variable</th>
<th>dTGR</th>
<th>dTGR+EK</th>
<th>SD</th>
<th>p=</th>
<th>ANOVA p=</th>
</tr>
</thead>
<tbody>
<tr>
<td>ED1</td>
<td>0.88 ± 0.13*#</td>
<td>0.37 ± 0.05*</td>
<td>0.13 ± 0.02*</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>ED2</td>
<td>0.40 ± 0.06*#</td>
<td>0.22 ± 0.03*</td>
<td>0.09 ± 0.01*</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>CD4</td>
<td>0.38 ± 0.07*#</td>
<td>0.21 ± 0.03*</td>
<td>0.12 ± 0.01*</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>CD8</td>
<td>0.48 ± 0.07*</td>
<td>0.42 ± 0.06*</td>
<td>0.22 ± 0.03*</td>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td>ICAM-1</td>
<td>0.61 ± 0.09*#</td>
<td>0.12 ± 0.02*</td>
<td>0.19 ± 0.03*</td>
<td>0.001</td>
<td></td>
</tr>
</tbody>
</table>

6.1.1.4 Urinary catecholamines and renal COMT activity

The renal COMT activity was significantly inhibited by approximately 40% in 7-week-old entacapone-treated dTGRs when assessed 12 h after the last dosage (Fig. 9a). No statistically significant difference between the renal cortex COMT activity was noted between the 4-week-old dTGRs (597 ± 32 pmol/mg per min) and SD rats (574 ± 49 pmol/mg per min). The COMT activity was evaluated from the whole renal cortex homogenate. However, the COMT expression in the kidney in dTGRs was increased perivascularly by over 500% (Fig. 9b and 9c). This marked increase in COMT expression was found especially in the vascular smooth muscle cells and inflammatory cells of the vascular wall (renal afferent arteries) (Fig. 9b). Interestingly, vascular COMT expression was negligible in 4-week-old dTGR and SD rats,
Results

suggesting a time-dependent increase in renal COMT expression (Fig. 5e and 5f).

![Bar graphs showing renal COMT activity](image)

![Bar graph comparing COMT expression](image)

Figure 9. a) Representative bar graphs showing the effects of 3-week entacapone treatment on renal COMT activity in double-transgenic rats (dTGRs). b) Bar graph comparing the COMT expression in dTGRs, in entacapone-treated 7-week old dTGRs, and in Sprague-Dawley control animals. Representative photomicrographs of COMT expression in the kidney of dTGR (c) and SD rats (d). Mean ± SEM are given, n = 10 in each group, *p<0.05.

We also evaluated the urinary catecholamines to assess the renal dopaminergic tonus. Urinary DA excretion was decreased by 50% in dTGR compared with SD rats (Fig. 10a). Neither acute nor chronic treatment with entacapone normalized the urinary DA excretion in dTGRs. Urinary NA excretion did not differ between dTGR and SD rats and was not influenced by entacapone (Fig. 10b). In normotensive SD rats entacapone treatment did not influence urinary DA or NA excretions (p>0.05, data not shown).
6.1.2 Role of COMT in long-term blood pressure regulation in mice (II)

The effects of genetic lack of COMT and COMT enzyme inhibition were studied in mice during normal (NaCl 0.8% w/w) and high-sodium (6%) diets. The COMT-deficient (-/-) mice and their genetic COMT (+/+ ) littermates were fed a normal diet for 1 week, after which the animals were fed high-sodium diet for 3 weeks. At the end of the study, a subgroup of COMT +/+ mice were kept on the high-sodium diet and treated with nitecapone (30 mg/kg i.p. b.i.d.) for 8 days.

6.1.2.1 Effects of genetic COMT deficiency and high-sodium diet on blood pressure and heart rate

During the normal sodium diet in a baseline situation, the blood pressures of COMT (-/-) and COMT (+/+ ) mice were similar and no differences in heart rate were noted (II: Fig. 4a and 4b). The absolute daytime and night-time systolic blood pressure values in COMT (-/-) mice (120.3 ± 1.1 and 130.3 ± 1.4 mmHg, respectively) were comparable to those of wild-type controls (121.1 ± 1.3 and 129.6 ± 1.2 mmHg, respectively) (II: Fig. 2a).
Results

After a 1-week normal diet, the mice were fed the high-sodium (NaCl 6%) diet for 3 weeks. During the high-sodium diet the blood pressure of COMT (+/+) mice increased significantly during the night. A high sodium intake for 3 weeks increased the night-time systolic and diastolic blood pressures in wild type mice by 7.4 mmHg and 2.0 mmHg (p<0.05 compared with baseline values; Fig. 11a), whereas only a tendency towards decreased blood pressure was noted in daytime blood pressures. Importantly, no significant changes in COMT (-/-) mice night- or daytime blood pressures were noted. However, the heart rate in COMT (-/-) mice was significantly lower after 2 weeks on a high-sodium diet (Fig. 4a and 4b). It should be stressed that rodents are nocturnal animals that are active at night and which rest during the day. The blood pressure in both COMT (-/-) and (+/+) mice displayed a 24-h rhythm characterized by several night-time peaks, the first of which occurred immediately at the start of the dark period (Fig. 3a-d).

6.1.2.2 Effects of pharmacological COMT inhibition on blood pressure and heart rate

To further evaluate the influence of COMT inhibition on blood pressure, a subgroup of wild-type (COMT +/+) mice were maintained on a high-sodium diet for an additional 8 days and were treated either with the COMT inhibitor nitecapone (30 mg/kg i.p. b.i.d) or a vehicle. Nitecapone prevented the salt-induced changes in the blood pressure of COMT (+/+) mice (Fig. 11b). No effects in heart rate were noted (data not shown). Vehicle injection had no effects on blood pressure or heart rate of COMT (+/+) mice (data not shown).

![Fig. 11. Effects of genetic (COMT -/-) (a) and pharmacological (nitecapone) (b) COMT enzyme blockade on night-time blood pressures (BPs) in mice during normal high-sodium diet. The baseline values (week 0) represent the night-time blood pressures during normal diet (a). The effect of 8 days treatment with nitecapone to night-time systolic blood pressure in wild type (WT) mice receiving high-sodium diet for three weeks (b). Mean ± SEM are shown in the figures. N= 7-8 in both groups, *p<0.05.](image-url)
Results

6.1.2.3 Urinary and kidney catecholamines and renal COMT activity

At baseline, 24-h urinary excretion of L-DOPA, DA and NA was 145%, 85% and 74% higher in COMT (-/-) mice than in wild-type COMT (+/+) controls (Table 4). The high-sodium diet increased urinary excretion of L-DOPA by 405% and 660%, reflected as 102% and 212% increases in excretion of DA in COMT (-/-) and wild-type mice, respectively. The absolute amounts of urinary L-DOPA and DA remained 60% and 20% greater in COMT (-/-) mice. The cortical NA content in the kidney of COMT (-/-) mice was 2-fold greater in COMT deficient mice than in COMT (+/+) mice (4.24 ± 0.20 μg/g compared with 1.72 ± 0.22 μg/g kidney tissue, respectively; P < 0.001).

The high-sodium diet increased NA excretion by 104% and 188% in COMT (-/-) and wild-type mice, respectively. An additional experiment assessing the effects of COMT inhibition on catecholamine excretion in COMT (+/+) mice was also conducted. In this study, in which COMT (+/+) mice received the normal diet, acute treatment with nitrocapone increased urinary excretion of NA by 160% (5.37 ± 0.40 nmol/day compared with 2.07 ± 0.22 nmol/day; p<0.001) and urinary excretion of DA by 50% (6.40 ± 0.63 nmol/day compared with 4.35 ± 0.72 nmol/day; p<0.05), and tended to increase urinary excretion of L-DOPA (2.43 ± 0.46 nmol/day compared with 1.99 ± 0.67 nmol/day; p=0.27, see Table 4).

Table 4. Urinary catecholamine (L-DOPA, NA and DA) concentrations (nmol/day). Only the results of nitrocapone-treated COMT +/- mice are shown in the table. The values of the urinary catecholamine excretion of their control groups are in the text (see 6.1.2.3). The results of the nitrocapone treatment are from an additional study. The results are shown mean ± SD, n = 6-10 in each group.* and # p<0.05 compared to COMT +/- animals, ¤ p<0.05 compared to untreated COMT +/- mice.

<table>
<thead>
<tr>
<th>Urinary catecholamine excretion (L-DOPA, NA, and DA nmol/day)</th>
<th>Baseline</th>
<th>6% NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urinary: COMT +/- COMT +/- + Nitrocapone COMT +/- COMT +/-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-DOPA 1.0 ± 0.4 2.4 ± 1.8 2.4 ± 1.5 7.5 ± 2.6a 12.1 ± 4.0a#</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NA 2.1 ± 0.8 3.7 ± 1.0a 5.4 ± 1.0a 6.2 ± 1.2a 7.6 ± 1.1a#</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DA 4.5 ± 1.9 8.3 ± 4.0a 6.4 ± 1.9a 12.9 ± 3.8a 15.6 ± 2.0a #</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

6.1.2.4 Sodium excretion and urine volumes

There were no differences between the strains of mice with respect to 24-h sodium excretion or urine volumes during either low-sodium or high-sodium diets (II: Table 1). Sodium excretion correlated with DA excretion when the mice were fed the normal sodium diet (r = 0.58, p<0.05). Serum creatinine and creatinine clearance were similar in COMT (-/-) mice (32.6 ± 1.4 μmol/l and 0.13 ± 0.01 ml/min per g kidney wet weight, respectively) and COMT (+/+) controls (32.9 ± 1.0 μmol/l and 0.16 ± 0.02 ml/min per g kidney wet weight,
6.1.3 Effects of COMT inhibition on prevention of L-DOPA-induced homocysteinemia (III)

The effects of the COMT inhibitor, entacapone (3, 10 or 30 mg/kg per day) on prevention of hyperhomocysteinemia were studied. The baseline value of homocysteine in Wistar rats was approximately 7-8 μMol/l. The acute dosage of L-DOPA (10, 30 or 100 mg/kg per day i.p.) + the DDC inhibitor carbidopa (2.5, 7.5 or 25 mg/kg per day i.p.) increased homocysteinemia. The combined L-DOPA + DDC inhibitor therapy dose-dependently increased the homocysteine formation in an acute study when measured 2 h after drug administration. The highest dosages (100 mg/kg of L-DOPA + 25 mg/kg of carbidopa) increased the homocysteine formation roughly 2-3 fold in 2 h (III: Table 1). The homocysteinemia peaked in two hours but was still above the baseline levels 8 h after the administration of L-DOPA (III: Figure 1).

In a subchronic study, the animals were treated with L-DOPA (100 mg/kg per day x 3 p.o) + carbidopa (25 mg/kg per day x 3 p.o.). The plasma homocysteine levels were assessed 2 h and 8 h after the last doses. Entacapone (30mg/kg/day x 3 p.o.) alone did not influence the baseline homocysteine levels (III: table 2). Combined L-DOPA + DDC inhibitor therapy again increased homocysteine formation by approximately 3-fold, as measured 2 h after administration. Entacapone prevented the L-DOPA-induced homocysteine formation almost completely at 2 h time point (III: Table 2). In addition, the homocysteine accumulated in the subchronic study. When measured on the fifth day 8 h after the last dose, the homocysteine levels were still nearly 2-fold higher than the baseline levels (III: Table 2).

6.1.4 Inflammation-induced effects on COMT activity and expression in the CNS (IV)

6.1.4.1 LPS-induced inflammation in the SN

The paranigral LPS injection (10 μg) induced inflammation in the injected area. Activation of the microglial cells and subsequent inflammation were confirmed with the assessment of nigral PDE-I activity, a marker of activated microglia/macrophages. The PDE-I activity in the SN was increased by over 1300% during the first 2 days and declined from then on; it was still increased by approximately 400% 2 weeks after the LPS injection (Fig. 12a). The activation and proliferation of microglia were also evident in the immunohistochemical studies, which showed an extensive time-dependent increase of OX-42-ir in the SN (Fig. 12b). OX-42 is known to label microglia and other macrophage cell line cells. The OX-42-ir cells were identified by their morphology as predominantly activated microglial cells. In addition, the more intense OX-42-ir in individual microglial cells (Fig. 12c and 12d), the
Results

number of OX-42-ir cells in the SN was also significantly increased, suggesting extensive proliferation of microglial cells in the SN area.

![Graph](image)

**Figure 12.** Representative bar graph of phosphodiesterase-I (PDE-I) activity in the substantia nigra (SN) (a). Immunohistochemical photographs of OX-42 immunoreactivity (-ir) in the SN in the control side (b) and lipopolysaccharide (LPS)-injected side 2 days (c) and 14 days (d) after the injection.

### 6.1.4.2 Neuronal injury

The paranigral LPS injection (10 μg) induced marked neurodegeneration of the nigrostriatal pathway. The dopaminergic neurodegeneration was confirmed with three different methods: decreased striatal DA and TH concentrations as well as significant decrease of TH-positive neurons in SN. The striatal DA concentration was time-dependently decreased by over 50% in LPS-infused rats 1 and 2 weeks after the paranigral LPS injection (**IV**: Fig. 1b). The striatal TH activity was also decreased significantly 1 and 2 weeks
after the LPS injection, supporting the degeneration of nigrostriatal dopaminergic neurons (IV: Fig. 2). The significantly decreased amount of TH-positive neurons in the SN was confirmed in immunohistochemical studies (IV: Fig. 3).

6.1.4.3 Inflammation-induced up-regulation of COMT activity and expression

LPS-induced inflammation and subsequent neurodegeneration of the nigrostriatal pathway were accompanied by marked increase in nigral COMT activity and expression. The inflammation up-regulated COMT activity by over 200% in 2 weeks (Fig. 13a). The Western blotting studies were assessed to evaluate the protein expressions of the COMT isoenzymes MB-COMT and S-COMT, showing significant increases in both MB-COMT and S-COMT expressions (Fig. 13b). However, the S-COMT expression was increased significantly more than the MB-COMT expression (Fig. 13b). In immunohistochemical studies, we located this increased COMT expression in OX-42-ir activated microglial cells by double-staining the same sections for both COMT and OX-42 (Fig. 14a and 14b).

![Figure 13](image)

Figure 13. Representative bar graph of time-dependent increase in nigral COMT activity (a). The COMT isoenzyme expressions were evaluated with Western blotting 2 and 14 days after the nigral LPS or vehicle injections. The corresponding blot is represented in the figure (b).
Results

6.1.4.4 Localization of increased perivascular COMT expression in the CNS

Substantial increases in perivascular COMT-ir (Fig. 14a) as well as OX-42-ir (Fig. 14b) were noted 2 weeks after the paranigral LPS injection. In the double staining of the brain, the COMT-ir and OX-42-ir colocalized almost completely (Fig. 14a and 14b), whereas complementary staining patterns between the COMT-ir and GFAP-ir in the SN were noted (IV: Fig 8a and 8b).

Figure 14. Representative higher magnification photomicrographs (a and b) from the rat substantia nigra (SN) 14 days after paranigral lipopolysaccharide infusion. The double-stained nigral sections show a complementary perivascular staining pattern of COMT (a) and OX-42 (b).

6.1.4.5 Effects of COMT inhibition in LPS-induced neuronal injury

Since the LPS-induced dopaminergic neurodegeneration is associated with inflammation and increased oxidative stress, the effects of COMT inhibitors possessing anti-inflammatory and antioxidant properties were investigated. The effects of two different COMT inhibitors, entacapone (30 mg/kg i.p. b.i.d. for 2 weeks) and tolcapone (15 mg/kg i.p. b.i.d. for two weeks), were studied. No significant neuroprotective or neurodegenerative effects were noted (IV: Fig. 10).
7. DISCUSSION

Although the COMT enzyme was discovered almost five decades ago, only in recent years has this O-methylating enzyme drawn increasing amount of attention as a possible etiological factor for various diseases. Evidence regarding the role of COMT in pathophysiology has been accumulating. However, despite recent advances the pathophysiological role of COMT at biochemical level is still more or less obscure. This doctoral thesis was conducted to provide novel information and theories on the role of the COMT enzyme and COMT inhibition in the CNS and in cardiovascular and renal pathophysiology using experimental animal models.

We investigated the role of COMT and possible changes in COMT expression and activity in rats in Ang II-induced tissue and organ damage, during CNS inflammation, L-DOPA treatment and in mice predisposed to excess sodium intake. The role of COMT was further defined with the use of pharmacological and genetic COMT enzyme blockade.

Majority of the discussion about the strengths and limitations of the studies will be discussed in the chapters considering the findings. However, some most important points are discussed next.

These studies reported new roles of COMT enzyme as well as novel effects of COMT inhibition both in the CNS as well as in the peripheral tissues. The use of experimental animal models can provide tools to investigate the possible biochemical reactions and behaviour of certain biochemical factor, like COMT in this case, in an in vivo environment, in study designs, which can not be conducted in humans. Experimental animal models can also provide results and new hypothesis, which can possibly be later tested in humans. No direct conclusions about the humans can be drawn merely based on experimental animal studies. All the speculation about the possible correlation in humans is hypothetical until the results reported have also been confirmed in clinical trials.

The animal models used in these studies have been well established and used also by other laboratories in several studies. The three first studies (I, II, III) were built to answer a specific question using simple and straightforward study design. The main aim of the fourth study was intentionally somewhat broader, but still fairly specific: to clarify the possible changes in COMT enzyme status during inflammation. The strengths of these studies include that the main aims of the studies could be answered based on the study design. The fact that the results also raised multiple new questions, however, can not be considered as a limitation but rather a consequence of successful study on a new area of investigation.

The strengths of these studies also include the use multiple variables to confirm the critical conclusion in the studies. In the injury models (I, IV) the damage was always confirmed with more than just one variable. Changes in
Discussion

the COMT enzyme status were also confirmed with three different methods: immunohistochemistry, enzyme activity assay as well as western blot analysis (IV). The battery of inflammatory and oxidative stress markers was fairly extensive and the methods were previously reported reliable assays (I). The immunohistochemical and morphological semi-quantitative methods have some clear limitations related to the fact that human evaluation takes place. However, the evaluation of the kidney and the heart samples was conducted by qualified pathologist from multiple samples with pre-established and previously reported grading scale. Furthermore, all samples were blindly evaluated by same person to reduce the possibility of errors.

In the work investigating the blood pressure changes in COMT deficient mice we used state of art method utilising radio telemetric blood pressure surveillance and computer analysis (II). Also, the other laboratory methods used in our studies are mainly well established and reliable assays, which have been widely used in previous publications by our as well as other laboratories.

Limitation of our studies include a few instances were some conclusion had to be drawn based on only one variable like renal dopaminergic tone in the dTGR (I). The urine catecholamine excretion was used as a marker of renal dopaminergic tonus. Although, the urinary DA excretion has been widely used as a marker of renal dopaminergic tone, its limitations reduce our possibilities to make any strong statements about the DA signalling in the renal tubular cells. Even though the method by itself is reliable, some concern about the urinary DA as a marker of renal dopaminergic tone has to be raised. Microdialysis study in rats by Wang and co-workers suggested that large portion of tubular DA would not be transferred to the urine but renal interstitial fluid instead [Wang et al., 2001]. Since no microdialysis assay was conducted this methods leaves some speculation whether changes in DA concentration in the renal interstitial fluid could have been noted. However, the possibility that changes in DA concentration would be notable only in one of these two renal compartments seems somewhat unlikely and the conclusions made based on this assay were cautious. Furthermore, since metabolic period the nitrocatechol dosing was conducted only at the beginning of the experiment, small temporal changes in urinary DA excretion might have been obscured by the 24h collection period.

The lack of dose-response studies in I, II and IV manuscripts using COMT inhibitors could also be considered a small limitation. However, in both studies the reason for use of COMT inhibitors was to evaluate their effects towards tissue injury. In both studies high dosages were thus chosen. In the dTGR model (I) the dose-response study could have possibly increased our knowledge concerning the role of direct COMT inhibition in the study. However, since our main aim was to investigate whether COMT inhibitors protected against the injury, single high dose seemed adequate. Furthermore, one could always argue that the b.i.d. dosing of COMT inhibitor was not optimal and dosing should have been conducted more often with lower doses to achieve more constant COMT inhibition. This kind of study design could have produced more information about the mechanism of action of COMT
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inhibitors. However, this dosing was chosen based on previous pharmacokinetic studies and provided an answer for our main phrasing of a question. If the COMT deficient or knockout rats would be in use, the study design based on these animals may have solved some of these probes like was shown in the second study (II). However, the study with COMT deficient mice (II) showed that with b.i.d. dosing of COMT inhibitor, a physiologically adequate COMT inhibition can be achieved. In the LPS induced inflammatory model, on the other hand, the use of lower dosages seemed futile since not even the high doses were able to cause any changes in the injury markers.

The fact that these studies report no direct evidence of causality could also be considered a limitation. However, the cast-iron evidence of causality is always extremely hard to prove. The strong associations/correlations noted in most of our studies can hardly be considered as a limitation but rather strength of these studies, especially, since these works covered an area not investigated before. It was thus likely that new question would raise and we could not answer to all of those within these publications.

In the following chapter (7.1), our findings concerning the effects of COMT inhibitors in the CNS and in cardiovascular pathophysiology will be first discussed. The second chapter (7.2) contemplates the role of the COMT enzyme from the perspective of physiology and pathophysiology, based on the results reported in our studies. The future perspectives and the possible clinical relevance of these findings are also discussed.

7.1 Effects of COMT inhibition in the brain and in the cardiovascular and renal pathophysiology (I-IV)

The effects of COMT inhibitors were examined in four different study designs (I, II, III, IV, Table 1). First, the COMT inhibitor entacapone was shown to protect against the Ang II induced renal damage and prevent the mortality in dTGR (I). Second, the genetic and pharmacological COMT inhibitions were shown to protect against the blood pressure-elevating effects of high sodium diet in mice (II). Third, the COMT inhibitor was shown to ameliorate the hyperhomocysteinemia during L-DOPA-induced homocysteinemia both acutely and subchronically in rats (III). Finally, since the COMT inhibitors had protected against the Ang II-induced inflammation and renal damage, their effects were studied in an inflammatory hemiparkinsonian animal model. Although, there has been some speculation that the enhanced catechol auto-oxidation occurs after COMT inhibition and that this might lead to increased oxidative stress and potentially amplify dopaminergic neurodegeneration, neither tolcapone nor entacapone increased LPS induced neurotoxicity in this model (IV). The results and conclusions of these studies are discussed next in detail.
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7.1.1 Entacapone protects against the Ang II-induced renal injury (I)

The effects of COMT inhibition by entacapone in Ang II-induced renal damage were investigated (I). The dTGRs harbouring the human renin and angiotensinogen genes develop hypertension, cardiac hypertrophy, and renal damage due to increased local Ang II formation [Luft et al., 1999; Mervaala et al., 1999b, 2000; Müller et al., 2000]. Ang II induces tissue damage in dTGR both by blood pressure-dependent as well as -independent mechanisms [Mervaala et al., 2000]. Enhanced Ang II production in dTGRs inhibits natriuresis in the kidney by decreasing renal blood flow and by increasing tubular sodium reabsorption via the AT₁ receptors, which eventually leads to hypertension [Matsusaka and Ichikawa, 1997]. The renal dopaminergic system has contrasting effects on sodium excretion [Pelayo et al., 1983; Siragy et al., 1989; Baines et al., 1992; Chen and Lokhandwala, 1993; José et al., 1998a; Aperia, 2000; Carey, 2001a; Pedemonte et al., 2005] promoting natriuresis and diuresis through inhibition of sodium transport proteins such as Na⁺/K⁺-ATPase and Na⁺-H⁺-exchanger in the PTCs. Furthermore, the D₁ receptor activation down-regulates the AT₁ receptor expression in the PTCs [Cheng et al., 1996; Wang et al., 2003]. Ang II overproduction also generates ROS, activates the redox-sensitive transcription factors such as NFkB and AP-1 and induces severe perivascular inflammation as well as adhesion molecule overexpression via AT₁ receptor stimulation in dTGRs [Luft et al., 1999; Mervaala et al., 1999a, 2000; Müller et al., 2000]. Since COMT inhibitors possessing antioxidant properties could potentially enhance the renal dopaminergic system as well as ameliorate oxidative stress in dTGRs, the effects of entacapone were studied.

The main findings were that the COMT inhibitor entacapone completely prevented cardiovascular mortality in dTGRs, which was 25% in untreated dTGR rats at the age of 7 weeks. Furthermore, entacapone decreased the albuminuria by approximately 85% compared with untreated dTGRs (Fig. 6). COMT inhibitor treatment also increased creatinine clearance and decreased the serum creatinine levels (Table 2). Entacapone also significantly ameliorated Ang II-induced vascular and glomerular lesions in the kidneys (Fig. 7). These findings clearly indicate that entacapone exerts renoprotective effects in dTGRs. Also, treatment with another COMT inhibitor, nitecapone (30 mg/kg i.p. b.i.d. for 3 weeks), resulted with similar effects on all the injury markers discussed above [Helkamaa et al., unpublished data].

To evaluate the protective mechanisms of entacapone treatment, several blood pressure-dependent and -independent variables were evaluated. Importantly, despite the remarkable effects of entacapone treatment on cardiovascular mortality and renal function in dTGRs, entacapone only tended to ameliorate the development of hypertension. The blood pressure in entacapone-treated dTGRs remained significantly higher than that in the nontransgenic control SD rats, suggesting that the protective effects of entacapone were mediated by blood pressure-independent mechanisms. This was also supported by the finding that COMT inhibition only tended to
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Ameliorate cardiac hypertrophy, which correlates very closely with the systemic blood pressure level in this animal model.

Since entacapone did not evoke any significant changes in the blood pressure-dependent variables, we assessed other possible therapeutic mechanisms of entacapone. We investigated the markers of oxidative stress as well as the renal aminegenic tone by evaluating the 24-h urinary catecholamine excretion.

The present study demonstrated that the dTGRs showed attenuated renal dopaminergic tone, based on the 24-hour urinary DA excretion (Fig 10). COMT inhibitors have been reported to increase DA content in the kidney, as measured using a microdialysis technique [Wang et al., 2001] and there is also accumulating evidence suggesting that renal dopamine production and/or DA receptor function are defective in arterial hypertension, as well as in renal parenchymal diseases [José et al., 1998a; Aperia, 2000; Carey, 2001a]. Entacapone could thus be speculated to mediate some of its beneficial effects via increased renal dopaminergic tone, which could among other effects also down-regulate the AT1 receptors in the proximal tubules [Cheng et al., 1996; Wang et al., 2003]. However, chronic entacapone treatment did not affect the Ang II-induced decrease in urinary DA excretion, even though entacapone inhibited the renal COMT activity by approximately 40% in 7-week-old entacapone-treated dTGRs when assessed 12 h after the last dosage (Fig. 9a; I: Fig. 5a). To further elucidate the role of COMT in regulation of renal DA metabolism in dTGRs, we examined the acute effects of entacapone on urinary DA excretion in young dTGRs without manifest renal damage. Consistent with the data from our chronic experiment, acute entacapone treatment likewise did not increase urinary DA excretion in young dTGRs. Although we were not able to confirm the increased dopaminergic tone in entacapone-treated dTGRs, this possibility cannot be completely ruled out. Entacapone causes a short-lasting, 50% maximal, increase in urine DA excretion in anaesthetized rats and produces natriuresis when given acutely [Eklöf et al., 1997; Odlind et al., 1999, 2001a; Vieira-Coelho et al., 2001; Wang et al., 2001]. It is evident that during our 24-h urinary collection period we were unable to detect any short-lasting increases in urinary DA excretion. Furthermore, since catecholamines were collected only from the urine, this study does not provide information about the possible changes of DA in the renal interstitial tissue were major portion of the DA has been suggested to be transported from the PTCs during COMT inhibition in rats [Wang et al., 2001].

Interestingly, the COMT inhibitors may also possess direct D1 receptor affinity [Vieira-Coelho et al., 2001]. COMT inhibitors have been shown to produce a profound natriuresis without increasing the urinary DA excretion. It was, thus, speculated that they may possess direct D1 receptor affinity. This is interesting, since the direct D1 receptor affinity of COMT inhibitors could also explain the protective effects of entacapone in dTGRs, for example, due to down-regulation of PTC AT1 receptors [Cheng et al., 1996; Wang et al., 2003]. D1 receptor activation could thus inhibit Ang II-induced effects and ameliorate Ang II-induced renal inflammation. On the other hand, so could DA mediated D1 receptor activation. However, the direct D1 affinity of COMT inhibitors has
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not been reliably confirmed and it is unlikely that the tissue levels of entacapone would be high enough to have significant effects on D1 receptors.

Previous studies have also revealed that in addition to COMT inhibition, entacapone and nitecapone exert anti-inflammatory properties [Suzuki et al., 1992], inhibit transcription factor NFkB [Suzuki and Packer, 1994], scavenge ROS [Suzuki et al., 1992; Marocci et al., 1994], act as iron chelators [Haramaki et al., 1995; Orama et al., 1997], and inhibit lipid peroxidation [Suzuki et al., 1992; Haramaki et al., 1995] at low micromolar concentrations. COMT inhibitors also provided protection against the development of streptozotocin-induced diabetic nephropathy [Lal et al., 2000]. Increased Ang II production in dTGRs mediates some of the harmful effects in the kidney via increased generation of ROS and activation of the redox-sensitive transcription factors NFkB and AP-1. In addition, Ang II induces severe perivascular inflammation and adhesion molecule overexpression through AT1 receptor stimulation [Luft et al., 1999; Mervaala et al., 1999a, 2000; Müller et al., 2000; Schiff and Touyz, 2003]. Thus, the effects of entacapone on markers of oxidative stress and inflammation were also evaluated.

We used four different approaches to evaluate the influence of entacapone treatment on oxidative stress (Fig. 8). First, we measured serum 8-isoprostane concentrations, using an enzyme-linked immunosorbent assay (ELISA). The isoprostanes are a unique series of prostaglandin-like compounds formed in vivo from the free radical-catalysed peroxidation of arachidonic acid, independent of the cyclo-oxygenase enzyme [Morrow and Roberts, 1996]. Quantification of F2-isoprostanes is used as a reliable marker of lipid peroxidation in vivo [Cracowski et al., 2002]. Second, we used tissue nitrotyrosine staining as a footprint of NO interaction with O₂⁻ [Vaziri et al., 2002]. Third, 8-OH-DG formation was used as a marker for oxidative DNA damage [Kasai, 2002]. Finally, the renal mRNA expression of the NADPH oxidase subunit p22phox was assessed. The p22phox mRNA expression was increased by over 1000% in dTGR rats compared with SD rats (Fig. 9d). The p22phox mRNA expression was also ameliorated by entacapone treatment (Fig. 9d). Entacapone thus normalized serum 8-isoprostane concentration in dTGRs, and also ameliorated Ang II-induced increases in tissue nitrotyrosine, 8-OH-DG, as well as p22phox mRNA expressions.

Furthermore, Ang II-induced renal damage is also associated with increased glomerular capillary pressure and increased glomerular permeability to proteins [Remuzzi and Bertani, 1998]. Excessive reabsorption of filtered proteins by PTCs may, in turn, trigger the activation of inflammatory genes via NFkB-dependent and -independent mechanisms, ultimately leading to renal scarring [Remuzzi and Bertani, 1998]. In the present study, entacapone effectively ameliorated Ang II-induced albuminuria by over 85% (Fig. 6). Entacapone also exerted clear protection against Ang II-induced glomerular and vascular damage (I: Fig. 2a, 2b and 2e). Since COMT inhibitors inhibit NFkB activation [Suzuki and Packer, 1994] and albuminuria could activate NFkB, leading to renal damage [Zoja et al., 1998; Remuzzi and Bertani, 1998], it is possible that the renoprotective effects of entacapone were
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These results thus support the hypothesis that the beneficial effects of entacapone on Ang II-induced glomerular and vascular injury may be related to its antioxidant properties. Decreased oxidative stress, in turn, leads to inhibition of NFκB activation and decreased activation of genes encoding chemokines, cytokines and other inflammatory mediators. Previous studies revealed that COMT inhibitors scavenge peroxyl radicals, NO and O₂⁻ and inhibit lipid peroxidation, as well as NFκB activation [Suzuki et al., 1992; Suzuki and Packer, 1994; Marcocci et al., 1994; Haramaki et al., 1995; Orama et al., 1997]. This would also be in line with the previous study showing that COMT inhibitor nitecapone protects against the streptozotocin-induced diabetic nephropathy via its anti-oxidant properties [Lal et al., 2000] rather than increased renal dopaminergic tonus. The marked anti-inflammatory effects of entacapone found in the present study may thus be secondary to its antioxidant properties. However, the release of renal DA also directly into the renal vein and plasma has been previously demonstrated in rabbits [Noshiro et al., 1995]. Since microdialysis studies [Wang et al., 2001] suggested that most of the increased renal DA during COMT inhibitor treatment also in rats is transported into the renal interstitial tissue instead of the urine, we cannot rule out the possibility that entacapone may have increased renal dopaminergic tonus after all, since we measured the DA only in the urine.

In conclusion, the COMT inhibitor entacapone was shown to protect against Ang II-induced renal damage and cardiovascular mortality via ameliorating the renal inflammation. Our results suggest that the effects were at least partially mediated via the antioxidant and anti-inflammatory properties of the COMT inhibitors. However, based on methods used, we can not exclude or confirm the possible direct beneficial effects of entacapone treatment in the renal dopaminergic system.

7.1.2 Effects of pharmacological and genetic COMT enzyme blockade on regulation of blood pressure and sodium homeostasis (II)

Based on the existing literature the COMT enzyme plays a significant role in the metabolism of renal DA and its precursor L-DOPA. DA promotes natriuresis by inhibiting the sodium transport proteins in the renal tubules [José et al., 1998a; Aperia, 2000; Carey, 2001a; Pedemonte et al., 2005]. Only about 1% of the filtrated sodium is normally excreted and the rest is reabsorbed via sodium transport proteins. Approximately 60-80% of the reabsorption takes place in the proximal tubules [Weiner, 1990; Adroqué and Madias, 2007]. Approximately half of this basal renal Na⁺ excretion has been estimated to be mediated by the paracrine action of renal DA [Pelayo et al., 1983; Siragy et al., 1989] and COMT inhibitor increase natriuresis by enhancing the renal dopaminergic tone and DA signalling via D₁ receptors [Eklöf et al., 1997; Hansell et al., 1998; Odlin et al., 1999; Wang et al., 2001; Vieira-Coelho et al., 2001]. Renal sodium handling plays a major role,
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especially in renal long-term blood pressure regulation [Guyton, 1991]. However, the role of the COMT enzyme or the effects of COMT blockade in long-term blood pressure regulation during sodium loading had not been investigated previously. For this reason, the effects of genetic and pharmacological COMT enzyme blockade during normal and high-sodium diets in mice were investigated (II).

No differences in blood pressures during a normal diet were noted between the COMT-deficient (COMT -/-) mice and their genetic wild-type littermates (Fig 12a). However, high-sodium diet (6% NaCl) increased the blood pressure in control mice but not in COMT-deficient mice. The difference in blood pressure lasted until the end of the study (3 weeks, Fig. 11a), after which a subgroup of COMT (+/+) mice were maintained on the high-sodium diet and treated with the COMT inhibitor nitecapone for an additional 8 days. The pharmacological COMT inhibition normalized the blood pressure in 8 days in COMT (+/+) mice, despite the continuous high-sodium intake (Fig. 11b). Our study was the first report on the association of inborn or pharmacological COMT blockade and blood pressure regulation. More importantly, recent studies have supported our findings and the significant role of COMT enzyme in blood pressure regulation was also found in humans [Hagen et al., 2007; Kamide et al., 2007]. The functional COMT polymorphism affecting the overall tissue COMT activity has been reported to increase the blood pressure in high-activity Val allele subjects. The Val/Val genotype of the COMT enzyme correlated with higher systolic blood pressure among a large Scandinavian population sample free of any major self-reported cardiovascular diseases [Hagen et al., 2007]. In addition, another smaller study investigating hypertensive genes in a Japanese population showed that the high-activity Val allele correlated marginally with the hypertension (p<0.07) [Kamide et al., 2007]. This study also reported some other novel single-nucleotide polymorphisms of the COMT gene, which showed an even stronger association with hypertension in Japanese hypertensive subjects [Kamide et al., 2007]. This study was also interesting, since the Japanese population is known to use a high amount of sodium in their diet. The same research group previously associated the COMT gene as a possible candidate gene for hypertension in Dahl salt-sensitive rats [Okuda et al., 2004], further supporting role of COMT enzyme, especially in salt-sensitive hypertension.

To evaluate the mechanism of sodium resistance we assessed the renal sodium and catecholamine excretion in COMT-deficient mice and their genetic COMT +/+ litters. As expected the high-sodium diet increased sodium excretion and urine volume in both mouse strains. No differences in the 24-h sodium excretion between the genotypes were noted during normal or high sodium diet (II: Table 1). However, it should be stressed that the COMT-deficient mice were able to excrete similar amounts of sodium with lower systolic blood pressure. These results were not in line with the previous study by Odlind and coworkers (2002), suggesting that COMT-deficient mice may have impaired natriuresis response to sodium loading. The basic importance of blood pressure in sodium excretion was, on the other hand, established by the results of our control mice (II) as well as in the acute sodium-load study with COMT-deficient mice [Odlind et al., 2002]. In our study, the control mice
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needed to increase the blood pressure according to the pressure-natriuresis curve to excrete the excess sodium (II). On the other hand, in the study by Odlind and coworkers the impaired sodium excretion in COMT -/- mice could have been related to a decrease in blood pressure in COMT-deficient mice of approximately 20 mmHg compared with 7 mmHg in control mice. Odlind and co-workers (2002) concluded that the COMT-deficient mice had impaired sodium excretion during acute sodium loading; however, the marked decrease in blood pressure found in the study was not discussed in their article, although it could affect the sodium excretion. The reason for decreased blood pressure during their acute experiment may have been due to the use of anesthesia and the route of administration of sodium chloride.

We also analyzed the catecholamine excretion in the urine and noted that the urinary L-DOPA and DA concentrations were significantly increased in COMT-deficient mice compared with wild type mice during normal as well as high-sodium diets (Table 4). At baseline, the 24-h urinary excretions of L-DOPA and DA in COMT-deficient mice were markedly increased compared with wild-type controls. In COMT (-/-) as well as in wild-type mice, the high-sodium diet increased the urinary L-DOPA and DA excretions extensively and the absolute excretion rates of urinary L-DOPA and DA remained 60% and 20% greater in COMT (-/-) mice (Table 4). This is also supported by the results undertaken in another laboratory, showing a similar type of increase in urinary DA excretion in COMT-deficient mice [Odlind et al., 2002]. Furthermore, nitecapone increased the catecholamine excretion in COMT (+/+) mice to nearly the same level as in COMT-deficient mice (Table 4), supporting the possibility that this increased urinary L-DOPA and DA excretion may have been due to COMT deficiency. Since urinary excretion of DA has been used as a marker for renal dopaminergic tone, these results suggest that both genetic and pharmacological COMT blockades may be associated with increased dopaminergic tone.

The increased sodium intake and hyperactive sympathetic nervous system are known to be associated with hypertension [Okuguchi et al., 1999] and long-term sodium excess has been suggested to increase sympathetic activity. Since L-DOPA, the precursor of renal DA synthesis, originates from the spill-over of sympathetic nerves, sodium loading may increase DA synthesis and dopaminergic tonus in the kidney [Grossman et al., 1990, 1991]. In our study, we showed that urinary NA concentrations in COMT (-/-) mice as well as in nitecapone treated COMT (+/+) mice were also elevated. Odlind and coworkers (2002) also reported increased NA excretion in COMT-deficient mice in their study. They also showed that the renal α1-adrenoceptor expression was similar in the genotypes [Odlind et al., 2002]. Since renal NA originates from the sympathetic nerve endings, this increased urinary NA excretion may point to increased sympathetic activity in COMT-deficient mice during normal diet as well as chronic sodium excess [II; Odlind et al., 2002]. This is important, since subsequently increased L-DOPA spill-over from the sympathetic nerve endings provides an additional explanation in addition to lack the of O-methylation for the increased renal dopaminergic activity in COMT-deficient mice. However, this may also simply reflect the decreased O-methylation of NA in the renal tissue in COMT (-/-) mice as well as

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Nitecapone-treated COMT (+/+) mice. This is supported by the observation that during normal diet, no differences in the blood pressures between the mouse strains were noted. However, it could also suggest that some other mechanisms such as increased dopaminergic tone may counterbalance the increased sympathetic activity in COMT-deficient mice.

As with any studies performed with genetically manipulated animals, the conclusions that can be drawn from experiments are often limited since compensatory mechanisms are likely to develop. However, no such compensatory mechanisms were reported in the study, which investigated the possible compensatory changes in protein levels of catecholamine-synthesizing (TH, DDC, dopamine β-hydroxylase) and catecholamine-metabolizing enzymes (MAO-A/B, phenylsulfotransferase) in COMT-deficient mice [Huotari et al., 2002a]. However, it should be noted that these compensatory mechanisms have been evaluated only in the brain and not in renal or any other peripheral tissues.

It is concluded that the sodium resistance in COMT-deficient mice was due to increased renal dopaminergic tone. The increased renal DA could shift the pressure-natriuresis curve to the left and enable the more efficient natriuresis at a lower blood pressure level in COMT (-/-) mice. These results underline the important role of COMT in the long-term regulation of blood pressure and sodium homeostasis. To the best of my knowledge, this was the first reported study to show a correlation between the COMT enzyme activity and blood pressure regulation (II). Most importantly, the crucial role of the COMT enzyme in blood pressure regulation has also since been confirmed in humans [Hagen et al., 2007; Kamide et al., 2007].

7.1.3 COMT inhibition and homocysteine during L-DOPA treatment (III)

Most of the methylation reactions, such as O-methylation, use SAM as a methyl donor. As a result, the SAM is degraded via its intermediate SAH to homocysteine (Fig. 2), which is a known individual risk factor for cardiovascular diseases such as coronary artery disease [McCully, 1969; Clarke et al., 1991; Boushey et al., 1995; Hankey and Eikelboom, 1999; Lentz, 2005], promoting endothelial dysfunction and vascular smooth muscle cell proliferation [Castro et al., 2006]. It was also suggested that SAH may be an even more sensitive risk indicator for cardiovascular diseases than homocysteine [Kerins et al., 2001]. Furthermore, homocysteine has been associated with the etiology of numerous neurologic and neuropsychiatric disorders such as stroke, dementia, depression, peripheral neuropathy, AD and PD [Reutens and Sachdev, 2002; Rogers et al., 2003; Müller et al., 2004a; Obeid and Herman, 2006]. L-DOPA is the standard medication used in the treatment of motor dysfunction in PD. Although L-DOPA therapy has been speculated to increase homocysteine formation the effects of COMT inhibition in reduction of homocysteinemia had not been studied. For this reason the effects of the COMT inhibitor entacapone in prevention of hyperhomocysteinemia in rats were investigated.
The effects of entacapone were studied during acute and subchronic L-DOPA + DDC inhibitor treatments. Entacapone significantly decreased homocysteine formation both acutely and subchronically (III). Furthermore, during the subchronic L-DOPA+DDC inhibitor treatment, there was accumulation of homocysteine (III) and the COMT inhibition ameliorated this phenomenon. Since the functional COMT gene polymorphism alters subjects’ COMT activity [Boudikova et al., 1990; Lotta et al., 1995; Chen et al., 2004; Sullivan Doyle et al., 2004], it is tempting to speculate that Parkinsonian subjects with the high-activity Val allele may have higher homocysteine levels during L-DOPA treatment. This could especially predispose the high-activity Val allele Parkinsonian subjects to a greater risk for cardiovascular diseases. L-DOPA-induced hyperhomocysteinemia increases the prevalence of coronary artery disease in PD [Rogers et al., 2003]. In the quartile with the highest homocysteine levels (>17.7 μMol/l), a nearly 2-fold apparent increased risk for coronary artery disease was shown in L-DOPA-treated PD patients. There were no differences in vitamin B12 (cobalamin) plasma levels between the L-DOPA-treated and control groups, suggesting that L-DOPA-induced hyperhomocysteinemia, and not plasma vitamin B12 levels, was more closely linked to the observed increase in coronary artery diseases [Rogers et al., 2003]. In a meta-analysis of 27 studies, it was concluded that 10% of the risk for coronary artery disease was due to elevated homocysteine levels [Boushey et al., 1995]. Furthermore, taking into account the possible role of COMT in salt-sensitive hypertension (see 7.1.2 and 7.2.3), these results suggest that the PD patient and particularly PD patients with high-activity Val alleles may benefit from the use of adjunct COMT inhibitor therapy during L-DOPA therapy in the form of reduced risk for cardiovascular diseases.

Since our study, the prevention of hyperhomocysteinemia by COMT inhibition has also been confirmed in several other studies in humans [O’Suilleabhain et al., 2004; Lamberti et al., 2005; Ostrem et al., 2005; Valcovic et al., 2005; Zesiewicz et al., 2006]. Interestingly, some recent studies in contrast to Rogers and coworkers (2003), suggested that vitamin status may play a more important role in homocysteine formation than COMT in a North American population [Miller et al., 2003; Postuma et al., 2006]. For example, Postuma and coworkers (2006) reported that the entacapone treatment had no effect on homocysteine formation during L-DOPA treatment. However, this may have been related to mandatory folic acid fortification of grains in North America as the authors discussed [Postuma et al., 2006; Zesiewicz et al., 2006]. Folic acid fortification of grains may blunt the effects of L-DOPA in homocysteine formation and explain the overall lower homocysteine levels in North American studies [Postuma et al., 2006; O’Suilleabhain et al., 2004].

In addition to ameliorating the homocysteinemia, the adjunct COMT inhibitor therapy would also be beneficial during L-DOPA therapy from the homocysteine formation perspective by allowing the reduction in L-DOPA dosage. The adjunct COMT inhibition during L-DOPA treatment leads to enhanced L-DOPA bioavailability and prolongs the duration of L-DOPA action [Müller et al., 2006]. Thus, lower dosages of L-DOPA can be used, leading alone to reduced homocysteine formation.
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Our study was the first to show that L-DOPA-induced homocysteine formation can be prevented by adjunct COMT inhibitor therapy in rats (III). Our results from the cardiovascular risk-assessment point of view support the use of adjunct COMT inhibitor treatment in patients with PD using L-DOPA, since homocysteine is an individual risk-factor for cardiovascular diseases. Broader use of adjunct COMT inhibitor therapy during L-DOPA treatment is also rational, since previous studies have indicated that homocysteine may associate with the etiology of various neurologic and neuropsychiatric diseases like PD itself.

7.1.4 Effects of COMT inhibition in LPS-induced dopaminergic neurodegeneration (IV)

The effects of COMT inhibition were also studied in an LPS-induced hemiparkinsonian animal model. Intranigral as well as systemic LPS administration causes an indirect dopaminergic neurodegeneration of the nigrostriatal pathway in rats [Castano et al., 1998; Herrera et al., 2000; Iravani et al., 2005; De Pablos et al., 2005; Qin et al., 2007]. Unlike the direct cell death of dopaminergic neurons by agents such as MPP+ or 6-OHDA, the neurodegeneration induced by LPS is indirect, due to the inflammatory response and activation of microglial cells [Castano et al., 1998; Kim et al. 2000]. LPS is a potent activator of microglial cells (Burrell, 1990) and SN has the highest density of microglial cells in the brain [Lawson et al., 1990]. In LPS-induced neurodegeneration, the activation of microglia and subsequent ROS formation appear to play a crucial role, since prevention of microglial activation also protects against LPS-induced dopaminergic neurodegeneration [Liu et al., 2000; Lu et al., 2000; Castano et al. 2002; Arai et al., 2004; Tomas-Camardiel et al., 2004; Peng et al., 2005; De Pablos et al., 2005]. Furthermore, the LPS-induced dopaminergic neurodegeneration is DA-dependent, since also α-methyl tyrosine, an inhibitor of TH activity, attenuates the neurodegeneration in this model [De Pablos et al., 2005]. COMT enzyme has been suggested to play a role in DA auto-oxidation, which could promote neurotoxicity [Offen et al., 2001; Ogburn et al., 2006]. This hypothesis suggests that COMT inhibition might increase DA auto-oxidation and enhance ROS formation endorsing neurotoxicity. Since we had previously shown that COMT inhibitors provided protection against Ang II-induced renal inflammation and damage via antioxidant mechanisms (I), we also studied the effects of COMT inhibition in the CNS in LPS-induced inflammation and dopaminergic neurodegeneration.

We assessed the effects of two different COMT inhibitors, tolcapone and entacapone. We estimated that the tolcapone dose used here was high enough to inhibit the COMT activity, based on previous kinetic studies with COMT inhibitors [Männistö et al., 1992a; Forsberg et al., 2003]. Even though entacapone at high doses can penetrate through the BBB and inhibit CNS COMT activity [Forsberg et al., 2003] and disruption of BBB after paranigral LPS injection [Tomas-Camardiel et al., 2004] would even further ameliorate this penetration, the entacapone was used more as an additional control for
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tolcapone. Nevertheless, in the present study COMT inhibition did not significantly increase LPS-induced neurotoxicity nor did it protect from it (IV: Fig 10). Therefore we state that changes in COMT activity do not affect LPS-induced dopaminergic neurotoxicity. These results were in line with previous in vivo studies showing that COMT inhibition in vivo, in contrast to the DA auto-oxidation theory, does not seem to have pro-oxidative effects, but if any, has rather antioxidative net effects [I; Lal et al., 2000; Lyras et al., 2002; Forsberg et al., 2004]. This lack of in vivo effect is most likely due to the localization of COMT (see 3.2.2.1).

7.2 Role of the COMT enzyme in the brain and in the cardiovascular and renal pathophysiology (I-IV)

In addition to studying the effects of COMT inhibition, another general aim here was also to provide novel information and theories on the role and behaviour of COMT enzyme under pathological conditions. We showed that the COMT enzyme, and especially the S-COMT isoform, was up-regulated during inflammation (I, IV). The upregulation of COMT was found to occur in inflammatory and perivascular cells, both in the brain as well as in the kidney. These changes in perivascular COMT may have clinical relevance from the perspective of L-DOPA therapy in Parkinsonian patients (I, III, IV). We also showed that the COMT deficiency protected against the blood pressure-elevating effects of sodium (II), which might have clinical implications from the perspective of salt-sensitive hypertension. These main findings and their possible clinical implications are discussed in the following chapters.

7.2.1 Up-regulation of COMT activity and expression during inflammation (I, IV)

The expression and activity of the COMT enzyme may increase in activated microglial cells during pathological situations [Reenilä et al., 1997]. This is interesting, since no significant COMT activity is normally found in the microglial cells, whereas astroglial cells and postsynaptic dendritic spines are considered to be the primary places of O-methylation in the brain [Kastner et al., 1994; Karhunen et al., 1995]. Based on the preliminary results of Reenilä and coworkers (1997), we further investigated the relationship of COMT and inflammation.

At first, the COMT expression was assessed in dTGRs, which harbour human angiotensinogen and renin genes and are associated with Ang II overproduction (see 7.1.1). In dTGRs, renal inflammation was associated with an over 500% increase in local perivascular COMT expression (Fig. 9b). This interesting finding also led us to assess the effects of inflammation on COMT expression and activity in the CNS (IV).
Discussion

We thus characterized the activity and expression of the COMT enzyme in the SN during local inflammation induced by LPS (IV, see 7.1.4). The local administration of LPS in the vicinity of SN induced microglial activation, which was seen as an acute over 1300% increase in PDE-I, a microglial activation marker (Fig. 12a), as well as a time-dependent increase in OX-42-ir, a microglial/macrophage marker, which peaked 14 days after LPS infusion (Fig. 12b, 12c and 12d). These results were in line with previous studies [Reenilä et al., 1997; Castano et al., 1998; Arimoto and Bing, 2003; Iravani et al., 2005]. The microglial activation was followed by an extensive and delayed dopaminergic neurodegeneration of the nigrostriatal pathway. This dopaminergic injury was confirmed with three different methods 2 weeks after the infusion. First, TH immunostaining showed a substantial decrease of TH-positive cells in the SN (IV: Fig. 3). Second, the striatal TH activity was decreased significantly (IV: Fig. 2). Third, the neurotransmitter of dopaminergic cells, DA, was decreased significantly in the nerve ending area striatum (IV: Fig 1b). These results were in line with previous results from other laboratories [Castano et al., 1998; Herrera et al., 2000; Iravani et al., 2005].

The COMT activity, protein expression and immunoreactivity were assessed from the brain. The LPS-induced microglial activation was associated with an over 200% increase in COMT activity in the SN, which was time-dependent and peaked in 2 weeks (Fig 13a). In the striatum, we observed no significant changes in COMT activity even though a significant neurodegeneration of the nigrostriatal pathway was confirmed. This supports previous findings that the dopaminergic terminals do not contain significant amounts of COMT enzyme [Karhunen et al., 1995; Rivett et al., 1983; Kaakkola et al., 1987; Lundström et al., 1995]. The increased COMT expression colocalized with the OX-42-ir microglial cells and perivascular cells (Fig 14a and 14b). In addition to microglia, OX-42 is also known to label macrophages, monocytes, dendritic cells, granulocytes, as well as pericytes [Robinson et al., 1986; Ford et al., 1995], of which the microglia and pericytes are known to reside normally in the CNS. The microglial cells are the brain resident macrophages [Davis et al., 1994; Gehrmann et al., 1995; Kreutzberg, 1996], whereas the pericytes are located around the microvasculature and are one of the cell types participating in the formation of the BBB [Shepro and Morel, 1993; Balabanov et al., 1998]. Interestingly, the pericytes are known to be relatively undifferentiated cells that can differentiate into macrophages in some situations [Balabanov et al., 1996, 1999; Dore-Duffy et al., 2006]. This is interesting, since LPS has also previously been shown to induce a strong macrophage/microglia reaction in the rat SN, with a characteristic clustering of macrophage-like cells around blood vessels [Herrera et al., 2000]. These perivascular cells, like the ones we noted, may thus be pericytes or pericytes that have transformed into macrophage-like cells. Some of the OX-42-ir cells could also be invading peripheral macrophages or granulocytes from the circulation [Lawson et al., 1992; de Vries et al., 1997; Cheng et al., 1998; Whitton, 2007]. Since the differentiation of microglial cells from these other perivascular macrophages and pericytes is extremely difficult [Guillemin and Brew, 2004] and was not the primary aim of our study, we merely identified the cells as OX-42-ir perivascular cells.
COMT has been considered to be a constitutively active enzyme that is not easily induced or suppressed [Männistö and Kaakkola, 1999]. However, since the thorough review article by Männistö and Kaakkola (1999), many recent studies have shown that COMT expression may be more easily upregulated or suppressed than previously believed, especially during pathophysiological events. There is compelling evidence for association of altered COMT expression and activity as well as functional COMT gene polymorphism with the etiology of various diseases from neuropsychiatric [Lachman et al., 1996; De Lisi et al., 2002; Takahashi et al., 2003; Potash et al., 2003; Palmatier et al., 2004; Gogos and Gerber, 2006; Abdolmaleky et al., 2006] and cardiovascular diseases [Hagen et al., 2007; Kamide et al., 2007] to cancers [Weisz et al., 1998, 2000; Tenhunen et al., 1999; Sasaki et al., 2003; Salama et al., 2006; Tanaka et al., 2006, 2007]. Both increased and decreased COMT expression can interfere with normal cell physiology and the physiology of the dopaminergic systems. However, little is known about the mechanisms leading to altered COMT expression or the role of the COMT enzyme during pathological situations.

In our studies we showed that inflammation in the CNS as well as in the peripheral tissues led to up-regulated COMT expression in the perivascular and inflammatory cells (I, IV). Thus, this increased COMT expression and activity appears to be a more general reaction to inflammation. This is supported by previous studies showing that other situations associated with inflammation, such as oncogenesis, also appear to upregulate the COMT enzyme [Tenhunen et al., 1999; Weisz et al., 1998, 2000; Sasaki et al., 2003; Salama et al., 2006]. Glialtoxins and neurotoxins such as fluorocitrate (Reenilä et al., 1997) and Angel’s salt [Helkamaa et al., unpublished data], also increase COMT activity in the CNS. Furthermore, very recent study reports that also traumatic brain injury upregulates COMT expression also in hippocampal microglial cells [Redell and Dash, 2007]. Interestingly, analysis of the COMT gene promoter area revealed multiple sites for NFkB, which is a well-known proinflammatory transcription factor. In a recent study, the tumor necrosis factor-α (TNF-α) was suggested to up-regulate COMT expression in the myometrial cells via the NFkB pathway [Wentz et al., 2006; Salama et al., 2006]. Inhibition of NFkB attenuated the TNF-α-induced up-regulation of COMT expression in myometrial cells [Wentz et al., 2006]. Interestingly, LPS-induced microglial activation is also mediated via the NFkB pathway [Moriyama et al., 2006]. Furthermore, the renal damage and inflammation associated with the increased perivascular COMT expression (I) in dTGRs have been shown to be mediated via NFkB and AP-1 [Luft et al., 1999; Mervaala et al., 1999a, 2000, 2001; Müller et al., 2000; Schiff and Touyz, 2003]. These studies, together with our results, suggest that the increased COMT expression and activity in the microglial and perivascular cells could be a more general reaction to inflammation and/or cellular injury in the neuronal tissue [IV; Reenilä et al., 1997; Helkamaa et al., unpublished data] as well as in the peripheral tissues [I; Salama et al., 2006; Wentz et al., 2006] and that up-regulation of COMT expression and activity during inflammation observed in our studies (I, IV) may be mediated via the NFkB pathway.
Discussion

The physiological significance of this increased expression and activation of COMT in inflammatory and perivascular cells during inflammation (I, IV) may be related to inactivation of catecholamines released at the site of tissue injury and subsequent inflammation. The increased amount of catechols released during inflammation and tissue damage could increase the formation of ROS and oxidative stress. Some oxidation metabolites of catechols, such as the hydroxylated catechol estrogens, increase oxidative DNA damage in the cells [Zhu and Conney, 1998; Weisz et al., 1998, 2000, Hirose et al., 2007]. Furthermore, the oxidation of catechols by MAO increases the formation of $O_2^-$ and $H_2O_2$. The catechols themselves can also auto-oxidize and form $O_2^-$ as well as reactive quinones. All these can cause cellular damage, DNA damage and eventually lead to apoptosis or carcinogenesis [Miyazaki et al., 2006; Hirose et al., 2007]. In this respect, enhanced O-methylation via COMT could decrease ROS formation, ameliorate the oxidative stress and protect the cells during inflammation. The up-regulated COMT expression and activity in inflammatory cells, as in glial cells as well as perivascular cells, may thus be a protective mechanism against the increased oxidative stress in inflamed tissues. This hypothesis would be logical since the COMT enzyme is known only to metabolize endogenous and exogenous catechol compounds.

Furthermore, many (brain) diseases as well as CNS inflammation can alter the functionality and the integrity of BBB. Mostly this results in increased BBB permeability [De Boer and Gaillard, 2006]. The situation is similar to that encountered in the peripheral tissues where inflammation also leads to increased vascular permeability. In this perspective, the up-regulation of COMT, especially in perivascular cells (I, IV), would be logical. The COMT enzyme plays a major role in the enzymatic barrier formed by the cells of the vascular wall, especially in the BBB. Increased glial and perivascular COMT activity could thus protect the brain and also other tissues against circulating catechols, in a corresponding manner to the high COMT activity in the liver that protects the body against exogenous catechol compounds.

The up-regulated COMT expression in the brain was further characterized with Western blotting to evaluate the effects of inflammation on COMT isoenzyme expressions (IV). In 2 weeks, the LPS-induced inflammation resulted in a 255% increase in S-COMT protein expression, while there was only 86% increase in MB-COMT protein expression (Fig 13b). If the up-regulation of COMT during pathological situations is indeed a cellular protective mechanism, the S-COMT isoenzymes particularly would be more suitable. The S-COMT has a higher $K_m$ value for most of the main substrates and also a much higher $V_{max}$ than MB-COMT [Guldberg and Marsden, 1975; Roth, 1992; Männistö and Kaakkola, 1999]. Although, the higher $K_m$ value may not be an advantage in all situations, it makes the S-COMT a safer isoenzyme to be up-regulated since the $K_m$ values of S-COMT for most substrates are well beyond the normal tissue catecholamine concentrations. For this reason, the up-regulated S-COMT would not interfere with normal neurotransmission as much as the up-regulation of MB-COMT. S-COMT could also metabolize catechols more efficiently during pathological tissue catechol concentrations, due to the much higher capacity.
Discussion

7.2.2 Hypothesis on the clinical significance of upregulation of COMT in CNS pathophysiology

The increased COMT activity and expression in the brain during inflammation would be especially interesting, since inflammation may play a role in the etiology of neurodegenerative and neuropsychiatric diseases such as PD and schizophrenia [McGeer et al., 1988; Akiyama and McGeer, 1989; Floyd 1999; Hong, 2005; Hirsch et al., 2005; Ouchi et al., 2005; Wojtera et al., 2005; Ashdown et al., 2006; Foster et al., 2006; Müller and Schwarz, 2006; Kim and Yoh, 2006]. It is thus tempting to speculate that inflammation-induced changes in COMT activity in the brain (IV) may also have clinical relevance. Functional COMT polymorphism may increase the risk of schizophrenia in high-activity Val allele subjects [Shifman et al., 2002; Glatt et al., 2003; Kremer et al., 2003; Horowitz et al., 2005; Gothelf et al., 2007; McIntosh et al., 2007]. Microglial activation and inflammation may also appear in the PFC of schizophrenic patients [Foster et al., 2006]. Based on these studies and results for up-regulated COMT expression and activity in microglial cells (IV; Reenilä et al., 1997; Redell and Dash, 2007), it is tempting to speculate that inflammation in the PFC, where O-methylation is already the primary metabolic route for DA [Karoum et al., 1994], could offer a possible novel explanation for cognitive dysfunction and negative symptoms in some schizophrenic patients. Also, since tolcapone has been shown to enhance cognitive performance both in rats (Tunbridge et al., 2004) as well as in humans (Apud et al., 2007) new CNS penetrating COMT inhibitors might be an interesting drug development targets for schizophrenia in the future because of their ability to increase the DA levels selectively in PFC, but not so much in subcortical or other brain areas.

Furthermore, this up-regulated glial and perivascular COMT expression and activity in the brain (IV; Reenilä et al., 1997; Redell and Dash, 2007) may affect the bioavailability of L-DOPA, which would be clinically relevant from the perspective of Parkinsonian patients undergoing L-DOPA therapy. The L-DOPA molecules contact several types of cells before being taken up by dopaminergic neurons on their way from the blood vessels to striatum: first, the endothelial cells of brain capillaries, then the other cells forming the BBB and finally the glial cells deeper in the brain parenchyma. Since activated microglial cells are a common finding in Parkinsonian brains [Imamura et al., 2003; Ouchi et al., 2005; Gerhard et al., 2006] and inflammation may play a role in the etiology of PD [McGeer et al., 1988; Akiyama and McGeer, 1989; Floyd 1999; Członkowska et al., 2002; Wojtera et al., 2005], it could be that up-regulation of COMT in activated microglial cells and perivascular cells may also occur in Parkinsonian brains. The increased COMT activity in BBB and in microglial cells would inevitably affect the amount of L-DOPA reaching the dopaminergic cells and could thus reduce the L-DOPA response and increase the motor fluctuations noted in PD during L-DOPA treatment. However, to the best of my knowledge, the up-regulation of COMT activity and expression has never been investigated in postmortem PD patient brains.
7.2.3 Hypothesis on the role of the COMT enzyme in cardiovascular and renal pathophysiology

Hypertension affects approximately 25% of the adult population. The etiology of hypertension is multifactorial and sodium, the main extracellular cation, has long been considered the pivotal environmental factor in hypertension [Karppanen and Mervaala, 2006; Adrogué and Madias, 2007]. Although all humans are somewhat sensitive to the blood pressure-elevating effects of sodium, some individuals are apparently more sensitive to the hypertensive effects of sodium than others. Interestingly, these salt-sensitive subjects appear to be at high risk of dying of cardiovascular diseases. It was previously shown that even normotensive salt-sensitive subjects had survival that was no different for that of hypertensive subjects [Weinberger, 2002]. Thus, any further understanding of the etiology of salt-sensitivity would offer novel important diagnostic tools for clinical cardiovascular risk assessment. Based on our results and the recent human studies (II; Hagen et al., 2006; Kamide et al., 2006), it is tempting to hypothesize that the COMT enzyme might play a role in salt-sensitive hypertension. Genetically (functional COMT polymorphism or COMT-deficient mice) or pharmacologically (COMT inhibitors) produced decrease or lack of COMT activity may provide at least partial protection against salt-sensitive hypertension by shifting the pressure-natriuresis curve to the left, due to increased renal DA concentration/production and enhanced renal dopaminergic tone. Hypothetically this would lead to a new balanced situation in which the increased amount of sodium could be excreted at the same (normal) mean arterial pressure level. Our results of increased natriuresis despite unchanged arterial pressure in COMT-deficient mice support this possibility (II).

It could be that the decreased COMT activity may increase the importance of the renal dopaminergic system, in sodium handling especially during sodium load. Instead of the increased pressure natriuresis the COMT-deficient mice and possibly also low-activity Met allele subjects may be able to excrete sodium excess due to more efficient renal dopaminergic natriuresis. Increased sodium loading increases plasma volume, which in turn causes a decrease in renin production and inactivation of the RAA system. This further emphasizes the importance of neurohumoral systems shifting the pressure-natriuresis curve to left, like dopaminergic system. The hypothesis on the role of COMT in salt-sensitive hypertension is also in line with previous studies. A recent study reported a correlation between COMT and salt sensitive hypertension in Salt-sensitive Dahl-rats [Okuda et al., 2004]. Renal DA production in response to high sodium intake has been suggested to be blunted in salt-sensitive hypertension [Pestana et al., 2001]. Salt sensitivity is more common in African-Americans than Caucasians [Morris et al., 1999; Brownley et al., 1999]. Interestingly, among African-Americans, the high-activity Val allele of the COMT gene is also more prevalent than in the Caucasian population (see 3.1.5.2). Furthermore, natural as well as surgical menopause also increases salt-sensitivity [Pechere-Bertschi and Burnier, 2004; Schulman et al., 2006]. Since estrogens down-regulate COMT activity [Xie et al., 1999], it is tempting to speculate that COMT may contribute to menopause induced salt-sensitivity. Based on these and our own results showing salt resistance in COMT-
deficient mice (II), it could be that the COMT enzyme activity of subjects may play at least a partial role in the etiology of salt-sensitive hypertension.

Interestingly, renal inflammation has been recently associated with hypertension. De Magalhaes Sartim and co-workers (2006) reported that experimental pyelonephritis in rats resulted with elevated blood pressure. The COMT enzyme activity or expression were not studied, but if inflammation increases COMT more generally as have been discussed in this thesis, an enhanced renal COMT expression could provide a possible explanation. This possibility is even further supported by the fact that sodium loading increased this elevation in blood pressure even further in pyelonephritic group but not in sham operated control animals. The authors concluded that the chronic renal inflammation may promote an inability of renal tubules to handle sodium when exposed to sodium overload [de Magalhaes Sartim et al., 2006]. This hypothesis would be inline with the hypothesis presented in this thesis.

We also reported that adjunct COMT inhibition during L-DOPA therapy reduced plasma homocysteine levels significantly (III), suggesting that increased 0-methylation via COMT can lead to increased homocysteine formation. As discussed earlier (See 7.2.1) the inflammation seems to increase especially perivascular COMT expression and activity. Since homocysteine has been shown to promote endothelial dysfunction and smooth muscle proliferation as well as increase the risk for coronary arterial disease, this perivascular increase in COMT expression during inflammation might be significant finding. Inflammation and homocysteine formation have been suggested to play a role in etiology of atherosclerosis [Castro et al., 2003; Mahmoudi et al., 2007], thus, it is tempting to speculate that pharmacological COMT inhibition might also have beneficial effects in the prevention of homocysteine-induced atherosclerosis and coronary artery disease. Interestingly, a recent study reported that subjects with low-activity Met/Met genotype of COMT gene had a decreased risk for myocardial infarction compared to those with high-activity Val-Val alleles [Eriksson et al., 2004].
Figure 15. A schematic illustration of hypothetical effects of inflammation and/or tissue damage on COMT expression and subsequent pathophysiological effects in the body. COMT, catechol-O-methyltransferase; L-DOPA, levodopa; DA, dopamine; PFC, prefrontal cortex; BBB, blood-brain-barrier; CNS, central nervous system; NFκB, nuclear factor kappaB, a transcription factor.
8. CONCLUSIONS

This doctoral thesis was designed to provide novel information on the role of the COMT enzyme and COMT inhibition in the brain and in cardiovascular and renal systems.

The COMT inhibitor entacapone was shown to protect against perivascular inflammation and renal damage and prevent the cardiovascular mortality completely in dTGRs, an animal model of Ang II-dependent hypertonia and target organ damage.

Both genetic as well as pharmacological COMT enzyme blockade were shown to protect against the blood pressure-elevating effects of high sodium intake in mice. The sodium-resistant effects of the COMT blockade were mediated via enhanced renal dopaminergic tone. Our findings suggest that renal COMT participates in the regulation of blood pressure and sodium homeostasis and may also play an important role in the pathogenesis of salt-sensitive hypertension.

The COMT inhibitor entacapone was also shown to ameliorate the L-DOPA-induced hyperhomocysteinemia in rats both acutely and subchronically. These results may also suggest a reduced risk of cardiovascular diseases in L-DOPA-treated Parkinsonian patients using additional COMT inhibitor therapy due to reduced homocysteinemia.

The LPS-induced inflammation following delayed dopaminergic neurodegeneration was accompanied by up-regulation of COMT expression and activity in microglial cells as well as perivascular cells. Similar up-regulation of COMT expression was also noted in the inflamed renal tissue in dTGRs, suggesting that inflammation may up-regulate COMT expression and activity. Furthermore, this increased glial and perivascular COMT activity in the CNS during inflammation may have even clinical relevance from the perspective of PFC functioning and the bioavailability of L-DOPA.

In conclusion, this thesis provided novel evidence that inflammation may upregulate COMT enzyme expression and activity, established association between COMT and sodium-induced hypertension and demonstrated the beneficial effects of COMT enzyme inhibitors in prevention of L-DOPA-induced hyperhomocysteinemia and Ang II induced renal failure.
Acknowledgements

9. ACKNOWLEDGEMENTS

The present work has been carried out in the Institute of Biomedicine, Pharmacology, University of Helsinki, during the years 2000-2007.

First, I would like to thank Professor Heikki Vapaatalo MD and Professor Esa Korpi MD, the Heads of the Department during these years, for providing me with the facilities in the Department and the encouragement and hard enough push on the back to get this work done.

Then, I sincerely owe my deepest gratitude to my two magnificent supervisors Docent Pekka Rauhala, MD, and Professor Eero Mervaala MD. It is impossible to find adequate words to thank these two distinguished gentlemen. Pekka first took me under his supervision in 2000 and hands-on taught me the laboratory and in vivo skills needed for this work. His wide-ranging knowledge concerning the field of neuroscience was invaluable asset to me and the scientific and non-scientific discussions we had will always stay in my mind. He guided me through these years with his firm and honest Ostrobothnian attitude and I have the highest respect for him as a scientist and an individual. Soon after beginning of this thesis I was privileged to have Professor Eero Mervaala appointed as my other supervisor. His solid expertise on the field of cardiovascular biochemistry is unparalleled and I feel deeply honoured to been given a chance to work under his supervision. His effectiveness and the ability to always find the essential as a scientist never stops to amaze me. Regardless of the astonishing amount of work they both do, I can sincerely say that whenever I had a new phenomenal, or usually less so, idea in my head, I was privileged to have two supervisors, who both always found time and patience to correct me. Without their vast knowledge and guidance the completion of this thesis would have been impossible. I wish to express my humblest gratitude and respect for both of you.

I thank Ewen MacDonald PharmD, and Docent Jouni Sirviö, the official pre-examiners of this thesis, for their encouragement, intelligent comments and their constructive criticism. I’m also honoured to have distinguished Professor Markku Koulu as my opponent.

I would also like express my utmost gratitude to Ilkka Reenilä PhD, who guided me into the intriguing world of HPLC. I especially like to thank Ilkka for his bright comments and the preliminary revision of this thesis. His inexhaustible knowledge about the COMT was always one of the supporting pillars to carry me through this journey alive. Thank you. My profuse thanks also belong to my research colleagues Antti Väänänen, MD PhD, Saara Merasto MSc and Piet Finckenberg PhD. Antti, with whom I also studied in and graduated from the same year class in the Medical Faculty, was always willing and able to help me with even with the smallest technical issues I had. His fresh knowledge about the doctoral thesis project and dissertation undoubtedly saved me from multiple problems. I also owe my ample thanks to Saara whose expertise in the world of immunohistochemistry was immeasurably valuable. She was also irreplaceable during surgical
Acknowledgements

operations. Piet deserves my highest respect and gratitude for his help in immunohistochemical, morphological and pathological studies. I had my best laughs with you guys. I most sincerely thank all of you.

I respectfully thank Professor Raimo K. Tuominen MD for his guidance in the Western blot analysis and his invaluable comments on the studies. I also sincerely thank Professor Pekka Männistö MD, for his help with the work on COMT-deficient mice and for sharing his immense knowledge about the COMT enzyme. I respectfully thank Professor Seppo Soinila MD, for his help with the CNS immunohistochemistry and for his contribution on the studies. I owe my special thanks to all my collaborators and co-authors Docent Erkki Nissinen, Helena Nissinen MD PhD, Henriikka Larjomaa MSc, Marjut Louhelainen MSc, Marko Huotari PhD, Docent Risto Lapatto MD, Carola Tilgmann PhD, Professor Maria Karayiorgou MD, Professor Joseph A. Gogos MD, Professor Friedrich C. Luft MD, Dominik N. Müller PhD, and Zhong Jian Cheng PhD. I also like thank Kristiina Haasio PhD, Ms Kati Nurminen and Jouko Levijoki MSc, from the Orion Pharma for the help in telemetric studies.

I owe warmest thanks to Mrs Vuokko Pahlsten, Mrs Anneli von Behr, Ms Sari Laakonen, Ms Pirjo Hänninen and Ms Kati Puputti for their invaluable and splendid help and guidance in the laboratory. I am also grateful to Mrs Eeva Harju for all the information and practical help concerning the Department’s official matters during these years. I warmly thank all of you. I’d also like to express my sincere gratitude to all the colleagues and other personnel in the department for creating a pleasant atmosphere to work in.

I extend my thanks to all of my dear friends, with whom I’ve been privileged to be able to relax and enjoy life as it should be enjoyed. Thank you all for every memorable moments we have spent together during these years. Your company is invaluable to me.

I owe my deepest gratitude for my family and especially for mom and dad, who have always supported me in everything I’ve decided to do.

Finally, my dearest and warmest thanks go to you, Anu. Although, you’ve also been busy with your own doctoral thesis project, I apologize for all the inhuman working-hours I’ve spent doing this thesis during last year. I sincerely thank you for standing by me and for all the help and love you’ve given me during these years.

Helsinki, August 2007

Teemu Helkamaa

This study was supported by grants from the Farmos Research and Science Foundation, the Finnish Parkinson Foundation, the Päivikki and Sakari Sohlberg Foundation, the Academy of Finland, the Sigrid Jusélius Foundation and the Paulo Foundation. Thank you for the needed support.
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10. REFERENCES


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