New insights to the brain functions of prolyl oligopeptidase

IIDA PELTONEN

ACADEMIC DISSERTATION

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**ABSTRACT**

Prolyl oligopeptidase (PREP, also known as POP; EC 3.4.21.26) is an 80-kDa serine protease hydrolyzing peptides smaller than 30-mer at the carboxyl side of an internal proline-residue. PREP is widely distributed in the body and the cytosolic form is abundantly expressed also in the brain. Several neuropeptides have been suggested to be hydrolyzed by PREP, such as arginine-vasopressin (AVP), oxytocin and substance P (SP), through which PREP has been connected to central nervous system (CNS) functions including learning, memory and mood. However, the break-down of these peptides by PREP has not been conclusively demonstrated *in vivo*. One of the major criticisms against the neuropeptide theory has been the different locations of intracellular PREP and the mainly extracellular neuropeptides. Although PREP has mostly been studied as a neuropeptide-cleaving enzyme, there are also other connections between PREP and brain diseases, such as the altered levels of PREP in the plasma of patients with CNS disorders and the location of PREP in neurons connected to important brain functions. Recent studies show that PREP may have effects on intracellular protein aggregation via protein-protein interactions.

The purpose of this study was to find novel actions and elucidate the relevance of proposed physiological roles of PREP in the rodent brain using different neurochemical and behavioural methods. Brain penetrating PREP inhibitors were used as tools in many of the studies. First, we studied the PREP inhibition capabilities of common psychoactive drugs. Since the pathophysiology of CNS diseases is not fully understood, we tested whether PREP could be a physiological target for these compounds. Second, we examined the colocalization and interaction of PREP with several neurotransmitter systems by lesioning the major nuclei of these transmitters with neurotoxins and then studying the effects of the lesions on PREP activity and immunoreactivity in the respective projection areas. Third, the colocalization and functional interaction of PREP with neurotensin and its receptor NTS1 were studied in the midbrain dopaminergic pathways. Finally, the effect of PREP inhibition on behaviour was tested in animal models of memory, locomotor activity and Parkinson’s disease.

We found that psychoactive compounds did not considerably inhibit PREP at concentrations achieved in the human therapy. Lesioning of the major neurotransmitter nuclei did not affect PREP activity or immunoreactivity in the projection areas indicating that PREP is not present in the long projection neurons or not connected to their functions. PREP was highly colocalized with neurotensin and NTS1 in the ventral tegmental area (VTA) and with NTS1 in the substantia nigra (SN). In addition, PREP inhibitor was shown to increase the dopamine levels in the SN and VTA in a NTS1-dependent manner. We found also that PREP inhibition increases locomotor activity in rats, but does not improve memory or affect rotational behaviour in a rat model of Parkinson’s disease.

Conclusively, although the precise role of PREP in the brain remains unclear, our studies substantiated the view that the major physiological role for PREP in the brain is not the improvement of memory. Interestingly, our results suggested that PREP may be involved in motor functions and in the neurotensin-mediated dopaminergic signalling in the SN and VTA through intracellular mechanisms.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>ACTH</td>
<td>Corticotropin</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AVP</td>
<td>Arginine-vasopressin</td>
</tr>
<tr>
<td>BNST</td>
<td>Bed nucleus of the stria terminalis</td>
</tr>
<tr>
<td>CCK</td>
<td>Cholecystokinin</td>
</tr>
<tr>
<td>CD</td>
<td>Carbidopa</td>
</tr>
<tr>
<td>CRH</td>
<td>Corticotropin-releasing hormone</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>5,7-DHT</td>
<td>5,7-Dihydroxytryptamine</td>
</tr>
<tr>
<td>DOPAC</td>
<td>3,4-Dihydroxyphenylacetic acid</td>
</tr>
<tr>
<td>DSP-4</td>
<td>N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein-coupled receptor</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-Hydroxytryptamine (serotonin)</td>
</tr>
<tr>
<td>HVA</td>
<td>Homovanillic acid</td>
</tr>
<tr>
<td>i.c.</td>
<td>Intracerebral</td>
</tr>
<tr>
<td>i.c.v.</td>
<td>Intracerebroventricular</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IP₃</td>
<td>Inositol-1,4,5-triphosphate</td>
</tr>
<tr>
<td>LD</td>
<td>L-Dopa</td>
</tr>
<tr>
<td>LDCV</td>
<td>Large dense core vesicle</td>
</tr>
<tr>
<td>MFB</td>
<td>Medial forebrain bundle</td>
</tr>
<tr>
<td>MPOA-AH</td>
<td>Medial preoptic-anterior hypothalamic area</td>
</tr>
<tr>
<td>MPTP</td>
<td>1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NAcc</td>
<td>Nucleus accumbens</td>
</tr>
<tr>
<td>NKA</td>
<td>Neurokinin A</td>
</tr>
<tr>
<td>NKB</td>
<td>Neurokinin B</td>
</tr>
<tr>
<td>NTS1</td>
<td>Neurotensin receptor 1</td>
</tr>
<tr>
<td>6-OHDA</td>
<td>6-Hydroxydopamine</td>
</tr>
<tr>
<td>OTR</td>
<td>Oxytocin receptor</td>
</tr>
<tr>
<td>PFC</td>
<td>Prefrontal cortex</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>POMC</td>
<td>Pro-opiomelanocortin</td>
</tr>
<tr>
<td>PPI</td>
<td>Pre-pulse inhibition of the startle response</td>
</tr>
<tr>
<td>PREP</td>
<td>Prolyl oligopeptidase</td>
</tr>
<tr>
<td>RER</td>
<td>Rough endoplasmic reticulum</td>
</tr>
<tr>
<td>s.c.</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SN</td>
<td>Substantia nigra</td>
</tr>
<tr>
<td>SP</td>
<td>Substance P</td>
</tr>
<tr>
<td>SSV</td>
<td>Small synaptic vesicle</td>
</tr>
<tr>
<td>Tβ4</td>
<td>Thymosin beta-4</td>
</tr>
<tr>
<td>TRH</td>
<td>Thyrotropin-releasing hormone</td>
</tr>
<tr>
<td>VPA</td>
<td>Valproate</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
</tr>
</tbody>
</table>
LIST OF ORIGINAL PUBLICATIONS

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


II Peltonen I, Myöhänen TT, Männistö PT: Association of prolyl oligopeptidase with conventional neurotransmitters in the brain. CNS Neurol Disord Drug Targets, 10(3):311-318, 2011


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1. INTRODUCTION

The term neuropeptide was first used by David de Wied in the early 1970’s to describe peptides in the brain without any endocrine functions (Bohus and De Wied 1966; De Wied 1971). He noticed that the brain was sensitive to peptide hormones and their derivatives. Eventually, his pioneering work led to the discovery of corticotropin (ACTH). Thereafter, research on neuropeptides has been carried out for over 40 years. Hundreds of neuropeptides have been found, and their functional significance has been intensively studied (Hökfelt et al. 2000).

Currently, there is no effective treatment for the majority of central nervous system (CNS) diseases, such as Alzheimer’s disease, Parkinson’s disease, schizophrenia, depression and bipolar disorder (Malavolta and Cabral 2011). One goal of the neuropeptide research has been the development of drugs for CNS diseases. However, there are several problems related to peptidergic pharmacotherapy including in vivo stability, route of administration, poor blood-brain barrier penetration and complexity of manufacture (Malavolta and Cabral 2011). Therefore, several receptor-specific non-peptide ligands with nanomolar affinity have been developed, which have helped to define functional roles for neuropeptides (Hökfelt et al. 2003). Another approach to affect neuropeptidergic systems has been the modulation of neuropeptide degradation by inhibiting neuropeptide-hydrolyzing enzymes. With enzyme inhibitors, the stability and penetration problems of neuropeptide receptor ligands may be avoided (Fricker 2005).

Prolyl oligopeptidase (PREP; EC 3.4.21.26; also known as prolyl endopeptidase, PEP, POP or PO) was identified by Walter et al. 1971 from bovine uterus as an oxytocin cleaving enzyme. Thereafter PREP has been connected to the hydrolysis of neuropeptides. PREP is an 80 kDa serine protease belonging to the family S9 of the serine carboxypeptidase clan (Rawlings and Barrett 1994). Its special feature is the hydrolysis of small (shorter than 30 amino acids) peptides containing proline, such as the neuropeptides arginine-vasopressin (AVP), neurotensin, oxytocin and substance P (SP). PREP cleaves the -Pro-Xaa- bond at the carboxyl side of a proline residue, where Xaa is any amino acid other than proline (Cunningham and O’Connor 1997; Polgar 2002). PREP family is widely distributed in organisms ranging from bacterial and archaeal species to mammals (Venäläinen et al. 2004). PREP is found in most rat and human tissues, but the highest activities have been measured in the brain (Kato et al. 1980; Irazusta et al. 2002; Myöhänen et al. 2009). PREP is considered to be mainly an intracellular and cytosolic enzyme constitutively expressed in all cells. However, a membrane-bound form has been described, too (O’Leary and O’Connor 1995; García-Horsman et al. 2007; Tenorio-Laranga et al. 2008). PREP activity has also been measured in plasma and cerebrospinal fluid suggesting the existence of an extracellular form or secretion of the enzyme, which has not been proved (Brandt et al. 2007).

In the rat brain, high PREP immunoreactivities have been found in the cerebral cortex, especially primary motor cortex and somatosensory cortex, the striatum, hippocampus, lateral septum and cerebellum (Myöhänen et al. 2008b). High amounts of PREP messenger ribonucleic acid (mRNA) have been found in the cerebellum and hypothalamus (Bellemere et al. 2004). Highest PREP activity levels in the rat brain have been
measured from the cortex, striatum and cerebellum (Irazusta et al. 2002; Agirregoitia et al. 2005). Generally, PREP has been present in neurons using gamma-aminobutyric (GABA) and acetylcholine (ACh) as neurotransmitters, but to a lesser extent in dopaminergic neurons (Myöhänen et al. 2008b). Due to its location in the brain and suggested importance in neuropeptide break-down, PREP has been connected to CNS diseases related to neuropeptidergic malfunction (for review, see García-Horsman et al. 2007 and Männistö et al. 2007).

PREP has mainly been studied as a memory-modulating enzyme, since many of the putative substrate peptides, including AVP, thyrotropin-releasing hormone (TRH) and SP, have been shown to possess memory-enhancing effects (Brandt et al. 2007). Several PREP inhibitors have been developed with the purpose to prevent and treat memory disorders by inhibiting the degradation of promnesic neuropeptides and thus elevating their levels in the brain (Männistö et al. 2007). Despite the intensive research, the final conclusions about the role of PREP in memory and learning and neuropeptide break-down (reviewed by García-Horsman et al. 2007 and Männistö et al. 2007 are yet to be drawn.

A large amount of evidence about the role of PREP in CNS diseases comes from studies about the levels of PREP activity in the plasma or serum of patients with psychiatric or neurological disorders (Maes et al. 1994; Maes et al. 1995; Mantle et al. 1996; Maes et al. 1999; Maes et al. 2001), which correlated with the severity of the disease or with the drug treatment. For example, Maes et al. 1994 and 1995 reported that PREP activity in plasma was significantly lower in patients with major depression than in healthy volunteers. Antidepressant fluoxetine and the mood stabilizer valproate (VPA) restored the altered PREP activity levels to normal. Interestingly, VPA was also shown to inhibit PREP activity (Cheng et al. 2005). In Parkinson patients, the activity of PREP (Mantle et al. 1996) and the levels of a putative PREP substrate neurotensin (Fernandez et al. 1996) have been altered. Active PREP has also been found to increase the aggregation of α-synuclein, the main component in Lewy bodies, the hallmarks of Parkinson’s disease (Brandt et al. 2008; Myöhänen et al. 2012).

Interestingly, novel roles for PREP independent of its hydrolytic activity have been proposed, including the involvement in the inositol-1,4,5-trisphosphate (IP₃) signalling (Williams and Harwood 2000), in intracellular trafficking and protein secretion (Schulz et al. 2005), and neuronal growth via protein–protein interactions (Di Daniel et al. 2009).

The present study aimed to gain new insights about the functions of PREP in the brain by studying PREP as a target of psychopharmaceutical compounds, the localization of PREP in the projection neurons of major classical neurotransmitters, the interaction of PREP with the neuropeptide neurotensin in the midbrain dopaminergic pathways and the effects of PREP inhibition in animal models of behaviour. The literature part of the thesis will focus on the characteristics and effects of putative PREP substrate peptides AVP, neurotensin, oxytocin, somatostatin and SP on behaviour. As neuropeptides act as neuromodulators in the CNS, their behavioural effects are complex and difficult to interpret. However, since the physiological function of PREP and particularly its role in behaviour are not known, it is important to review the conceivable behavioural effects of putative PREP substrates.
2. REVIEW OF THE LITERATURE

2.1 Basic characteristics of neuropeptides

Neuropeptides represent the largest and most diverse class of signalling molecules in the CNS. Currently, hundreds of different neuropeptides are known and the discovery period does not seem to be over yet (Hökfelt et al. 2000). With the growing number of known peptides and small proteins affecting neurons, the exact definition to pinpoint the essence of neuropeptides has lacked behind. Although there are common hallmarks for neuropeptides, such as the biosynthesis by neurons, regulated release and the ability to modulate or mediate neural functioning by acting on receptors, there is no exact definition of the term neuropeptide (Burbach 2010). For example, neuropeptides can be defined as proteineous substances produced by neural cells and acting on any neural or non-neural substrate or target. That definition covers all neural secretory peptides and proteins of the brain regardless of their size, such as growth factors, chemokines and peptide hormones of the endocrine system (Burbach 2010). However, a more conservative definition of neuropeptides takes into account also the size of the peptides.

Currently, neuropeptides are mostly considered to be typically composed of 3-40 amino-acid residues and they act as neurotransmitters or neuromodulators in the CNS (Salio et al. 2006). Every neuropeptide has a unique amino acid sequence which determines its biological functions (Hook et al. 2008). Although the effects of neuropeptides on target neurons are diverse, they most commonly modify the membrane excitability (Salio et al. 2006). They can also regulate gene transcription, local blood flow, synaptogenesis and glial cell architecture (Theodosis et al. 1986; Cauli et al. 2004; Landgraf and Neumann 2004).

After the 1970s, it has been understood that neurons are able to manufacture and utilize more than a single molecule for signalling (Hökfelt et al. 2000). Neuropeptides often coexist in neurons with classical small-molecule neurotransmitters such as glutamate, catecholamines, ACh and 5-hydroxytryptamine (serotonin; 5-HT) (Hökfelt et al. 2000). However, neuropeptides are in many ways different from them (Hökfelt et al. 2000). They are about 50 times larger than the classical neurotransmitters giving them many more recognition sites for receptors (Salio et al. 2006). Therefore neuropeptides bind to their receptors with a higher affinity than the classical neurotransmitters and they can produce biological effects even when released in low quantities. Neuropeptides are also more stable in the extracellular space of the brain compared to classical neurotransmitters (e.g. the half-lives for oxytocin and AVP are about 20 min) (Mens et al. 1983). Furthermore, neuropeptide actions are not targeted or associated with particular synapses, since they can diffuse far away from the releasing site. Pharmacokinetically, neuropeptides are between the classical neurotransmitters and hormones in the neuron-to-neuron communication (Salio et al. 2006). The co-existence of neuropeptides and other messengers allows the neurons to communicate fast and slowly, with neurons close by and far away.
2.1.1 Neuropeptide families and their distribution and expression in the CNS

Bioactive peptides are found in the whole animal kingdom, but also in plants (Hökfelt et al. 2000). Peptide families have ancient origins. Examples of neuropeptides and their families in the CNS are listed in Table 2-1. The best studied neuropeptide family includes two hypothalamic magnocellular neurosecretory peptides oxytocin and AVP (Hökfelt et al. 2000).

Intensive research has provided information about the distribution of neuropeptides in the CNS. They can be found in all parts of the CNS and peripheral nervous system (PNS), but all peptides have their own distribution patterns. Some peptides are present at high levels under normal circumstances, whereas others are normally expressed at very little or undetectable levels and require a specific stimulus, e.g. a nerve injury, for their expression to increase (Hökfelt et al. 2000). There are also peptides expressed only in early stages of development. In general, peptides can be expressed in one or several of the above-mentioned manners, which can also be neuron type specific.

2.1.2 Coexistence of neuropeptides with other messengers

When neuropeptides were discovered, it was believed that peptidergic systems were separate from the neurons containing classical neurotransmitters. Thereafter, it has been understood that neurons are able to manufacture and utilize more than a single molecule for signalling (Hökfelt 1991). Releasing a mixture of messenger molecules provides a neuron the possibility to signal with variable velocities and functions (Kupfermann 1991). This coexistence is now considered a common trait of all neurotransmission (Merighi 2002).

Neuropeptides can coexist with several other types of neurotransmitters, such as other neuropeptides, classical neurotransmitters, the gaseous neurotransmitter nitric oxide and certain growth factors (Salio et al. 2006). It is generally believed that when a neuropeptide coexists with a classical neurotransmitter, the principal messenger molecule is the latter one. Therefore neuropeptides are considered to be the modulators of the actions of the classical neurotransmitters (Hökfelt et al. 2000). The coexistence is so common, that it is more of an exception if there are no classical neurotransmitters present in the neuropeptidergic neurons. This seems to be the case in the hypothalamic magnocellular neurons, where oxytocin and AVP are the principal messengers. However, there is no rule about the colocalization combinations of neurotransmitters and neuropeptides. Examples of the coexistence of neuropeptides with classical neurotransmitters are given in Table 2-2.
Table 2-1. Examples of neuropeptides in the CNS, their families and amino acid sequences. The sequences of the neuropeptides susceptible to hydrolysis by PREP in vitro are highlighted in bold. Modified from Hökfelt et al. 2000 and Salio et al. 2006.

<table>
<thead>
<tr>
<th>Neuropeptide</th>
<th>Amino acid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hypothalamic peptides</strong></td>
<td></td>
</tr>
<tr>
<td>Arginine-vasopressin</td>
<td>Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH₂</td>
</tr>
<tr>
<td>Oxytocin</td>
<td>Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH₂</td>
</tr>
<tr>
<td><strong>Hypothalamic releasing and inhibiting peptides</strong></td>
<td></td>
</tr>
<tr>
<td>LHRH</td>
<td>pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂</td>
</tr>
<tr>
<td>Somatostatin</td>
<td>Ser-Ala-Asn-Ser-Asn-Pro-Ala-Met-Ala-Pro-Arg-Glu-Arg-Lys-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-NH₂</td>
</tr>
<tr>
<td>TRH</td>
<td>pGlu-His-Pro-NH₂</td>
</tr>
<tr>
<td><strong>Neuropeptide Y and related peptides</strong></td>
<td></td>
</tr>
<tr>
<td>Neuropeptide Y</td>
<td>Tyr-Pro-Ser-Lys-Pro-Asp-Asn-Pro-Gly-Glu-Asp-Ala-Pro-Ala-Glu-Asp-Leu-Ala-Arg-Tyr-Ser-Ser-Leu-Arg-His-Tyr-Ile-Asn-Leu-Ile-Arg-Gln-Arg-Tyr-NH₂</td>
</tr>
<tr>
<td><strong>Opioid peptides</strong></td>
<td></td>
</tr>
<tr>
<td>Dynorphin A</td>
<td>Tyr-Gly-Gly-Phe-Leu-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln</td>
</tr>
<tr>
<td>Met-enkephalin</td>
<td>Tyr-Gly-Gly-Phe-Met</td>
</tr>
<tr>
<td>Leu-enkephalin</td>
<td>Tyr-Gly-Gly-Phe-Leu</td>
</tr>
<tr>
<td><strong>Tachykinins</strong></td>
<td></td>
</tr>
<tr>
<td>Neurokinin A</td>
<td>His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH₂</td>
</tr>
<tr>
<td>Neurokinin B</td>
<td>Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met-NH₂</td>
</tr>
<tr>
<td>Substance P</td>
<td>Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH₂</td>
</tr>
<tr>
<td><strong>VIP-glucagon family</strong></td>
<td></td>
</tr>
<tr>
<td>VIP</td>
<td>His-Ser-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-NH₂</td>
</tr>
<tr>
<td><strong>Other peptides</strong></td>
<td></td>
</tr>
<tr>
<td>ACTH</td>
<td>Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Val-Gly-Lys-Lys-Arg-Pro-Arg-Pro-Val-Val-Lys-Val-Tyr-Pro-Asn-Gly-Ala-Glu-Glu-Pro-Ser-Ser-Ser-Ser-Glu-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe</td>
</tr>
<tr>
<td>CCK</td>
<td>Asp-Tyr(SO₃H)-Met-Gly-Trp-Met-Asp-Phe-NH₂</td>
</tr>
<tr>
<td>Endomorphin-1</td>
<td>Tyr-Pro-Trp-Phe-NH₂</td>
</tr>
<tr>
<td>Endomorphin-2</td>
<td>Tyr-Pro-Phe-Phe-NH₂</td>
</tr>
<tr>
<td>α-MSH</td>
<td>Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH₂</td>
</tr>
<tr>
<td>Neurotensin</td>
<td>pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Pro-Tyr-Ile-Leu</td>
</tr>
</tbody>
</table>

ACTH, corticotropin; CCK, cholecystokinin; CRH, corticotropin-releasing hormone; LHRH, luteinizing-hormone-releasing hormone; α-MSH, α-melanocyte-stimulating hormone; TRH, thyrotropin-releasing hormone; VIP, vasoactive intestinal polypeptide.
Table 2-2. Examples of the coexistence of neuropeptides with classical neurotransmitters in the CNS. Modified from Binder et al. 2001 and Salio et al. 2006.

<table>
<thead>
<tr>
<th>Neuropeptide</th>
<th>Classical neurotransmitter</th>
<th>CNS area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substance P</td>
<td>5-HT</td>
<td>Medulla oblongata</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dorsal raphe nuclei</td>
</tr>
<tr>
<td>Noradrenaline</td>
<td></td>
<td>Medulla oblongata</td>
</tr>
<tr>
<td>Aspartate</td>
<td></td>
<td>Spinal cord</td>
</tr>
<tr>
<td>GABA</td>
<td></td>
<td>Hypothalamus</td>
</tr>
<tr>
<td>Glutamate</td>
<td></td>
<td>Retinal ganglion cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Spinal cord</td>
</tr>
<tr>
<td>Somatostatin</td>
<td>GABA</td>
<td>Visual cortex</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Entorhinal cortex</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Suprachiasmatic nucleus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Amygdala</td>
</tr>
<tr>
<td>Acetylcholine</td>
<td></td>
<td>Hippocampus</td>
</tr>
<tr>
<td>Neurotensin</td>
<td>Dopamine</td>
<td>VTA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Substantia nigra</td>
</tr>
<tr>
<td></td>
<td>GABA</td>
<td>NAcc shell</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NAcc core</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Striatum</td>
</tr>
</tbody>
</table>

CNS, central nervous system; GABA, gamma-aminobutyric acid; 5-HT, 5-hydroxytryptamine; NAcc, nucleus accumbens; VTA, ventral tegmental area.

Co-existence and co-release of classical neurotransmitters and neuropeptides is often called co-transmission regardless of the existence of a true interaction. Indeed, based on the differences in the synthesis, storage, release and targets of classical neurotransmitters and neuropeptides, it is possible that interaction does not always occur (Yang et al. 1996). On the other hand, multiple transmitters released to the extracellular space can result in interactive actions even though they would not be released from the same neuron.

2.2 Neuropeptides - from synthesis to signalling

2.2.1 Biosynthesis, storage and release

There are several common features in the biosynthesis of most neuropeptides, including the synthesis as a part of larger inactive protein precursors known as proneuropeptides or prohormones (Fig. 2-1) (Kitabgi 2006a). The synthesis of neuropeptides begins with the translation of the mRNAs and the generation of preproneuropeptides (Hook et al. 2008). Thereafter, the proteolytic processing starts in the rough endoplasmic reticulum (RER), where the proneuropeptide is released form the preproneuropeptide. The proneuropeptides are routed through the Golgi apparatus and packaged into large dense core vesicles (LDCV, also known as large granular vesicles) together with the processing proteases (Seidah 2011). Processing enzymes recognize the specific cleavage sites, which are often flanked by clusters of basic residues (Kitabgi 2006a). The proneuropeptides may
contain copies of only one or several different peptides in their sequence. As an example, TRH is cleaved from a proneuropeptide containing five copies of TRH, whereas proopiomelanocortin (POMC) precursor is cleaved into ACTH, α-melanocyte stimulating hormone (α-MSH) and β-endorphin, which is an opioid peptide (Fig. 2-1) (Hökfelt et al. 2000). Also other enzymatic modifications can happen in the LDCVs, such as glycosylation, C-terminal amidation, acetylation, phosphorylation and sulfation (Hökfelt et al. 2000). Finally, LDCVs can contain one or more cleavage products from the same precursor polypeptide.

LDCVs are usually targeted to axon terminals, but also some LDCVs are transported to dendrites (Merighi et al. 2011). In the terminals, there are also small synaptic vesicles (SSVs) containing classical neurotransmitters. LDCVs differ from them in the way how they are stored and released from the presynaptic neuron (Fig. 2-2). SSVs are usually located in large amounts near the synaptic axon terminal. Their release requires a high elevation in the intracellular Ca\(^{2+}\) concentration close to the Ca\(^{2+}\) channels of the synapse.

![Diagram of Proenkephalin, Proopiomelanocortin, ProNPY, Progalanin, ProCRF, and Provasopressin](image)

**Figure 2-1.** Example of the structure of proneuropeptides (neuropeptide precursor proteins). Neuropeptides are synthesized as proneuropeptides and processed proteolytically from mono- (R), di- (KR, RK, RR, KK) and multibasic (KKRR) sites to release the active neuropeptide. Some proneuropeptides, like the proneuropeptides of neuropeptide Y (NPY), galanin, corticotropin-releasing hormone (CRF) and arginine-vasopressin (AVP), contain only one copy of the active neuropeptide. Whereas the proneuropeptide proenkephalin contains several copies of (Met)enkephalin (M), one copy of (Leu)enkephalin (L), and one copy of related opioid peptides (ME-Arg-Phe; H) and ME-Arg-Gly-Cleu (O). Additionally some proneuropeptides carry copies of discrete peptides, such as the proneuropeptide pro-opiomelanocortin (POMC), which produces corticotropin (ACTH), α-melanocyte-stimulating hormone (α-MSH) and β-endorphin (Hook et al. 2008).
LDCVs are stored away from the synaptic membrane in variable amounts, and their release is triggered by a diffuse increase in the Ca$^{2+}$ concentration inside the terminal (Verhage et al. 1991). Interestingly, LDCVs are released anywhere at the terminal membrane. It has been suggested that approximately 50% of the release events occur at extra-synaptic locations, also in dendrites (de Wit et al. 2009). Co-stored neuropeptides are released at the same time, but the neuron can regulate the relative proportions of individual neuropeptides when they are synthesized. Apparently also the different dissolving rates of the co-stored neuropeptides from the LDCVs may regulate the speed of peptide secretion. Vesicles empty their contents to the extracellular space by either classical exocytosis, where the vesicle membrane is completely fused with the plasma membrane, or with a so called “kiss and run” principle, where a transient pore is formed to release part of the transmitters. LDCVs typically release their content using exocytosis (Harata et al. 2001).

Figure 2-2. A simplified figure about the synthesis and transport of neuropeptides. First, the inactive precursor proneuropeptide is synthesized in the endoplasmic reticulum (ER). Then the precursor is transferred to the Golgi apparatus for packaging. The proneuropeptides are packaged in large dense core vesicles (LDCV) together with processing enzymes which then cut out the bioactive peptides on the way to the synaptic terminal. Neuropeptide-containing LDCVs reside in variable amounts in the terminal area, whereas the small synaptic vesicles (SSV) containing classical neurotransmitters cluster close to the synaptic axon terminal. TGN, trans-Golgi network. Modified from Bean et al. 1994.
2.2.2 Neuropeptide receptors

Neuropeptides signal through specific G-protein-coupled receptors (GPCRs) that are coupled to various intracellular signalling cascades. Their localization follows distinct patterns generally away from the synaptic cleft and usually also away from the ligand peptides, resulting in the so-called peptide/receptor mismatch (Merighi et al. 2011). The expression of neuropeptide receptors can be functionally regulated. For example, activity-dependent translocation of neuropeptide receptors from LDCVs’ to terminals’ membranes occurs at least in the kappa opioid receptors of the hypothalamus (Shuster et al. 1999). The differences in the expression of neuropeptide receptors can in turn change the overall effects of released neuropeptides. After agonist binding, neuropeptide GPCRs are internalized and recycled back to the cell membrane.

2.2.3 Neuromodulatory functions in the CNS

As mentioned in chapter 2.1.2, neuropeptides are generally considered to modulate the actions of classical neurotransmitters. The modulation of fast neurotransmission can occur pre- and postsynaptically affecting both excitatory and inhibitory neurotransmission (Merighi et al. 2011). There are different ways for the neuropeptides to modulate neurotransmission, although the intracellular mechanisms are not entirely understood. The simplest kind of interaction occurs in the postsynaptic membrane when a neuropeptide GPCR alters the signal transduction properties of the ion channel receptor opened by a classical neurotransmitter (Merighi et al. 2011). This can change the affinity of the receptor for the neurotransmitter. Also, the number of receptors on the plasma membrane can be altered, resulting in an increase or decrease in the neurotransmission. Presynaptic modulation exists usually through presynaptic autoreceptors of one or more neurotransmitters which control their own release. As neuropeptides can diffuse far away from the site that they have been released, there is a possibility for them to control neurotransmission also at different synaptic sites.

2.2.4 Neuropeptide-processing enzymes

Peptidases play several pivotal roles in the life of neuropeptides: on one hand they are responsible for neuropeptide synthesis, but on the other hand they metabolize and inactivate neuropeptides determining the range and time span of neuropeptidergic signalling. Generally, the biosynthetic processing occurs intracellularly and the metabolism extracellularly. The processing enzymes are called exopeptidases, if they cleave amino acids from the end of the peptide chain, whereas endopeptidases break peptide bonds of nonterminal amino acids. The peptide precursor molecules contain basic amino acids separating the bioactive and spacer regions (Fig. 2-1). Two endopeptidases (proprotein convertase 1, EC3.4.21.93; and proprotein convertase 2, EC3.4.21.94) appear to be involved in the cleavage of the precursor molecules generating intermediate peptides with C-terminal basic residues (Fricker 2005). Thereafter, the basic residues are removed by carboxypeptidase E (EC 3.4.17.10). Sometimes the peptide needs further processing, such as C-terminal amidation by peptidylglycine-α-amidating monooxygenase (EC 1.14.17.3).
N-terminal acetylation or other modifications like glycosylation and phosphorylation can occur. However, the enzymes responsible for these modifications are not well characterized.

Neuropeptide signalling can be terminated by overlapping mechanisms, including peptide degradation by peptidases on the surface of the cell, receptor uncoupling from heterotrimeric G-proteins and receptor endocytosis (Defea et al. 2000). The peptidases and the mechanisms how neuropeptides are cleaved are highly conserved in both at the level of protein sequence and catalytic properties (Isaac et al. 2009). Neuropeptide-metabolizing enzymes are usually localized extracellularly; some of them are attached to the extracellular surface with transmembrane segments or they are secreted to the extracellular space without any anchors. Commonly, these enzymes belong to a group of thermolysin-like metalloendopeptidases including neprilysin (neutral endopeptidase/enkephalinase EC3.4.24.11; EP 24.11), angiotensin-converting enzyme (EC3.4.15.1; ACE), neurolysin (EC3.4.24.16; EP 24.16), endothelin-converting enzyme (EC3.4.24.71; ECE) and EP 24.15 (EC3.4.24.15; thimet oligopeptidase, TOP) (Kim et al. 2003).

A common feature for the metabolism of neuropeptides is that the specificity is wide; several different peptidases can participate on the degradation of a certain neuropeptide and a certain peptidase can degrade several neuropeptides (Fricker 2005). For example, neurotensin is cleaved in the synaptic membranes of the rat brain by EP 24.15, EP 24.11 and EP 24.16 (Kitabgi 2006b). Unfortunately, the wide specificity of the processing enzymes has hindered the development of enzyme inhibitors as drug candidates. Since a relatively small number of peptidases are responsible for the processing of majority of neuropeptides, the inhibition of one enzyme affects several different neuropeptides and fails to produce specific effects. Neuropeptide degradation can also produce shorter but still active peptides. That seems to be the case with SP, which C- and N-terminal fragments are known to possess different effects (Nikolaus et al. 2000). Interestingly, neuropeptides are released in relatively high (micromolar) concentrations. Given the affinity of neuropeptides to their receptors, even a 100-fold decrease in these concentrations in the extracellular space by a peptidase would still leave a sufficient number of intact neuropeptide molecules to reach the receptors (Landgraf and Neumann 2004).

2.3 Common brain neuropeptides that are also putative PREP substrates

Neuropeptides play an important role in the normal function of CNS. Although there are several neuropeptides present in the CNS, this chapter will focus on those known as putative PREP substrates, at least in vitro (for review, see García-Horsman et al. 2007 and Männistö et al. 2007). In theory, PREP can cleave short peptides with an internal proline residue (Cunningham and O’Connor 1997). However, as mentioned in the Introduction, the relevance of PREP in neuropeptide metabolism in vivo has been questioned. Still, it is important to understand the effects of the putative PREP substrates in the brain. The basic characteristics of the behaviourally most interesting putative PREP substrate peptides are reviewed here, and their effects on behaviour are discussed in chapter 2.4.
2.3.1 AVP

AVP was first detected 1895 by Oliver and Schäfer as a neurohypophysial hormone that altered blood pressure (Caldwell et al. 2008). This 9 amino acid peptide is biologically active in the periphery and in the CNS. It is mainly synthesized in the magnocellular cells of the supraoptic nucleus and paraventricular nucleus which project to the posterior pituitary (Brownstein et al. 1980). These neurons secrete AVP to the blood stream where it acts on the vascular system. However, there are also small AVP containing neuron populations, mainly in the paraventricular nucleus, bed nucleus of the stria terminalis (BNST), medial amygdala and suprachiasmatic nucleus, whose projections remain in the brain (Fig. 2-3). Also the distribution of AVP receptors suggests that the peptide has a role as a neuropeptide transmitter in the brain. Of the three known receptor types for AVP, V1A is located in the periphery (liver, kidney and vasculature) and widely in the brain. V1B is prominent in the anterior pituitary and in many other tissues and throughout the brain. V2 is mainly located in the kidney (Caldwell et al. 2008). AVP is known to regulate various behaviours, especially social behaviours related to affiliation, social and non-social memory, as well as mood and aggression (Caldwell et al. 2008). It is also known to control the circadian hormone secretion (Kalsbeek et al. 2010).

Figure 2-3. Major arginine-vasopressin (AVP) nuclei and their projections in a sagittal section of the rat brain (modified from McEwen 2004). BNST, bed nucleus of the stria terminalis; LC, locus coeruleus; LS, lateral septum; VTA, ventral tegmental area.

2.3.2 Oxytocin

The nonapeptide oxytocin is synthesized in neurons of the supraoptic and paraventricular nuclei of the hypothalamus, which project to the posterior pituitary and release oxytocin into the bloodstream (Landgraf and Neumann 2004). This peripherally acting oxytocin is critical for the processes of lactation and parturition. Oxytocin neurons of the paraventricular nucleus also send projections to many regions within the brain, including
the hippocampus, amygdala, and hypothalamus (Fig. 2-4). Oxytocin excerts its effects via oxytocin receptors (OTR), GPCRs which are located in discrete brain regions, such as the central nucleus of the amygdala and the ventromedial nucleus of the hypothalamus (Tribollet et al. 1992; Bale et al. 2001). Oxytocin is involved in various behaviours such as maternal care, aggression, pair bonding, sexual behaviour, social memory and anxiety-related behaviour. Although the behavioural effects of oxytocin have been extensively studied, they seem to differ depending on the species, strain, gender, behavioural test conditions, mode of drug administration and other experimental conditions (Neumann 2008).

![Figure 2-4. Sagittal illustration of major oxytocin nuclei and their projections in the rat brain (modified from McEwen 2004). MFB, medial forebrain bundle; MS, medial septum; SN, substantia nigra.](image)

### 2.3.3 Neurotensin

Neurotensin is a tridecapeptide found in the CNS as well as in the gastrointestinal tract. The gene encoding preproneurotensin also contains the sequence of a related 6 amino acid peptide neuromedin N (Kitabgi et al. 1992). Neurotensin-producing neurons as well as their projections are abundantly expressed in the brain (Fig. 2-5), giving the peptide a wide range of effects. Most abundantly neurotensin is found in the amygdala, lateral septum, BNST, SN and ventral tegmental area (VTA) (Uhl 1982). Neurotensin exerts its effects through two GPCRs, the high-affinity NTS1 and the low-affinity NTS2 (Mustain et al. 2011). NTS1 is found throughout the CNS, especially in the dopaminergic neurons of the midbrain, in the small and large intestine, liver and in various malignancies. NTS2 is located more scatteredly in the CNS than NTS1, but not in the gastrointestinal tract. Also two single-transmembrane domain receptors bind neurotensin (NTS3 and sorLA/LR1), but their role is not fully understood.
Since its discovery in the 1970s, the effects of neurotensin have been studied extensively. Neurotensin acts as a neurotransmitter and a modulator of other signals. The close association between neurotensin and the mesocorticolimbic and nigrostriatal dopamine systems has served as a rationale to study interaction between these systems. Indeed, neurotensin has been well established as a modulator of dopaminergic neurotransmission (for a review, see Binder et al. 2001 and St-Gelais et al. 2006). Therefore, neurotensin has been implicated in CNS conditions such as schizophrenia, Parkinson’s disease and addiction, which are characterized by abnormalities in dopaminergic functions. Neurotensin can affect dopaminergic transmission via two mechanisms: it modulates the activity of dopaminergic neurons and controls the release of dopamine (St-Gelais et al. 2006). The activating effect occurs via a Ca\(^{2+}\)-dependent excitatory effect (St-Gelais et al. 2004) and the release of dopamine is controlled with a separate indirect antagonistic effect on dopamine D\(_2\) receptors (Binder et al. 2001; Jomphe et al. 2006; Fawaz et al. 2009). The synaptic localization of NTS1 and D\(_2\) receptors determines whether the overall dopaminergic signalling is enhanced or inhibited (reviewed by Binder et al. 2001, also summarised in Fig. 6-1).

Although neurotensin is best known for its effects on the dopaminergic transmission, there is also evidence about the connections of neurotensin with cholinergic, 5-HT, GABAergic and glutamatergic neurotransmission (St-Gelais et al. 2006). Neurotensin receptors are expressed in these neurons in variable amounts and neurotensin can have either stimulatory or inhibitory effects (Mustain et al. 2011). Furthermore, neurotensin has been reported to affect the activity of glial cells, such as astrocytes and microglia, suggesting a role for neurotensin in the inflammation or trauma processes.
2.3.4 Somatostatin

Somatostatin (somatotropin release-inhibiting factor, SRIF) is a cyclic peptide first known as an inhibitor of growth hormone release from the anterior pituitary (Siehler et al. 2008). Somatostatin is found in the CNS, but also in the endocrine tissues and in the gastrointestinal tract. It has a wide range of biological actions, including inhibition of the secretion of growth hormone, insulin, glucagon and gastrin, as well as other hormones secreted by the pituitary and gastrointestinal tract. There are two biologically active forms of somatostatin in mammals, the 14 amino acid peptide SRIF14 that is more predominant in the CNS, and a longer N-terminally extended form SRIF28. Both are produced by a same gene and cleaved from a common proneuropeptide (Vezzani and Hoyer 1999). In mammals, six somatostatin GPCRs (sst1, sst2A, sst2B, sst3, sst4 and sst5) have been cloned (Viollet et al. 2008). The distribution of somatostatin receptors is given in Table 2-3. According to the similarities in the structural and pharmacological features, somatostatin receptors have been divided in two groups (for a review, see Olias et al. 2004). In the CNS, somatostatin and its receptors are located in regionally varying amounts (Epelbaum 1986; Schindler et al. 1996).

High somatostatin immunoreactivity has been found in the hypothalamus, amygdala, preoptic area, hippocampus, striatum, cerebral cortex, olfactory regions, and brainstem. Somatostatinergic neurons can be classified in two subcategories, the long-projecting neurons and short GABAergic interneurons acting within microcircuits (Viollet et al. 2008). In addition to the role in the neuroendocrine processes, somatostatin is known to control neuronal activity in the CNS (Viollet et al. 2008). Via its receptors, somatostatin exerts several different functions (see Viollet et al. 2008), including the presynaptic inhibition of glutamate and GABA release. Therefore it has been connected to the modulation of sleep, eating and drinking, locomotor activity, cognition and nociception (Vezzani and Hoyer 1999).

<table>
<thead>
<tr>
<th>Brain area</th>
<th>Main somatostatin receptors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Olfactory bulb</td>
<td>sst2, sst3, sst4</td>
</tr>
<tr>
<td>Anterior olfactory nucleus</td>
<td>sst2</td>
</tr>
<tr>
<td>Neocortex</td>
<td>sst2, sst4</td>
</tr>
<tr>
<td>Hippocampus:</td>
<td></td>
</tr>
<tr>
<td>CA1</td>
<td>sst2, sst4</td>
</tr>
<tr>
<td>CA3</td>
<td>sst2</td>
</tr>
<tr>
<td>Dentate gyrus</td>
<td>sst2</td>
</tr>
<tr>
<td>Amygdala</td>
<td>sst2</td>
</tr>
<tr>
<td>Lateral hypothalamus</td>
<td>sst1, sst2</td>
</tr>
<tr>
<td>Locus coeruleus</td>
<td>sst2</td>
</tr>
</tbody>
</table>
2.3.5 SP

The undecapeptide SP is one of the best-known and most-studied neuropeptides. It was first discovered as early as in the 1930s (Mantyh 2002) from brain and intestine extracts, and later identified in the 1970s (Chang et al. 1971). SP is found in the CNS, with especially high levels in the hypothalamus and SN (Fig. 2-6), as well as in the spinal cord and PNS. SP belongs to the neuropeptide family called tachykinins together with neurokinin A (NKA) and B (NKB), which share the similar C-terminal amino acid sequence (Phe-X-Gly-Leu-Met x NH2) (Saria 1999; Hökfelt et al. 2001). They are encoded by two genes, preprotachykinin I and preprotachykinin II (Hökfelt et al. 2001). NKB is only produced by the latter gene. Three different tachykinin receptors are known: NK1, NK2 and NK3 receptors. Tachykinin peptides bind to these receptors in the following order of affinity; SP binds with highest affinity to the NK1 receptor, whereas NKA prefers NK2 receptors and NKB preferably binds to the NK3 receptors (Saria 1999). NK1 receptors are highly expressed in the striatum, nucleus accumbens (NAcc), hippocampus, lateral nucleus of the hypothalamus, habenula, interpeduncular nucleus, nucleus of the tractus solitarius, raphe nuclei and medulla oblongata (Otsuka and Yoshioka 1993).

Based on the anatomical distribution in the CNS and PNS, SP and its receptors have been connected to several pathophysiological conditions, including pain, movement disorders, memory and learning, anxiety, emesis and depression (Quartara and Maggi 1998). In the CNS, SP frequently resides the same neuron as other tachykinins and classical transmitters such as dopamine, ACh, 5-HT, GABA and glutamate (Hasenöhrl et al. 2000). Indeed, SP has been shown to regulate the release of these neurotransmitters or their effects on the target neurons (for a review, refer to Otsuka and Yoshioka 1993). Many antagonists have been developed for the SP-preferring NK1 receptor, some of which penetrate to the brain after oral administration (Quartara and Maggi 1998). However, the species variation in the primary sequence of the NK1 receptive site has hindered the research regarding the physiological functions of SP (Saria 1999). Furthermore, it has been suggested that SP may act as a precursor for two N- and C-terminal fragments having separate biological functions.

The different effects of SP and its fragments have been connected to selective and site-specific changes in the activities of classical neurotransmitters. For example, SP and its C-terminal fragment, but not the N-terminal sequence, increase extracellular dopamine in NAcc (Boix et al. 1992). Therefore, the behavioural effects of SP may be determined by the site of action within the brain and the way of the hydrolysis of the peptide. It has been also suggested, that the actions of SP or its fragments are terminated by an active uptake mechanism (Nakata et al. 1981).
As neuropeptides can act as neurotransmitters or modulators, regulators of hormonal secretion and cerebral blood flow, or as mediators of immune system and blood-brain barrier permeability (Malavolta and Cabral 2011), they must have an impact on behaviour. Furthermore, the malfunction of neuropeptidergic systems has been connected to several CNS disorders. However, due to the abundance of neuropeptides and the wide spectrum of their functions in the brain also make the interpretation of the effects of a single neuropeptide on a specific type of behaviour complicated. This chapter will focus on the data obtained about the behavioural effects of some of the most common neuropeptides that are also putative substrates of PREP in vitro (AVP, neurotensin, oxytocin, somatostatin and SP) on experimental animals. The chapter will concentrate on some effects of these neuropeptides on motor behaviour, memory and learning and affective behaviours, and some other behavioural effects relevant to this thesis.

The research about the behavioural effects of neuropeptides has been hindered by the lack of selective non-peptide receptor agonists/antagonists, the very limited permeability of these peptides across the blood-brain barrier, and their fast degradation. The studies have been mostly done by injecting the neuropeptides into the brain, since they commonly do not reach the CNS when administered by other routes. Neuropeptide receptor agonists or antagonists have also been developed, some of them able to reach the brain. In addition, some studies in knockout mice are reviewed. Some of the neuropeptides have structural similarities, such as oxytocin and AVP, and unspecificity...
between them may occur. Therefore, recently more specific techniques such as the overexpression of neuropeptides or their receptors with virally mediated gene transfer have been used. It is important to keep in mind that the manipulation of a single component is likely to affect several other systems. For example using knockout mice or chronic treatments can have long-term consequences making the data interpretation difficult. All techniques have their own advantages and pitfalls, but together they can contribute to understanding the behavioural effects of neuropeptides.

Table 2-4 summarizes the behavioural effects discussed in this chapter.

2.4.1 Motor behaviour

Several neuropeptides affect motor functions by direct or indirect mechanisms. Increased or decreased locomotor activity in an open field study can indicate multiple effects of a neuropeptide. For instance, locomotor activity can be decreased because of a sedative or anxiogenic effect.

Neuropeptides that have been reported to increase locomotor activity, include AVP and somatostatin. Intracerebroventricular (i.c.v.) and subcutaneous (s.c.) administration of AVP has been associated with an increase in locomotor activity in young adult rats, which could be related to an increase in dopaminergic activity (DiMichele et al. 1996). AVP can also modulate locomotion related to circadian clock (Li et al. 2009) or dehydrated state, via V1A receptors (Tsunematsu et al. 2008). Somatostatin receptors in the striatum and other basal ganglia nuclei are involved in regulation of motor behavior (Semenova et al. 2010). Somatostatin can modulate locomotor activity by activating sst1, sst2 and sst4 receptors in the ventral pallidum and sst2 and sst4 receptors in the striatum. However, the reports about the effects of somatostatin on motor functions have not always been uniform. I.c.v and local brain administrations of somatostatin increased the locomotor activity in several studies (Vecsei et al. 1984; Viollet et al. 2000). However, there are also studies reporting no effect of somatostatin on the locomotor activity (Vecsei et al. 1984).

The reports about the effects of SP and oxytocin on locomotor activity are more complex. I.c.v. injections of SP enhance stereotypical grooming and scratching behaviour in mice (Hall et al. 1987). NK1 receptor agonist GR 73632 has also increased locomotor activity in guinea pigs (Severini et al. 2002). Although the effects of intraperitoneal (i.p.) and s.c. injections of SP in mice have been variable, SP has decreased spontaneous locomotor activity and counteractedamphetamine-induced hyperactivity. Oxytocin can affect locomotor activity differently with different doses and in male and female rats (Uvnas-Moberg 1994). In an open field test, low dose of oxytocin increased the exploratory activity of rats in the centre of the field. In contrast, about 100-fold higher doses suppressed the locomotor activity (Uvnas-Moberg et al. 1994). In male rats, the s.c. injected oxytocin has sedative effects (Uvnas-Moberg et al. 1994), whereas in females, it increases or decreases locomotor activity depending on the oestrous cycle and sex hormones (Petersson et al. 1998). Oxytocin has also been reported to enhance locomotor activity induced by clonidine, an α2-receptor agonist. This finding connected the motor activity-increasing effects to the activation of noradrenergic neurons in the locus coeruleus (Petersson et al. 1999).
As a proposed endogeneous antipsychotic, neurotensin has been mostly studied in animal models related to schizophrenia, such as stimulant-induced hyperlocomotion or antipsychotic-induced hypolocomotion. However, also spontaneous locomotor activity has been studied. I.c.v. and systemic neurotensin or its analogue have reduced both spontaneous locomotor activity and amphetamine-induced hyperlocomotion (Casti et al. 2004; Caceda et al. 2006). However, only the stimulation of neurotensin system seems to affect locomotion, since neurotensin antagonists have not affected mouse spontaneous locomotor activity when administered i.p. (Casti et al. 2004). In addition, it has been shown that although single i.c.v. administration of neurotensin blocks the amphetamine-induced hyperlocomotion, chronic administration can potentiate it (Norman et al. 2008). However, pretreatment with a NTS1 antagonist has not modified the locomotor activity induced by stimulants, suggesting that endogenous neurotensin is not involved in stimulant-induced hyperlocomotion (Caceda et al. 2012). On the other hand, haloperidol-induced hypolocomotion was alleviated by neurotensin antagonists SR48692 and SR142948 (i.p.) (Casti et al. 2004).

2.4.2 Learning and memory

The ability to remember is a prerequisite for functioning in life. With great simplification, memory processes involve acquisition, consolidation, reinforcement and retrieval of information. The role of cholinergic system in learning and memory is well established (Gulpinar and Yegen 2004). However, these and other memory-related functions are modulated by a number of neuropeptides. Indeed, several neuropeptides, such as AVP, oxytocin, ACTH, CRF, somatostatin, neurotensin and SP have been suggested to modulate learning and memory (Kovacs and De Wied 1994). To be able to exert these effects, neuropeptides or their active fragments need to act at brain areas responsible for memory functions. Although the whole picture is not fully unveiled, there is basic understanding about the brain regions involved in cognitive processes, including the hippocampus, amygdala, medial septum and neocortex (Gulpinar and Yegen 2004). The best documented neuropeptides involved in memory and learning processes are AVP and oxytocin (Gulpinar and Yegen 2004). Next paragraphs summarize the data obtained about the effects of AVP, neurotensin, oxytocin, somatostatin and SP on learning and memory behaviours in experimental animals.

Promnesic neuropeptides

The neuropeptides with reported promnesic properties include AVP, somatostatin and SP (Kovacs and De Wied 1994). AVP in the ventral hippocampus is connected to information processing, memory consolidation, storage and retrieval (Gulpinar and Yegen 2004). Although i.c.v. injected AVP has been shown to improve learning and memory in avoidance paradigms, it has not been as clear if AVP enhances memory in other type of tests (Klimkiewicz 2001). The effects on memory have been proposed to be facilitated mainly via the AVP-mediated activation of hippocampal interneurons (Dietrich and Allen 1997). Some fragments of AVP (4-9 and 5-8) have been shown to be more potent than the native peptide (Dietrich and Allen 1997; Fujiwara et al. 1997). Indeed, it has been suggested, that the AVP₄.
fragment is the most important in memory processes (Klimkiewicz 2001). For example, s.c. injection of AVP$_{4-9}$ fragment has enhanced working and reference memory in the radial-arm maze (Dietrich and Allen 1997). Furthermore, AVP projections from the medial amygdala and BNST to the lateral septum are associated with social memory (Bluthe et al. 1990; Bluthe et al. 1993). Social memory and social recognition are important for an individual to distinguish a friend from foe according to the visual, olfactory or auditory cues, and to choose the appropriate behaviour for the situation. To support this, it has been shown in Brattleboro rats lacking AVP, that the injection of AVP into the lateral septum facilitates social memory (Engelmann and Landgraf 1994). Also in normal rats infusions of V1A antagonists or antisense V1A oligonucleotides into the lateral septum have this kind of memory impaired (Engelmann and Landgraf 1994; Landgraf et al. 1995). Knockout mice have been used to show that the deficiency of V1A impairs social memory which can be reversed with the overexpression of V1A in the lateral septum (Bielsky et al. 2004; Bielsky et al. 2005).

Although best known as a potential endogenous antipsychotic, neurotensin has also been connected to memory functions (Caceda et al. 2006). Neurotensin may affect learning and reinforcement especially through the NTS1 receptors in the central nucleus of amygdala (Laszlo et al. 2010). When neurotensin was administered bilaterally to this nucleus, it enhanced the passive avoidance learning in the rat possibly due to the modulation of mesolimbic dopaminergic system (Laszlo et al. 2012).

The role of somatostatin is well acknowledged in the memory consolidation and retention processes (Viollet et al. 2000). Early studies showed that i.c.v injected somatostatin and its analogue are able to reverse the avoidance response impairing effects of cysteamine, a somatostatin-depleting agent, in a conditioned active avoidance test (Schettini et al. 1988). Similar results have been obtained with passive avoidance learning test (Vecsei et al. 1984; Matsuoka et al. 1995). Matsuoka et al. 1994 showed that the memory-improving effects of somatostatin would be mediated via enhanced cholinergic transmission in the hippocampus. In particular, intrahippocampal injections of somatostatin enhance the rate of acquisition of a spatial discrimination task in a radial maze (Lamirault et al. 2001). Later, somatostatin has been shown to regulate the homeostasis in the hippocampus through modulation of sst2 and sst4 receptors, which seem to cooperate in the control of hippocampus- and striatum-mediated memory functions (Gastambide et al. 2010). Especially hippocampal sst4 is involved in the selection of memory strategies (Gastambide et al. 2009).

SP has been connected to memory processes in several studies (Hasenöhrl et al. 2000). The most important finding has been that the N- and C-terminal fragments of SP have distinct roles in learning and reinforcement (Hasenöhrl et al. 2000). The N-terminal fragment appears to be more important in memory promoting, whereas the C-terminal sequence mediates the reinforcement. These effects have been obtained both after the peripheral and central injections to the nucleus basalis of the ventral pallidum (Huston and Hasenohrl 1995). Also the injections of these fragments to the NAcc caused similar effects, where the N-terminal fragment facilitated learning (Gaffori et al. 1984).
Amnesic neuropeptides
Oxytocin exerts opposite effects on fear-motivated avoidance behaviour as AVP (Kovacs and De Wied 1994). Therefore it has been proposed that oxytocin is an amnestic neuropeptide. Oxytocin impairs memory consolidation when injected to the hippocampal dentate gyrus or dorsal raphe nucleus (Kovacs and De Wied 1994). Also, oxytocin injected to the Meynert nucleus has increased the latency of Morris water maze task in rats, indicating impairment on spatial learning (Wu and Yu 2004).

Taken together, there are several neuropeptides present at the brain areas related to memory and learning, such as the hippocampus, where they regulate neurotransmission. Therefore they can have various effects on memory processes via several different mechanisms. However, there are no particular neuropeptidergic mechanisms that could be responsible for the modulation of learning or memory processes (Kovacs and De Wied 1994). Instead, several different neuropeptides with different localization and origin are cooperating with each other and also with classical transmitters. In some instances a particular neuropeptide becomes more effective in a particular behavioural situation. Interestingly, some of these neuropeptides may contribute to plastic changes in the connectivity of neurons which are reconstructed during learning and memory formation.

2.4.3 Affective behaviours
Neuropeptides are considered to tune the direct communication of neurons, and this tuning may be important in mood. Many neuropeptides are located in the important brain areas for the generation and perception of emotion and stress, such as the hypothalamus, limbic structures, amygdalar nuclei, BNST and frontal cortex structures (Papathanassoglou et al. 2010). Indeed, several neuropeptides have been connected to affective behaviours or even to the pathophysiology of psychiatric disorders, such as schizophrenia, anxiety and depression (De Wied and Sigling 2002; Landgraf 2006). Especially neuropeptides considered to possess opiate-like, neuroleptic-like and amphetamine-like activity may be involved in these processes (De Wied and Sigling 2002). The following paragraphs focus on the effects of AVP, neurotensin, oxytocin, somatostatin and SP on affective behaviours, such as psychotic, anxious and depressive behaviours.

Psychotic behaviour
Of all neuropeptides, neurotensin is best characterized in the regulation of psychotic behaviour. After Charles Nemeroff published his paper about the role of neurotensin as an endogenous antipsychotic in 1980, the theory got a lot of attention (Nemeroff 1980). Nemeroff proposed that neurotensin inhibits dopaminergic neurotransmission like antipsychotics, and that schizophrenia is partially caused by a lack of neurotensin in the brain.

There are several facts supporting the role of neurotensin as an endogenous antipsychotic, mostly obtained from studies with experimental animals. First, neurotensin and its receptors are abundantly expressed in the dopaminergic neurons of the midbrain (Binder et al. 2001), where neurotensin is known to modulate dopaminergic transmission.
Second, studies where neurotensin has been injected in the VTA or NAcc have shown that the effects of neurotensin are similar as those of antipsychotics. For example, both neurotensin and antipsychotics increase the firing rate of dopaminergic neurons when injected to VTA (Hollerman et al. 1992), whereas injections to the NAcc cause the inhibition of D2 receptors (Li et al. 1995). The antipsychotic-like effect of neurotensin is also seen in the behaviour of the animals; it causes sedation, muscle relaxation, hypotermia and stereotypical chewing. Third, there are several studies showing a possible link between neurotensin and dopamine in the pre-pulse inhibition of the startle response (PPI) test which is a commonly used animal model of schizophrenia. It refers to the ability of a weak stimulus to inhibit the response to a following strong stimulus. PPI is altered in schizophrenic patients and the disruption of PPI can be produced in rodents by dopamine agonists or glutamatergic NMDA receptor antagonists. The injection of neurotensin to the NAcc reduces amphetamine induced PPI in rats (Feifel et al. 1999). However, the same effect is not seen with injections to the VTA (Feifel and Reza 1999). Similarly as atypical antipsychotic drugs, the systemically administered neurotensin agonists, NT69L and PD149163, have shown to be effective in pharmacologically induced PPI model. Feifel et al. 2011 showed that neurotensin agonist PD149163 elevated PPI also in brown Norway rats (a rat strain that has genetically deficits in PPI) similarly to clozapine. The behavioural effects of the systemically administered neurotensin agonists are similar as with the direct injections to the NAcc (Caceda et al. 2006). This suggests that postsynaptic neurotensin receptors in the NAcc are stimulated also after the peripheral administration. Furthermore, neurotensin knockout mice have a disrupted PPI (Kinkead et al. 2005).

There is less evidece about the effects of other neuropeptides on psychotic behaviour. The brain AVP system may have a role in both positive and negative symptoms of schizophrenia, probably due to interactions with the glutamatergic and dopaminergic pathways (Frank and Landgraf 2008). However, it has been suggested that AVP analogues would have potentially alleviating effects on schizophrenic behaviour (Matsuoka et al. 2005). Also compelling preclinical evidence indicates that oxytocin has a role in schizophrenia (Feifel 2012). Somatostatin fragments have been linked to schizophrenia and especially the positive symptoms of schizophrenia, since cysteamine, the somatostatin-depleting agent has reversed the amphetamine-induced deficits in PPI model (Feifel and Minor 1997). I.c.v administered somatostatin-28 has caused deficits in PPI, which was then partially reversed with a selective sst1 antagonist SRA-880 (Semenova et al. 2010). Although SP modulates dopamine release in the dopaminergic neurons of the midbrain, it has not been studied in the animal models of schizophrenia. However, it has been suggested that the NK1/NK3 antagonists may be effective in the treatment of schizophrenia (Catalani et al. 2011).

Anxiety and depression
Anxiety is a protective, if at times uncomfortable, emotion that is fundamental to adaptation and survival. However, excessive anxiety can be disabling and interfere with normal life. Several neuropeptides or their analogues have been reported to possess anxiolytic or anxiogenic effects.
A range of studies have shown that AVP has anxiogenic properties (Mak et al. 2012). Antagonists of V1A (Bleickardt et al. 2009) and V1B (Griebel et al. 2002) have reduced the anxiety-related behavior in the elevated plus-maze following i.p. administration. Similar results have also been found in AVP knockout mice (Bielsky et al. 2004). In i.c. administration studies, the exact anxiolytic-like effects of V1A antagonists have been connected to the ventral hippocampus, whereas the anxiolytic-like effects of V1B antagonism may be specific to the dorsal hippocampus (Engin and Treit 2008).

Brain oxytocin is an important regulator of fear and stress responses. Oxytocin is anxiolytic in female and male rats, and in mice, when administered centrally (Windle et al. 1997; Ring et al. 2006; Blume et al. 2008). However, the OTR antagonists have only produced anxiety in pregnant or lactating female rats (Neumann et al. 2000). Therefore it has been suggested that the activation of the oxytocin system during the peripartum period is required for the anxiolytic effect. The central amygdala (Bale et al. 2001) and hypothalamic paraventricular nucleus (Blume et al. 2008) have been connected the anxiolytic effects of oxytocin.

Recently, also somatostatin has been connected to affective behaviours, such as anxiety and depression (Yeung and Treit 2012). Somatostatin receptors are extensively expressed in limbic areas such as the amygdala, septum and hippocampus that are related to the affective behaviours. It has been shown that i.c.v. administered somatostatin produces anxiolytic effects in the elevated plus-maze and antidepressant effects in forced swim test in rats (Engin et al. 2008).

SP has been linked to anxiety and depression in several studies. First, SP may be involved in the action of antidepressants (Shirayama et al. 1996) and anxiolytic drugs (Brodin et al. 1994), because these conditions have low levels of SP in the brain. More importantly, the intracerebral (i.c.) injection of SP agonists have produced anxiogenic (De Araujo et al. 1999) and SP antagonists anxiolytic effects (File 1997) when studied in rats with different animal models. Therefore it seems the severity of anxiety and SP activity go hand in hand (Hasenöhrl et al. 2000). However, the effects are dependent on the specific brain area and the dose of SP (Nikolaus et al. 2000). SP and its C-terminal fragment have had anxiogenic effects when injected to the dorsal periaqueductal grey (De Araujo et al. 1999), but when injected to the ventral pallidum, both N- and C-terminal fragments of SP have produced anxiolytic effects (Nikolaus et al. 2000). With low doses, SP has been reported to be anxiolytic and with higher doses anxiogenic (Hasenohrl et al. 1998).

### 2.4.4 Other behavioural effects

**Affiliative behaviours**

Affiliation comprises social bonding between individuals, such as relationships between sexual partners or parents and infants. These behaviours have been studied using species that express monogamy and biparental care of the offspring, such as the monogamous prairie and pine voles. Interestingly, their behaviour has been compared to the non-monogamous species of the same genus *Microtus* (montane and meadow voles).

Young et al. 1999 showed that a species-specific distribution of V1A is important for the development of affiliative behaviours. It has also been shown that AVP
antagonists injected i.c.v. block the social bonding in voles measured by a partner preference test, whereas AVP infusions promote partner preference (Cho et al. 1999). Interestingly, intra-septal AVP increases and V1A antagonist decreases paternal behaviour in sexually naïve male prairie voles supporting the role of AVP in the affiliative behaviours (Wang et al. 1994). The mechanisms behind the effects are not fully understood. However, it has been shown that also AVP and dopamine interact, especially in the ventral pallidum, to mediate pair bonding (Young et al. 2008). These findings suggest that the interaction between AVP and dopamine systems is important in the regulation affiliative behaviours.

Aggressive behaviour
The AVP projections originating from the BNST and medial amygdala to the caudal lateral septum have been associated with aggressive behaviour in males, depending on the species examined (de Vries and Miller 1998). When V1A antagonists are given orally or injected to the medial preoptic-anterior hypothalamic area (MPOA-AH), they block the aggression-related flank-marking behaviour in the extensively studied Syrian hamsters indicating that MPOA-AH and V1A receptors are important in controlling aggressive behaviour (Ferris and Potegal 1988; Ferris et al. 2006). Interestingly, gonadal steroids control the biosynthesis of V1A (Young et al. 2000). However, there are some species differences in the AVP-mediated regulation of aggressive behaviours.

I.c.v. injection of SP has reduced fighting in mice that were made aggressive with isolation (Hall et al. 1987), and different peptide fragments of SP can have opposite effects on aggressive behaviour.

Table 2-4. A summary table about the effects of neuropeptides and putative in vitro PREP substrates, including arginine-vasopressin (AVP), neurotensin, oxytocin, somatostatin and substance P (SP), on behaviour, including locomotor activity, memory and learning, psychotic behaviour and anxiety and depression. See the text for further details. ↑, mostly increasing effect; ↓, mostly decreasing effect; ††, both increasing and decreasing effects reported; – effects not known.

<table>
<thead>
<tr>
<th>Type of behaviour</th>
<th>AVP</th>
<th>Neurotensin</th>
<th>Oxytocin</th>
<th>Somatostatin</th>
<th>SP</th>
</tr>
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<tbody>
<tr>
<td>Locomotor activity</td>
<td>↑</td>
<td>↓</td>
<td>††</td>
<td>††</td>
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<tr>
<td>Memory and learning</td>
<td>↑</td>
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<tr>
<td>Psychotic behaviour</td>
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<td>Anxiety and depression</td>
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</tr>
</tbody>
</table>
2.5 The importance of PREP in neuropeptide metabolism

The discovery of PREP as an oxytocin-cleaving enzyme in the bovine uterus set the course of the PREP research closely around neuropeptides for about 40 years. Throughout these years, PREP was strongly believed to participate in the neuropeptide degradation. This issue has been comprehensively reviewed by Männistö et al. 2007 and Tenorio-Laranga et al. 2011 and it will be discussed later also in the experimental part of this thesis. Therefore this chapter will cover only the main findings and problems related to PREP and neuropeptides.

There are a couple of important reasons why PREP has been proposed to be involved in the neuropeptide metabolism. First, the catalytic properties of PREP. PREP cleaves proline-containing neuropeptides shorter than 30 amino acids from the internal side of a C-terminal proline (Cunningham and O’Connor 1997). PREP cleaves the Pro-Xaa bond at the carboxyl side, where Xaa is any other amino acid other than proline (Cunningham and O’Connor 1997). Since proline is a unique amino acid with a peptide stabilizing cyclic structure, it reduces the susceptibility of a peptide for the proteolytic hydrolysis. As several broad selectivity peptidases can not hydrolyze proline-containing peptides, specific peptidases, like PREP, are needed. Many of the bioactive peptides, such as SP, TRH, AVP and neurotensin contain proline and are short enough to be degraded by PREP (García-Horsman et al. 2007). Second, PREP is expressed in several tissues, but most abundantly in the brain. Therefore it has been suggested that PREP plays an important role in the regulation of brain neuropeptide levels.

The development of PREP inhibitors has enabled the close investigation of the importance of PREP in neuropeptide metabolism and physiological processes. In the 1990s, PREP was mostly studied as a memory-modulating enzyme, since many of its proposed substrates have been connected to memory and learning (Cunningham and O’Connor 1997). In several studies, PREP inhibitors increased neuropeptide levels (Toide et al. 1995b; Toide et al. 1996; Bellemere et al. 2003) and improved memory (Toide et al. 1995a; Morain et al. 2002; Jalkanen et al. 2007). However, the increasing effects of PREP inhibitors on neuropeptide levels were not uniform. Usually these effects were observed after a single but not chronic administration, and they varied with the dose and brain area studied. Some studies failed to show any increase in the neuropeptide levels (Toide et al. 1995b; Jalkanen et al. 2007; Jalkanen et al. 2011) and in memory and learning. The conclusion was that PREP does not have a significant effect on the neuropeptide levels (Jalkanen et al. 2007; Männistö et al. 2007).

The major dilemma in the role of PREP in neuropeptide metabolism is the intracellular location of PREP. As mentioned above, the enzymes cleaving extracellular active neuropeptides are either anchored to the cell membrane or secreted to the extracellular space. Inside the cell, the enzymes participating in the biosynthesis of the neuropeptides are packaged inside the LDCVs with the precursor molecules. Cytosolic enzymes are not known to participate in neuropeptide processing and there is no evidence indicating that PREP would be inside the LDCVs. Even though PREP has been detected in the synaptosomal membranes and in plasma and cerebrospinal fluid, the quantities of PREP have been very small compared to the tissue levels (Tenorio-Laranga et al. 2008; Tenorio-
Laranga et al. 2010). Therefore, the direct role of PREP in the degradation of extracellular neuropeptides is currently unlikely.

However, Tenorio-Laranga et al. 2011 suggested a novel role for PREP in peptide processing. As a cytosolic enzyme, PREP could generate or degrade active peptides derived from cytosolic proteins. As an example, the tetrapeptide acetyl-N-Ser-Asp-Lys-Pro (Ac-SDKP) derived from the intracellular thymosin beta-4 (Tβ4), is an active molecule important in angiogenesis (Cavasin 2006). PREP can not cleave Ac-SDKP from the full length Tβ4, but following another peptidase PREP can detach Ac-SDKP which then promotes angiogenesis *in vivo* (Myöhänen et al. 2011).

2.6 Concluding remarks of the literature review

Neuropeptides can have various functions in the CNS, but they are best known as modulators of neurotransmission. They can affect diverse behavioural aspects in several parts of the brain. The complex involvement of neuropeptides in multiple processes makes it difficult to distinguish the role of a single peptide in a particular behaviour. Furthermore, it is difficult to affect the levels or functions of a single neuropeptide in a single area of the brain. This has to be taken into consideration when assessing the effects of PREP on the functions of neuropeptides. More interestingly, even the cleavage products of neuropeptides can act as individual signalling fragments, as it is the case with SP. Therefore, the inhibition or enhancement of neuropeptidergic signalling can have various and unpredictable effects. The phenomenon has also raised concerns about the possibility to use neuropeptide agonists or antagonists as drug candidates.
3. AIMS OF THE STUDY

Despite the intensive research, the physiological role of PREP is still unknown. This study aimed to discover novel and clarify the supposed functions of PREP in the brain with different biochemical and behavioural testing methods. The specific aims of this study were:

I To study the role of PREP as a target for various psychopharmacological compounds.

II To investigate the colocalization and interaction of PREP with major neurotransmitter systems in the brain.

III To explore the colocalization and functional connection of PREP with neurotensin system in the nigrostriatal and mesolimbic dopaminergic pathways.

IV To find out whether PREP inhibition affects behaviour in animal models of locomotor activity, memory and learning and Parkinson’s disease in rats.

Figure 3-1. The aims of the study in a simplified scheme. Solid lines represent proved interactions and dash lines putative interactions.
4. MATERIALS AND METHODS

4.1 Animals

Animals were purchased from Harlan Laboratories, the Netherlands (II, III, IV) and Biocenter 2 or 3, Helsinki, Finland (I). Invariably, the animals were housed under controlled conditions (temperature 20 ± 2°C, 12-h light/dark cycle, water available *ad libitum*). Animals had free access to food (Harlan Teklad Global Diets, The Netherlands) in all experiments except in the radial-arm maze study (IV). The detailed description of the restricted diet used in the radial-arm maze study is in paper IV. All animal experiments were performed according to the Council of Europe (directive 86/609) and Finnish guidelines and approved by the State Provincial Office of Southern Finland.

9-11 weeks old male Wistar rats (n = 8) were used for testing the effect of various CNS drugs on the activity of PREP (I). 15 male NMRI mice weighing 35-45 g were used for the *ex vivo* study with a single dose of thioridazine (I). In radial-arm maze experiments (IV), young (3-month-old; n = 31) and old (8- to 9-month-old, n = 51) male Wistar rats were used. Locomotor activity (IV) was studied with 11 young (3-month-old) male Wistar rats. The effects of selective neurotransmitter lesions on the activity and expression of PREP were studied with male Wistar rats (n = 65) weighing 250-350 g on the operation day (II). The effects of neurotensin receptor modulation and PREP inhibition on the levels of dopamine and the co-localization of PREP with neurotensin and NTS1 in the rat brain were studied with 128 and 6 male Wistar rats, respectively (III and unpublished data). The rotational behaviour was tested with 36 male Wistar rats weighing 260-350 g on the day of operation (unpublished).

4.2 Drugs

Suc-Gly-Pro-AMC (N-succinyl-glycyl-prolyl-7-amino-4-methylcoumarin), the substrate for PREP activity assay (studies I, II) was purchased from Bachem (AG, Bubendorf, Switzerland) and dissolved in assay buffer (0.1 M Na-K-phosphate buffer, pH 7.0). AMC (7-amino-4-methylcoumarin; Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was used as a fluorescence standard in the PREP activity assay (I, II) and dissolved in DMSO. For details of the psychoactive compounds used in the PREP activity assays of study I, see Table 1 in paper I.

PREP inhibitors JTP-4819 (2(S)-[(2(S)-(Hydroxyacetyl)-1-pyrrolidinyl]carbonyl]-N-phenylmethyl]-1-pyrrolidinecarboxamide) and KYP-2047 (4-phenylbutanoyl-L-prolyl-2(S)-cyanopyrrolidine) were synthesised by Dr. Erik Wallén (University of Eastern Finland, Department of Pharmaceutical Chemistry, Kuopio, Finland). JTP-4819 was dissolved in assay buffer or saline and used in PREP activity assays and in the *ex vivo* study (I). KYP-2047 was given to the rats in the radial-arm maze studies (IV), in the rotational experiments (unpublished) and in the study of the interaction of PREP and neurotensin (III). It was dissolved in 5% Tween 80 because of its low solubility in water. In rat brain, 3 mg/kg KYP-2047 causes 85% inhibition of PREP activity ten minutes after its i.p. administration.
(compound 2B in Venäläinen et al. 2006) and the inhibition lasts for several hours. NTS1 agonists JMV-449 (Tocris Bioscience, Bristol, United Kingdom) and PD149163 (NIMH Chemical Synthesis and Drug Supply Program) were dissolved in saline. Saline was also used to dissolve NTS1 antagonist SR142948 (Tocris Bioscience, Bristol, United Kingdom) (III).

For the neurotransmitter lesion study (II), 6-hydroxydopamine (6-OHDA) was obtained from Sigma-Aldrich and dissolved in 0.2 mg/ml ascorbic acid solution. Ibotenic acid (Sigma-Aldrich), 5,7-dihydroxytryptamine (5,7-DHT; Sigma-Aldrich) and DSP-4 (N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride; Sigma-Aldrich) were dissolved in saline. In the radial-arm maze study (IV), scopolamine hydrobromide (Sigma-Aldrich) dissolved in saline was used. For the ex vivo study (I), thioridazine hydrochloride (Sigma-Aldrich) was dissolved in saline. In the rotational experiments (unpublished), L-3,4-dihydroxyphenylalanine methyl ester hydrochloride (L-dopa, LD; Sigma-Aldrich) was dissolved in 0.25 % carboxymethylcellulose gel (Sigma-Aldrich). Carbidopa (CD; Orion Pharma Oyj, Espoo, Finland) was added in the same gel to give a suspension. Saline was used to dissolve apomorphine hydrochloride hemihydrate (Sigma-Aldrich).

All treatments were given i.p. in all other studies except in studies II and III where some of the neurotoxins (6-OHDA, 5,7-DHT and ibotenic acid) and NTS1 ligands (JMV-449 and SR142948) were injected i.c. In the rotation experiments, apomorphine was given s.c. Injection volumes (i.p. and s.c. injections) were 0.1 ml/100 g for rats, and 0.1 ml/10 g for mice. Injection volumes for i.c. injections are described in detail in II and III. Drug doses were calculated as free base.

4.3 Stereotaxic surgery (II, III)

Stereotaxic surgery was used to administer neurotoxins (6-OHDA, 5,7-DHT and ibotenic acid) in study II and NTS1 ligands (agonist JMV-449 and antagonist SR142948) in study III to specific areas of the rat brain. The rats were operated under general isoflurane anesthesia (4.5 % for induction, 2-3 % for maintenance; Baxter Oy, Helsinki, Finland) using a stereotaxic frame (Stoelting, Wood Dale, IL, USA). Lidocaine (Orion Pharma) was used to give a local anesthetic effect. After drilling a small hole to the skull, the injections of were given with a 10 μl microinjection syringe (Hamilton, Bonaduz, Switzerland). In the end of the operation, the rats received a single dose of tramadol (1 mg/kg, s.c.; Orion Pharma) for postoperative pain.

4.3.1 Administration of neurotoxins (II)

In study II, two cholinergic nuclei (the medial septum and Meynert nucleus), one 5-HT nucleus (the dorsal raphe) and a dopaminergic pathway (the medial forebrain bundle; MFB) were lesioned with neurotoxins. Only one nucleus was lesioned from each rat. Unilateral lesion of the MFB was also used to study the effects of PREP inhibitor on the rotational behaviour (unpublished). The details of the i.c. neurotoxin injections are given in Table 4-1. The coordinates of the injections were calculated relative to bregma according to the atlas of Paxinos and Watson 1997.
Table 4-1. Details of intracerebral injections (studies II and III).

<table>
<thead>
<tr>
<th>Injected chemical</th>
<th>Study</th>
<th>Injection site</th>
<th>Coordinates from bregma</th>
<th>Dose</th>
<th>Injection volume</th>
<th>Infusion rate</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dopaminergic neurotoxin (6-OHDA)</td>
<td>II, rotation</td>
<td>MFB</td>
<td>-4.4 +1.2 -8.3</td>
<td>10 µg</td>
<td>4 µl</td>
<td>0.5 µl/min</td>
<td>Intact side</td>
</tr>
<tr>
<td>5-HT neurotoxin (5,7-DHT)</td>
<td>II</td>
<td>Dorsal raphe</td>
<td>-7.08 0.4 -6.0</td>
<td>0.08 µg</td>
<td>0.2 µl</td>
<td>0.2 µl/min</td>
<td>Saline</td>
</tr>
<tr>
<td>Cholinergic neurotoxin (IBA)</td>
<td>II</td>
<td>Meynert nucleus</td>
<td>-1.32 3.4 -7.4</td>
<td>3.0 µg</td>
<td>3 µl</td>
<td>1 µl/min</td>
<td>Intact side</td>
</tr>
<tr>
<td>NTS1 agonist (JMV-449) or antagonist (SR142948)</td>
<td>II</td>
<td>Medial septum</td>
<td>0.72 0.0 -7.0</td>
<td>3.0 µg</td>
<td>3 µl</td>
<td>1 µl/min</td>
<td>Saline</td>
</tr>
<tr>
<td>NTS1 agonist (JMV-449) or antagonist (SR142948)</td>
<td>III</td>
<td>Striatum</td>
<td>+1.0 +3.0 -5.0</td>
<td>5.0 µg</td>
<td>5 µl</td>
<td>1 µl/min</td>
<td>Saline</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>NAcc</td>
<td>+1.7 +1.1 -7.0</td>
<td>5.0 µg</td>
<td>5 µl</td>
<td>1 µl/min</td>
<td>Saline</td>
</tr>
</tbody>
</table>

A/P, anterior/posterior; 5,7-DHT, 5,7-dihydroxytryptamine; D/V, dorsal/ventral; 6-OHDA, 6-hydroxydopamine; 5-HT, 5-hydroxytryptamine; IBA, ibotenic acid; L/M, lateral/medial; MFB, medial forebrain bundle; NAcc, nucleus accumbens; NTS1, neurotensin receptor 1.

4.3.2 Intracerebral injections of NTS1 agonist and antagonist (III)

NTS1 agonist JMV-449 or antagonist SR142948 was injected i.c. either to the striatum or NAcc (Table 4-1). 1 h after the injection the rats were decapitated, the brains were removed and dissected on ice. Tissue samples were collected from the striatum, NAcc, SN and VTA, frozen on dry ice and stored -80 °C before the high-performance liquid chromatography (HPLC) analysis of dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA). A group of rats received PREP inhibitor 5 mg/kg KYP-2047 30 min before the i.c. injection.

4.4 Intraperitoneal injections of NTS1 agonist and antagonist (unpublished)

NTS1 agonist PD149163 and antagonist SR142948 were injected i.p. (1 mg/kg). Control rats received an injection of saline. 15, 30 or 60 min after the administration the rats were decapitated and the brains were removed and dissected on ice (n = 6 in each treatment group/time point). The dissected tissues were frozen on dry ice and stored -80 °C until the HPLC analysis of dopamine, DOPAC and HVA.
4.5 The ex vivo study with a single dose of thioridazine (I)

Saline, thioridazine (10 mg/kg; an antipsychotic compound that also inhibits PREP with its therapeutical plasma concentrations) or JTP-4819 (10 mg/kg) was injected i.p. to mice. 1 h after the injection, the mice were decapitated, their brains were removed and the cortex, the cerebellum and the prefrontal cortex (PFC) were dissected on ice and stored in -80°C until the measurement of PREP activity.

4.6 Behavioural testing methods (IV)

4.6.1 Eight-arm radial maze (IV)

The protocol for the radial-arm maze experiment is described in detail in IV. Briefly, the rats’ task was to find pieces of chocolate breakfast cereal (SunMuro, TopFood Oy, Finland) at the end of four randomly chosen arms. The testing was done in a dark room, and the rats had to navigate in the maze just by looking at the visual cues, such as the furniture. To keep the rats motivated in the task, they were kept on a restricted diet. The rats were first habituated (Fig. 4-1, days 1 and 2) to the maze, trained the task (Fig. 4-1, days 3-11) and the experiments were done on days 12-15. The experiment started when the rat was placed on the central platform and ended when all rewards were eaten or if 10 min had passed. The training and the experiments were conducted twice a day with a 1 h gap in between. The same four arms were always baited for the same rat.

![Figure 4-1. Experimental design for the eight-arm radial maze experiments (study IV).](image)
On the testing days, each rat was injected i.p. twice 30 min before the first trial of the day. The treatment groups were saline + Tween (SAL-TWE; young, n = 8; old, n = 12), saline + 5 mg/kg KYP-2047 (SAL-KYP; young, n = 8; old, n = 13), 0.4 mg/kg scopolamine + Tween (SCO-TWE; young, n = 8; old, n = 13) or 0.4 mg/kg scopolamine + 5 mg/kg KYP-2047 (SCO-KYP; young, n = 7; old, n = 13). The experimenter was blind for the treatments and kept track of the time the rats spent in the maze, their visits in each arm and the number of eaten rewards. On testing day 3 (day 14), rats did the test only once and the gap was prolonged to 24 h so that the second test was on testing day 4 (day 15) without drug injections.

4.6.2 Locomotor activity (IV)

The effect of 5% Tween 80 or KYP-2047 (3 or 5 mg/kg) on the locomotor activity of 3-month old rats was measured for 60 min in the MED Associates open field activity monitor (43.2 x 43.2 x 30.5 cm; six boxes; St. Albans, GA, USA) directly after the 30 min habituation period and the i.p. injections. The distance travelled and rearings were registered via infrared photo beam interruptions. The locomotor activity experiments were conducted as a cross-over study meaning that all treatments were given to all rats. The washout period was one week.

4.6.3 Rotational behaviour (unpublished)

The rotational behaviour was studied two weeks after the 6-OHDA lesion to the MFB with an apomorphine (0.1 mg/kg, s.c.) control rotation (n = 36; experimental design in Fig. 4-2.) in the rotometer bowls (MED Associates Inc., GA, USA). Thereafter, two priming rotations with LD (10 mg/kg, i.p.) and CD (30 mg/kg, i.p.) were done three and four weeks post-lesion to rats that had more than 30 contralateral rotations in the apomorphine control rotation. Priming was done to sensitize the rats to LD (Brotchie 2005). Eight rats that rotated the most were selected to the final experiments. LD/CD-induced turning behaviour was monitored weekly for 120 min. The rats received KYP-2047 (3, 10 or 30 mg/kg), vehicle (5% Tween 80) or 10mg/kg KYP-2047 without LD/CD in a cross-over manner 30 min prior to the experiment. After the rotation experiments, the success of the lesion was confirmed by analyzing the dopamine levels with HPLC in both striata.

![Figure 4-2. Experimental design for the rotational experiments. APO, apomorphine; CD, carbidopa; LD, L-dopa; MFB, medial forebrain bundle.](image-url)
4.7 Analytical procedures

4.7.1 Tissue preparation (I, II)

For the enzyme activity measurements in study I, procedure described in Venäläinen et al. 2002 was used. The animals were decapitated, the brains were removed and homogenized with assay buffer (0.1 M Na-K-phosphate buffer, pH 7.0) with a mechanical (rats; Kontes Microtube Pellet Pestle Rod with Motor, Vineland, NJ, USA) or ultrasound (mice; Rinco Ultrasonics, Arbon, Switzerland) homogenizer. The protocol described in Myöhänen et al. 2008c was used to prepare the samples for enzyme activity measurements in study II. Two weeks post lesion the rats were deeply anesthetized with pentobarbital and then perfused intracardially with phosphate-buffered saline. The brains were removed, dissected on ice and the samples were cooled on dry ice. The homogenization was done to the assay buffer with an ultrasound homogenizer (the striatum, hippocampus and PFC samples) or a mechanical homogenizer (the cortex and cerebellum). Thereafter the homogenate was centrifuged at 10 000 g, 4°C, for 20 min. The supernatants were collected, diluted to the final concentration with assay buffer if needed and stored at -80°C until the enzyme activity assay.

For tissue preparation in paper II and III, a similar protocol as in Myöhänen et al. 2008c was used. Two weeks post lesion, the rats were deeply anesthetized with pentobarbital and perfused first with phosphate-buffered saline and then with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Next, the rats were decapitated and the brains were removed, post-fixed with 4% paraformaldehyde and sucrose and stored at -80°C until sectioning into 40μm cryosections with a microtome (Leica SM2010, Leica Microsystems Inc., Bannockburn, IL, USA).

4.7.2 PREP activity assay (I, II)

The PREP activity assays in studies I and II were performed as described earlier in Venäläinen et al. 2002. In brief, sample was preincubated with 0.1 M Na-K-phosphate buffer (pH 7.0) for 30 min at 30°C. The reaction was initiated by adding substrate (4 mM Suc-Gly-Pro-AMC) and the plate was incubated for 60 min at 30 °C. The reaction was terminated with 1 M Na-acetate buffer (pH 4.2). The formation of fluorescent AMC was measured with a Wallac 1420 VICTOR² fluorescence plate reader (PerkinElmer, Waltham, MA, USA) with the excitation and emission wavelengths of 355 and 460 nm, respectively. The reaction velocities are given as nmol AMC/min/mg protein.

4.7.3 Validation of PREP activity measurements (unpublished)

Earlier, the effects of reaction conditions, such as the solvents, pH and temperature have been studied by Venäläinen et al. 2002. The method with rat brain homogenate was validated by studying the effects of pipetting order and keeping reagents on ice during the experiment. Also the intra-assay variation, day-to-day variation and variation in the standards were studied. Intra-assay and day-to-day variation were calculated with a high (control) and low (1000 µM VPA) PREP activity level. In the intra-assay test, every second
well on the plate was a control well and every second a 1000 µM VPA well. Day-to-day variation was calculated by adding the same high and low activity wells into other measurement’s plates on ten consecutive experiment days. Mean, standard deviation and coefficient of variation (CV) were calculated from both PREP activity levels. Variation in the standard wells was calculated by measuring the same well plate three times with the fluorescence plate reader, and from ten different well plates. Mean, standard deviation and CV were calculated from the fluorescence values.

4.7.4 Kinetic measurements (I)

The PREP inhibition type and kinetic parameters (IC$_{50}$ and K$_i$) were measured from ketanserin, thioridazine and VPA with the PREP activity assay described in 4.7.2 using different substrate concentrations (50, 100, 200, 300 and 400 µM). IC$_{50}$ values were calculated with the sigmoidal dose-response equation using GraphPad Prism version 5.02 (GraphPad Software, San Diego, CA, USA). The intercept of the Y-axis in the same equation gave the K$_i$ values. The reversibility of PREP inhibition was tested by first incubating ketanserin, VPA and thioridazine with the brain homogenate in room temperature for 2 hr in a Pierce Slide-A-Lyzer Dialysis Unit (3,500 MWCO, Rockford, IL, USA) floating in the incubation buffer and then measuring PREP activity as described in chapter 4.7.2.

4.7.5 HPLC analysis of dopamine, DOPAC and HVA in tissue samples (III, rotational behaviour)

HPLC electrochemical detection method was used in study III and after the rotational experiments for the analysis of the levels of dopamine, DOPAC and HVA. The method is described earlier by Airavaara et al. 2006 and just minor changes were made to the method. The column was heated to 40°C. The mobile phase consisted of 0.1 M NaH$_2$PO$_4$ buffer (pH = 3), 220 mg/l of octane sulfonic acid, MeOH 6%, and 0.2 mM EDTA.

4.7.6 Immunofluorescence (II, III)

Immunofluorescence of free-floating sections was used in studies II and III. The protocol is described earlier by Myöhänen et al. 2008a and Myöhänen et al. 2008b. Information of used primary antibodies is presented in Table 4-2. Briefly, sections were first incubated in 10% normal goat serum (Vector laboratories, Burlingame, CA, USA) in tris-buffered saline followed by an overnight incubation with respective antibodies at room temperature. After washes with tris-buffered saline, the sections were incubated with secondary antibody (for choline acetyltransferase, ChAT, 5-HT and dopamine-β-hydroxylase, DβH; dilution 1:500; FITC-conjugated goat anti-rabbit IgG, Pierce Biotechnology, Rockford, IL, USA; for neurotensin; dilution 1:500; Texas-red conjugated goat anti-rabbit IgG polyclonal antibody, Thermo Scientific, Rockford, IL, USA; for NTS1; dilution 1:500; Texas-red conjugated goat polyclonal secondary antibody to guinea pig IgG, Abcam, Cambridge, UK; for PREP; dilution 1:500, FITC conjugated rabbit anti-chicken IgY, Pierce Biotechnology) for 2 h. The sections were placed to objective glasses and a mounting medium (Vectashield with 4’,6-diamino-2-phenylindole, DAPI; Vector laboratories) was used to demonstrate the nuclei of the cells.
Colocalization in paper III was calculated by counting the percentage of all neurotensin or NTS1 cells containing colocalization with PREP. The colocalization was classified as low (0-19 % of all neurotensin- or NTS1-containing neurons), moderate (20-49 %) or high (50 % or more).

4.7.7 Laser scanning microscopy (II, III)

Immunofluorescence stained sections were analyzed and photographed using Leica TCS SP2 AOBS (Leica Microsystems Inc.) equipped with an argon-He/Ne laser mounted on an inverted Leica DM IRE2 microscope (Leica Microsystems Inc.). Only brightness and contrast were adjusted in the pictures with Adobe Photoshop CS2 software (version 9.0, Adobe Systems Incorporated, San Jose, CA, USA).

4.8 Statistical analysis

4.8.1 Radial-arm maze (IV)

Results from the radial-arm maze experiments were analyzed with GraphPad Prism using two-way analysis of variance (ANOVA) and Bonferroni multiple group comparison test as a post hoc test.

4.8.2 Locomotor activity (IV)

Predictive Analysis Software (PASW) Statistics version 18 (SPSS Inc., Chicago, IL, USA) was used to analyze the data from the locomotor activity measurements (II) with one-way ANOVA for repeated measures and Tukey’s test as a post hoc test. The data in Fig. 5-7 was analyzed with one-way ANOVA and Tukey’s test as a post hoc test with GraphPad Prism.

4.8.3 Enzyme activity assays (I)

The results from the ex vivo study with a single dose of thioridazine were analyzed with one-way ANOVA and Tukey’s test as a post hoc test using PASW Statistics version 18.

4.8.4 Intracerebral injections in neurotensin study (III)

Two-way ANOVA was used to analyze the overall effects of NTS1 agonist and KYP-2047 on the levels of dopamine and dopamine metabolites (combined DOPAC+HVA) with PASW Statistics version 18. The pairwise comparisons (saline vs. NTS1 agonist groups; saline vs. saline KYP-2047 groups) were calculated with Student’s unpaired t-test.
Table 4-2. List of primary antibodies used in studies II and III.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>PREP</th>
<th>ChAT</th>
<th>5-HT</th>
<th>DBH</th>
<th>Neurotensin</th>
<th>NTS1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marker for Species</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PREP protein</td>
<td>Cholinergic cells</td>
<td>5-HT cells</td>
<td>Noradrenergic cells</td>
<td>Neurotensin protein</td>
<td>NTS1</td>
</tr>
<tr>
<td></td>
<td>Chicken IgY</td>
<td>Rabbit IgG</td>
<td>Rabbit IgG</td>
<td>Rabbit IgG</td>
<td>Rabbit IgG</td>
<td>Guinea pig IgG</td>
</tr>
<tr>
<td>Immunogen</td>
<td>Purified pig PREP</td>
<td>Synthetic peptide from porcine ChAT.</td>
<td>Whole-length 5-HT</td>
<td>Cloned from human DBH, synthetic CPTSQGRSPAGTVVSI-amide</td>
<td>Neurotensin conjugated to BSA</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sequence: H-GLFSSYRPLPHT QDLTVAQKSS-NH₂</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Manufacturer</td>
<td>Venäläinen et al. 2006</td>
<td>Millipore/Chemicon, Temecula, CA, USA</td>
<td>Sigma</td>
<td>Millipore/Chemicon</td>
<td>Millipore/Chemicon</td>
<td>Abcam</td>
</tr>
<tr>
<td>Product #</td>
<td>-</td>
<td>AB5042</td>
<td>SS545</td>
<td>AB1536</td>
<td>AB5496</td>
<td>AB66216</td>
</tr>
<tr>
<td>Dilution used</td>
<td>1:500</td>
<td>1:2000</td>
<td>1:4000</td>
<td>1:250</td>
<td>1:500</td>
<td>1:500</td>
</tr>
<tr>
<td>Specificity and reference</td>
<td>Myöhänen et al. 2007</td>
<td>Djebaili et al. 2005</td>
<td>Pre-incubation control, manufacturer's datasheet</td>
<td>Western blot, manufacturer's datasheet</td>
<td>Pre-incubation control; Williams and Beitz 1989; Dufourny et al. 1999</td>
<td>Manufacturer's datasheet</td>
</tr>
</tbody>
</table>

ChAT, choline acetyl transferase; DBH, dopamine-β-hydroxylase; 5-HT, 5-hydroxytryptamine (serotonin); NTS1, neurotensin receptor 1; PREP, prolyl oligopeptidase
5. RESULTS

5.1 PREP activity measurements (I, II)

5.1.1 Validation of PREP activity measurements (unpublished)

The pipetting order in the PREP activity assay affects the results possibly due to the difference in the incubation times in the wells; it is simply impossible to pipette all the reagents manually to the reaction with an exact speed. This was taken into account by dividing the wells containing different concentrations of compounds evenly on the 48-well plate. It was also noticed in the validation experiments, that keeping the rat brain homogenate on ice when pipetting reduces the variation, but keeping the whole plate on ice does not affect the results. Overall, there was very little variation in the intra-assay and day-to-day variation tests. The CVs in the intra-assay variation experiment were 3.76 % for control wells and 3.99 % for 1000 µM VPA wells (Fig. 5-1A). In the day-to-day variation test, the CV for control wells was 6.69 % and for 1000 µM VPA wells 6.14 % (Fig. 5-1B).

![Figure 5-1. Intra-assay (A) and day-to-day (B) variation in the PREP activity measurements studied with high (control wells) and low (1000 µM valproate) PREP activity levels using rat brain homogenate. In the intra-assay test (A), every second well on the plate was a control well and every second a valproate well. In the test of day-to-day variation the control and valproate wells were added into other measurement’s plates on ten consecutive experiment days. In intra-assay test, the fluorescence values decrease slightly towards the last wells of the plate in the pipetting order of substrate and rat brain homogenate. The means of PREP activities in control and valproate wells are represented as black lines in both figures.](image)

The fluorescence values of the standards varied day-to-day, but also in the intra-assay study where the same plate was measured in the fluorescence plate reader three times in a row. The CVs were around 18-27 %. The problem was later found to be related to the varying temperature in the standard wells and it was fixed by making sure that the temperature of all added liquids was equal before the reading of the plate.
5.1.2 The effects of psychoactive compounds on the activity of PREP (I)

Because changes in serum PREP activity have been linked to several psychiatric disorders and the exact mechanisms of actions of the commonly used psychopharmaceuticals are not entirely known, we tested their effects on the activity of PREP. Altogether 38 compounds were tested. The results of the PREP activity measurements are listed comprehensively in Table 1 in paper I and summarized in Table 5-1. In total, 18 compounds inhibited PREP activity over 20% from the control activity at 10 µM concentration (Table 5-1). Thioridazine and VPA inhibited PREP at their therapeutic plasma concentrations (Fig. 2 in paper I).

Table 5-1. Psychoactive compounds that inhibited PREP activity over 20% of the control activity at 10 µM concentration, their therapeutic plasma concentrations (if available) and brain/plasma ratios as reported in the literature. Valproate inhibited PREP only 3% at 10 µM concentration, but it is listed here because it inhibits PREP with its therapeutic plasma concentration.

<table>
<thead>
<tr>
<th>Compound</th>
<th>PREP inhibition (%) at 10 µM</th>
<th>Therapeutic plasma concentration (µM)</th>
<th>Reported brain/plasma ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buspirone</td>
<td>25</td>
<td>0.0026-0.010</td>
<td></td>
</tr>
<tr>
<td>Chlorpromazine #</td>
<td>55</td>
<td>0.095-0.47</td>
<td>11.5</td>
</tr>
<tr>
<td>Citalopram</td>
<td>27</td>
<td>0.03-0.61</td>
<td>10</td>
</tr>
<tr>
<td>Clozapine #</td>
<td>40</td>
<td>0.3-0.9</td>
<td>24</td>
</tr>
<tr>
<td>Desipramine #</td>
<td>20</td>
<td>0.038-1.9</td>
<td>20</td>
</tr>
<tr>
<td>Duloxetine</td>
<td>21</td>
<td>0.016-0.46</td>
<td></td>
</tr>
<tr>
<td>Escitalopram</td>
<td>39</td>
<td>0.063-0.2</td>
<td></td>
</tr>
<tr>
<td>Flupentixol</td>
<td>56</td>
<td>0.0023-0.035</td>
<td></td>
</tr>
<tr>
<td>Imipramine #</td>
<td>29</td>
<td>0.18-0.54</td>
<td>3-4, 25</td>
</tr>
<tr>
<td>Ketanserin</td>
<td>49</td>
<td>0.13-1.3</td>
<td>1</td>
</tr>
<tr>
<td>Lamotrigine</td>
<td>22</td>
<td>12-60</td>
<td>1</td>
</tr>
<tr>
<td>Levomepromazine</td>
<td>32</td>
<td>0.015-0.04</td>
<td>10</td>
</tr>
<tr>
<td>Prazosin</td>
<td>91</td>
<td>0.0026-0.08</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Prochlorperazine #</td>
<td>77</td>
<td>0.02-0.09</td>
<td>420</td>
</tr>
<tr>
<td>Promazine #</td>
<td>64</td>
<td>0.035-0.18</td>
<td>62.5</td>
</tr>
<tr>
<td>Risperidone</td>
<td>56</td>
<td>0.0073-0.05</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Ritalserin</td>
<td>33</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thioridazine *#</td>
<td>91</td>
<td>0.27-5.4</td>
<td>2.2, 1.4</td>
</tr>
<tr>
<td>Valproate *#</td>
<td>3</td>
<td>280-700</td>
<td>15.1</td>
</tr>
</tbody>
</table>

*, inhibits PREP with therapeutic plasma concentration; #, inhibits PREP 30-50% at presumably achievable brain concentration

Due to the lipophilicity of many psychopharmacological compounds, their concentrations in the brain may be higher than in plasma. With literature-based possible brain concentrations, chlorpromazine, clozapine, desipramine, imipramine, prochlorperazine and promazine inhibited PREP 30-50% from the control activity. The inhibition curves and IC<sub>50</sub> values of six most potent PREP inhibitors are shown in Fig. 1 in paper I.
5.1.3 Kinetic analysis of ketanserin, thioridazine and VPA (I)

The inhibition kinetics of ketanserin, thioridazine and VPA, some of the most potent putative PREP inhibitors, were calculated to determine their PREP inhibition type. The non-linear IC$_{50}$ values of ketanserin are characteristic of mixed inhibition (Fig. 5-2). For thioridazine, the IC$_{50}$ values remained constant as a function of substrate concentration, which is typical for a non-competitive inhibitor (Fig. 5-2). VPA was a competitive inhibitor, since the IC$_{50}$ values increased linearly as a function of substrate concentration (Fig. 5-2). In the test for the reversibility of PREP inhibition, ketanserin, thioridazine and VPA turned out to be reversible inhibitors, since the PREP activity was restored back to control levels after the 2 h incubation (data not shown).

![Figure 5-2](image)

**Figure 5-2.** The IC$_{50}$ values of ketanserin, thioridazine and valproate plotted against the substrate concentrations. The substrate was Suc-Gly-Pro-AMC, temperature 30° C and pH 7.0. Valproate is a competitive inhibitor, since its IC$_{50}$ values increased linearly with the increasing substrate concentration, typically for competitive inhibition. The IC$_{50}$ values of thioridazine remained nearly constant, which is typical for non-competitive inhibition. Ketanserin is a mixed type inhibitor. IC$_{50}$ values were calculated by a sigmoidal dose-response equation with GraphPad Prism version 5.02. The values are represented as mean (± SEM) of 3–4 experiments.

5.1.4 PREP activity after a single dose of thioridazine in the mouse brain (I)

Since thioridazine inhibited PREP in vitro, we conducted an ex vivo study in mice to test whether it could be potent enough to inhibit PREP in vivo. The single i.p. injection of thioridazine (10 mg/kg) did not inhibit PREP in the cerebellum, cortex or PFC 1 h after the administration (Fig. 4 in paper I). However, JTP-4819 (10 mg/kg, i.p.) inhibited PREP statistically significantly in all brain areas (JTP-4819 versus saline; p < 0.0001 in the PFC and the cerebellum; p < 0.001 in cortex).

5.1.5 PREP activity in projection areas after the lesions of selected neurotransmitter nuclei (II)

The purpose of this study was to find out whether PREP would be present in long cholinergic, 5-HT, noradrenergic or dopaminergic projection neurons. Chemical lesions were made in the rat medial septum, Meynert nucleus, dorsal raphe and locus coeruleus or the MFB, the terminals which contain mRNA, protein or activity of PREP. The activity of PREP in the respective terminal projection areas was measured. The projection areas were identified using Paxinos 2004. Table 5-2. summarises the effect of the lesions of selected
Table 5-2. The activity of PREP in the respective projection areas two weeks after a lesion of the dorsal raphe, locus coeruleus, medial forebrain bundle, medial septum or Meynert nucleus of the rat brain.

<table>
<thead>
<tr>
<th>Lesion sites</th>
<th>Treatment</th>
<th>Projection areas</th>
<th>Cortex</th>
<th>Hippocampus</th>
<th>Striatum</th>
<th>PFC</th>
<th>Cerebellum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dorsal raphe (5-HT)</td>
<td>SAL</td>
<td></td>
<td>1.4 ± 0.2</td>
<td>0.8 ± 0.04</td>
<td>1.0 ± 0.09</td>
<td>0.8 ± 0.06</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>5,7-DHT</td>
<td></td>
<td>1.1 ± 0.2</td>
<td>0.8 ± 0.05</td>
<td>1.0 ± 0.05</td>
<td>0.9 ± 0.03</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>Locus coeruleus (noradrenergic)</td>
<td>SAL</td>
<td></td>
<td>1.5 ± 0.08</td>
<td>0.7 ± 0.02</td>
<td>0.8 ± 0.04</td>
<td>0.7 ± 0.04</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>DSP-4</td>
<td></td>
<td>1.5 ± 0.06</td>
<td>0.8 ± 0.06</td>
<td>0.8 ± 0.04</td>
<td>0.7 ± 0.03</td>
<td>1.7 ± 0.08</td>
</tr>
<tr>
<td>Medial forebrain bundle (dopaminergic)</td>
<td>Control</td>
<td></td>
<td>-</td>
<td>-</td>
<td>1.2 ± 0.06</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>6-OHDA</td>
<td></td>
<td>-</td>
<td>-</td>
<td>1.1 ± 0.08</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Medial septum (cholinergic)</td>
<td>SAL</td>
<td></td>
<td>0.8 ± 0.04</td>
<td>0.9 ± 0.05</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>IBA</td>
<td></td>
<td>0.8 ± 0.02</td>
<td>1.0 ± 0.06</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Meynert nucleus (cholinergic)</td>
<td>Control</td>
<td></td>
<td>1.0 ± 0.04</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>IBA</td>
<td></td>
<td>1.0 ± 0.06</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

PFC = prefrontal cortex, SAL = saline, 5,7-DHT = 5,7-dihydroxytryptamine, DSP-4 = a noradrenergic neurotoxin, Control = intact side used as a control, 6-OHDA = 6-hydroxydopamine, 5-HT, 5-hydroxytryptamine (serotonin); IBA = ibotenic acid. All values are represented as mean ± SEM of 3-5 values.
neurotransmitter nuclei on the activity of PREP. Only the PREP activity values of lesioned and control samples of the same lesion’s projection areas are comparable. No statistically significant changes in the activity of PREP were observed in the projection areas (the cortex and hippocampus) after the cholinergic lesion of the medial septum.

The cholinergic lesion of the Meynert nucleus did not cause any changes to the activity of PREP in its projection area (the cortex). Also when the dopaminergic innervation of the MFB was lesioned, the activity of PREP in the striatum did not change. The lesion of the 5-HT nucleus (the dorsal raphe) did not affect the activities of PREP in the respective projection areas (the cortex, hippocampus, striatum, cerebellum and PFC), either. Finally, no effect on the PREP activity was seen in any of the projection areas after the lesion of the noradrenergic nucleus (the locus coeruleus) by DSP-4. The effects of the lesions on immunoreactive PREP are described in 5.2.1.

5.2 The expression of immunoreactive PREP (II, III)

5.2.1 The expression of immunoreactive PREP after neurotransmitter lesions (II)

The expression level of immunoreactive PREP protein was determined from the corresponding projection areas of the lesioned nuclei using immunofluorescence (Paxinos 2004). PREP immunofluorescence was studied from the hippocampus and cortex; two memory-related areas rich in PREP (Myöhänen et al. 2008b). The hippocampus was stained from the medial septum, dorsal raphe and locus coeruleus -lesioned rats, and the primary somatosensory cortex was stained after the lesion of Meynert nucleus. The neurotransmitter lesions did not affect the expression level of immunoreactive PREP in the corresponding projecting areas of the lesioned nuclei (Fig. 2 in paper II). The expression of PREP in the striatum after the MFB lesion was not changed either (data not shown) as previously described (Myöhänen et al. 2008b).

5.2.2 Colocalization of PREP and neurotensin or NTS1 in the striatum, the NAcc, the SN and the VTA (III)

PREP is located in the same brain areas as neurotensin and NTS1 in the nigrostriatal and mesolimbic dopaminergic pathways. Therefore we tested if there is colocalization between them. The colocalization of PREP with neurotensin and NTS1 in the rat striatum, NAcc, substantia nigra (SN) and VTA was studied with immunofluorescence. Neurotensin immunofluorescence was found in vesicle-like structures in the striatum, SN, NAcc and VTA (Fig. 1 in paper III). PREP and neurotensin immunoreactivities were highly colocalized in the VTA (Fig. 1P in paper III), but the colocalization was moderate in the striatum and SN (Fig. 1D and H in paper III). The colocalization was low in the NAcc (Fig. 1L in paper III). NTS1 immunoreactivity was detected in all brain areas (the striatum, SN, NAcc and VTA; Fig. 2 in paper III) also in vesicle-like formations. Colocalization of PREP and NTS1 was high in the SN and VTA (Fig. 2H and P in paper III), but low in the striatum and NAcc (Fig. 2D and L in paper III).
5.3 Effects of PREP inhibitor, NTS1 agonist and antagonist on the levels of dopamine and its metabolites in the striatum and NAcc (III)

In this study, our aim was to measure if there is a functional interaction of PREP and NTS1 in the nigrostriatal and mesolimbic dopaminergic pathways. We injected NTS1 agonist (JMV-449; 5 µg) and antagonist (SR142948; 5 µg) to the striatum or NAcc and measured the levels of dopamine and its metabolites in the striatum and SN, or NAcc and VTA. A group of rats received PREP inhibitor KYP-2047 (5 mg/kg; i.p.) 30 min before the i.c. injections.

5.3.1 Injection of NTS1 agonist and antagonist to the striatum (III)

The striatal injection of NTS1 agonist JMV-449 (5 µg) tended to increase the levels of dopamine in the striatum (saline vs. NTS1 agonist, p = 0.052; Fig. 5-3A) and in the SN (saline vs. NTS1 agonist, p = 0.149; Fig. 5-3B). The levels of dopamine metabolites were also slightly increased by the NTS1 agonist in the striatum and SN (Fig. 5-3C and D). NTS1 antagonist (SR142948, 5 µg) had a significantly different effect on the dopamine and dopamine metabolite levels in the SN (Fig. 5-3B and D) compared to the NTS1 agonist (NTS1 agonist vs. antagonist, p < 0.05).

Figure 5-3. The effects of intrastriatally administered saline, neurotensin receptor 1 agonist (5 µg JMV-449; NT<sub>ago</sub>) and antagonist (5 µg SR142948; NT<sub>ant</sub>) on the levels of dopamine (DA) in the rat striatum (STR; A) and substantia nigra (SN; B) 1 h after their injection. The levels of dopamine metabolites (DOPAC and HVA) were analyzed from the STR (C) and SN (D). PREP inhibitor KYP-2047 (5 mg/kg) was given intraperitoneally 30 min before the intracerebral injections. Overall KYP-2047 or neurotensin receptor 1 (NTS1) ligand effects were calculated with two-way ANOVA. Values are represented as mean ± SEM, n = 4-6. * p < 0.05 NT<sub>ago</sub> vs NT<sub>ant</sub> groups.
PREP inhibitor KYP-2047 treatment (5 mg/kg, i.p.) decreased the dopamine levels in the striatum significantly (overall KYP-2047 effect in the two-way ANOVA, p < 0.001; Fig. 5-3A), but not in the SN (Fig. 5-3B). The effects of NST1 agonist or antagonist on dopamine or dopamine metabolite levels were not modified by KYP-2047 in the striatum and SN (Fig. 5-3).

5.3.2 Injection of NST1 agonist and antagonist to the nucleus accumbens (III)

When injected to the NAcc, NST1 agonist JMV-449 increased the levels of dopamine in the VTA (saline vs. NST1 agonist groups, p = 0.026; Fig. 5-4B) and dopamine metabolites in the NAcc (saline vs. NST1 agonist groups, p = 0.048; Fig. 5-4C) and VTA (saline vs. NST1 agonist groups, p = 0.003; Fig. 5-4D). NST1 antagonist (SR142948) injection in the NAcc had a significantly different effect on the dopamine (NST1 agonist vs. antagonist groups, p = 0.015; Fig. 5-4B) and metabolite levels (NST1 agonist vs. antagonist groups, p = 0.011; Fig. 5-4D) in the VTA compared to the NST1 agonist. KYP-2047 treatment had a significant increasing effect on dopamine metabolites in the VTA (overall KYP-2047 effect in two-way ANOVA, p = 0.001; Fig. 5-4D).

Figure 5-4. The effects of saline, neurotensin receptor 1 agonist (5 µg JMV-449; NT_{ago}) and antagonist (5 µg SR142948; NT_{ant}) on the levels of dopamine (DA) in the rat nucleus accumbens (NAcc; A) and ventral tegmental area (VTA; B) 1 h after their injection to the NAcc. The levels of dopamine metabolites (DOPAC and HVA) were analyzed from the NAcc (C) and VTA (D). PREP inhibitor KYP-2047 (5 mg/kg) was given intraperitoneally 30 min before the intracerebral injections. Overall KYP-2047 or neurotensin receptor 1 (NST1) ligand effects were calculated with two-way ANOVA. Values are represented as mean ± SEM, n = 4-6, except in NAcc injection saline and NT_{ago} control groups n = 8-10. * p < 0.05, ** p < 0.01 Saline vs. NT_{ago} groups. # p < 0.05, ## p < 0.01 NT_{ago} vs. NT_{ant} groups.
5.3.3 Intraperitoneal injections of NTS1 agonist and antagonist (unpublished)

To avoid the stereotaxic operation, we tested if we can see changes in dopamine and dopamine metabolite levels in the striatum, SN, NAcc and VTA after the i.p. injections of saline and blood-brain barrier crossing NTS1 ligands (agonist PD149163 and antagonist SR142948; 1 mg/kg). The levels of dopamine or dopamine metabolites DOPAC and HVA did not change significantly in the striatum, NAcc, SN and VTA 60 min after the i.p. injections of either saline, NTS1 agonist (1 mg/kg PD149163) or antagonist (1 mg/kg SR142948) (Fig. 5-5A-C). We also studied the levels of dopamine, DOPAC and HVA 15 and 30 min after the injections, but found no changes (data not shown). Since no differences were observed, the impact of PREP inhibitor KYP-2047 on the NTS1 ligands’ effects was not tested.

![Figure 5-5. The effects of NTS1 agonist (PD149163 NT<sub>ago</sub>) and antagonist (SR142948, NT<sub>ant</sub>) 1 mg/kg, i.p. on the levels of dopamine (A), DOPAC (B) and HVA (C) in the rat striatum (STR), nucleus accumbens (NAcc), substantia nigra (SN) and ventral tegmental area (VTA) 60 min after the injection. Mean ± SEM, n = 6/treatment.](image-url)
5.4 The effects of PREP inhibition in behavioural models of CNS diseases (IV, unpublished)

The behavioural effects of PREP inhibitor KYP-2047 were tested in three animal models of CNS disorders; the eight-arm radial maze (study IV), the locomotor activity test (study IV) and in Ungerstedt’s turning model, which is a model for Parkinson’s disease (unpublished). Radial-arm maze study was performed to clarify the controversial role of PREP inhibitors in memory and learning. Locomotor activity was tested after the radial-arm maze experiments showed that PREP inhibition may increase the locomotor activity. Finally, the experiments with the turning model were performed to see the effects of PREP inhibition in dopaminergic signalling after the 6-OHDA lesion of the MFB.

5.4.1 The effect of PREP inhibitor KYP-2047 on the memory and learning of young and old rats in eight-arm radial maze (IV)

The purpose of this study was to measure the effects of PREP inhibitor KYP-2047 on the scopolamine-impaired memory in young and old rats in the eight-arm radial maze. All results of the experiment are presented in the Fig. 1, 2 and 3 in paper IV. PREP inhibitor KYP-2047 (5 mg/kg) did not have an effect on the memory and learning of scopolamine-treated young and old rats in the radial arm maze. KYP-2047 treatment did not significantly improve the error rates (Fig. 1 in paper IV) or the time spent in the maze (Fig. 2 in paper IV) in young and old rats. However, the young and old rats responded differently to scopolamine treatment and to the task in the maze.

The young rats were faster (Fig. 2 in paper IV) and they conducted statistically significantly (SAL-TWE groups young versus old; p < 0.05 on days 2-4) fewer errors in the maze than the old rats (Fig. 1 in paper IV). Scopolamine treatment increased the time spent in the maze (Fig. 2 paper IV) significantly in both young (SAL-TWE versus SCO-TWE, p<0.05 on day 1 trial 1 and day 3 trial 1) and old rats (SAL-TWE versus SCO-TWE, in all trials: p<0.0001) and the error rates of old rats (Fig. 1 in paper IV; SAL-TWE versus SCO-TWE; p < 0.05 on day 1 trials 1 and 2, and on the first trials of days 2 and 3). KYP-2047 increased the frequencies (arms visited/minute) of young rats statistically significantly (Fig. 5-6; SAL-TWE versus SAL-KYP, p < 0.05 on day 2 trials 1-2 and on day 3 trial 1).

5.4.2 Locomotor activity after a single administration of PREP inhibitor KYP-2047 (IV)

To test whether PREP inhibition affects the locomotor activity, vehicle or KYP-2047 (3 or 5 mg/kg) was given to young rats and the locomotor activity was measured for 60 min. KYP-2047 (3 mg/kg) increased the locomotor activity of young rats in 0-50 min significantly (p < 0.05) as seen in the activity curve of Fig. 4 in paper IV. The higher dose (5 mg/kg) did not quite cause a significant increase (p = 0.14) in the locomotor activity. The same data is presented in a cumulative form in Fig. 5-7.
**Figure 5-6.** Effects of PREP inhibitor KYP-2047 on frequencies (arms visited/minute) of young rats in the radial-arm maze. During testing days 1 and 2, the first trial was done 30 min after the i.p. injection of 5 mg/kg KYP-2047 (KYP), 0.4 mg/kg scopolamine (SCO), 5 % Tween 80 (TWE) or saline (SAL) combinations (SAL-TWE, SAL-KYP, SCO-TWE or SCO-KYP). The second trial was performed 1 h after the first trial. On testing day 3, the gap of trials 1 and 2 was prolonged to 24 h so that trial 2 was on the testing day 4. Values are represented as mean (± SEM). *p<0.05, **p<0.01 SAL-KYP group versus SAL-TWE group (Bonferroni multiple group comparison test). n = 8 in all groups except in SCO-KYP group n = 7.

**Figure 5-7.** The locomotor activity of young rats in 60 min measured immediately after the i.p. injection of vehicle (VEH; 5 % Tween 80) or PREP inhibitor KYP-2047 (3 or 5 mg/kg, i.p.). The study was done in a cross-over manner with one week washout period. In each group, n = 11, *p<0.05 as analyzed using one-way ANOVA and Tukey’s test as a post hoc test.
5.4.3 The effect of PREP inhibitor KYP-2047 on the rotational behaviour after an unilateral 6-OHDA-lesion of MFB (unpublished)

Since alterations in plasma PREP activity have been connected to Parkinson’s disease (Mantle et al. 1996), the effects of PREP inhibition on the LD/CD-induced rotational behaviour was measured once a week starting 5 weeks after the unilateral 6-OHDA lesion (10 µg) of the MFB. The LD/CD-induced turning behaviour rats was not affected by PREP inhibitor KYP-2047 treatment (3, 10 or 30 mg/kg; i.p. 30 min prior to the experiment). 3 mg/kg KYP-2047 had a tendency to decrease the contralateral rotations, but not significantly (Fig. 5-8A). We also tested whether KYP-2047 (10 mg/kg) alone would have an effect on the rotational behaviour. However, when given KYP-2047 without LD/CD, the rats did not practically rotate at all (data not shown). The HPLC analysis of striatal dopamine levels showed that the MFB lesions were successful since there was only approximately 3 % dopamine left in the lesion side striata compared to the striata on the intact side (Fig. 5-8B.). We also wanted to test whether PREP activity in the striatum is changed after the lesion, but there was no change (Fig. 5-8C).

![Figure 5-8](image_url)

**Figure 5-8.** Rotational experiments with KYP-2047 on unilaterally 6-OHDA A) L-dopa/carbidopa (10/30 mg/kg) –induced contralateral rotations in 120 min. Vehicle (5 % Tween 80; VEH) or KYP-2047 (3, 10 or 30 mg/kg) were injected to MFB-lesioned rats i.p. 30 min prior to the experiment. Rotational behavior was measured once a week in a cross-over manner. B) Dopamine levels in intact and lesioned (6-OHDA) striatum. Only approximately 3 % of dopamine was left in the lesioned striata confirming the success of the lesions. C) PREP activity in intact side and lesioned (10 µg 6-OHDA) striatum. 6-OHDA-lesion did not affect the activity of PREP in striatum. Results are represented as mean ± SEM, n = 8.
6. DISCUSSION

6.1 PREP as a potential physiological target of psychopharmacological compounds

Changes in plasma or serum PREP-like activity have been linked to several psychiatric diseases, such as depression, mania and schizophrenia (Brandt et al. 2007). PREP activity has been significantly lower in patients with major depression than in healthy volunteers (Maes et al. 1995). On the other hand, plasma PREP activity has been significantly higher in patients with mania and schizophrenia compared to healthy control subjects (Maes et al. 1995). Interestingly, the altered plasma PREP activity levels are restored to normal levels with the antidepressant fluoxetine and the mood stabilizer VPA. Even though the relevance of the plasma PREP measurements has been questioned, PREP has also been connected to mood disorders via other mechanisms. Interestingly, one possibility for PREP to affect mood is the interference of the phosphoinositol signalling cascade (Williams et al. 1999). Furthermore, the mood stabilizer VPA has been shown to inhibit PREP (Cheng et al. 2005). These above mentioned findings proposed that the connection of commonly used psychopharmacocigal substances and PREP activity should be more closely studied. Therefore, in study I we were interested to see whether PREP activity is inhibited by psychopharmaceuticals, such as the antipsychotics, anxiolytics, antidepressants, anticonvulsants or mood stabilizing drugs.

We studied extensively the effects of various psychopharmaceuticals on PREP activity and found several weak inhibitors, some of which could inhibit PREP also at their achievable brain concentrations. The most inhibitors were found in the group of antipsychotics, since all antipsychotics that we tested, except haloperidol and sulpiride, inhibited PREP. As a group, antipsychotics are often considered to be very ‘dirty’ drugs because of their actions on various neurotransmitter receptors, such as dopaminergic, α-adrenergic, muscarinic, 5-HT and histaminergic receptors (Table 1 in paper I). It seems that there is something in the chemical structure of antipsychotics that will also affect PREP activity. The antipsychotic thioridazine and anticonvulsant/mood stabilizer VPA were the only compounds that inhibited PREP with their therapeutic plasma concentrations. Interestingly, a common mechanism of action to all compounds that inhibited PREP in our experiments is the antagonism of adrenergic α-receptors, as listed in Table 1 in paper I.

Despite these in vitro results, it seems unlikely that CNS drugs would inhibit PREP in vivo, since their potency to inhibit PREP is considerably low (Ki values in µ-molar range) in relation to the therapeutic levels. Second, the CNS drugs work mainly on receptors or other target proteins on the membrane of the neurons. Although PREP activity has also been measured from body fluids such as plasma and cerebrospinal fluid, PREP is currently believed to be mostly intracellularly located. Therefore the compounds need to penetrate cell membrane before they are able to encounter PREP. In our studies, we used brain homogenate, where the cell membranes have been mechanically broken. Very little is known about the intracellular concentrations and effects of the CNS drugs even though they...
are known to be very lipophilic and penetrate membranes, such as the blood-brain barrier. Lastly, even the most potent PREP inhibitor in our studies, thioridazine, did not inhibit PREP in the ex vivo study with mice. However, the brain homogenate was diluted before measuring PREP activity and that can result in a false negative outcome. Still, our studies showed that these compounds may not be potent enough to inhibit PREP at their therapeutical concentrations.

6.2 The effects of selective neurotransmitter lesions on the activity and expression of immunoreactive PREP protein

In study II, we lesioned the major nuclei of the nerve tracts of common small-molecule neurotransmitters, and studied their effects on PREP activity and immunofluorescence expression in the respective projection areas. The purpose of this study was to investigate whether PREP is located in the terminal areas of ACh, 5-HT, noradrenaline and dopamine projection neurons. The lesioned nuclei were chosen based on their projections to areas known to contain PREP protein, PREP mRNA or high PREP activity, e.g. the cortex, striatum, cerebellum, hippocampus and hypothalamus (Fig. 1B-C in paper II) (Bellemere et al. 2004; Myöhänen et al. 2007; Myöhänen et al. 2008b). Our results showed that PREP activity and immunoreactivity did not change in the terminal areas after the lesions, suggesting that there is no PREP, or no direct connection between the ACh, 5-HT, noradrenaline and dopamine systems and PREP, at least in the long projection neurons. It has to be pointed out though that we did not study the immunoreactivity of PREP from all of the projection areas of the lesioned nuclei.

PREP has been linked to number CNS disorders, such as the cognitive, neurodegenerative and psychiatric conditions (Maes et al. 1995; Maes et al. 1996; Toide et al. 1998; Myöhänen et al. 2012), which are in turn often connected to abnormalities in the functions of conventional small-molecule neurotransmitters. For quite some time, PREP has been thought to affect the functions of neurotransmitters by cleaving the neuropeptides (Männistö et al. 2007), which usually coexist in neurons with one or more classic neurotransmitters as co-transmitters or modulators (Hökfelt et al. 2003). For instance, GABAergic neurons can use SP as a neurotransmitter (McGinty 2007). Therefore, by cleaving neuropeptides, PREP could change the functions of neurotransmitters. However, it has been shown that for example PREP and SP are only poorly colocalized in the brain (Myöhänen et al. 2008a). Also, as the role of PREP as neuropeptide-cleaving enzyme is questionable, PREP may not be connected to neurotransmission via neuropeptide hydrolysis (Männistö et al. 2007; Jalkanen et al. 2011).

Despite the intensive research in the animal models of cognition, there is a lack of studies about the direct effects of PREP on neurotransmitter functions, and the most data comes from the immunohistochemical studies. Myöhänen et al. 2007 have studied the localization of PREP in the rat and human brain and also its spatial association with neurotransmitters and neuropeptides (Myöhänen et al. 2008b). They found PREP in GABAergic, cholinergic and glutamatergic neurons in the brain, pointing to the fact that
PREP may be involved in excitatory and inhibitory neurotransmission (Myöhänen et al. 2008b). Also, a few studies have reported functional associations of PREP with neurotransmitters. For example, PREP inhibitor JTP-4819 has elevated ACh activity in the cortex of aged rats, suggesting that PREP is connected to cholinergic neurotransmission (Toide et al. 1995a). In another study, an antagonist of the glutamatergic NMDA receptor has increased PREP activity in the posterior cingulate/retrospenial cortices (Arif et al. 2007). Quite recently Jalkanen et al. 2012 studied the effects of two PREP inhibitors KYP-2047 and JTP-4819 with microdialysis and found that, if anything, PREP inhibitors decrease the levels of ACh and dopamine in the rat striatum.

Our results show that PREP is either not present in these long projection neurons or that there is no such signal coming from the presynaptic neuron that could affect PREP activity or expression in the postsynaptic neuron. Earlier immunohistochemical studies support the view that PREP is absent from these projection neurons of the ACh, 5-HT, noradrenaline and dopamine systems (Myöhänen et al. 2008b). It seems more probable that PREP is located in the cholinergic interneurons and in glutamatergic and GABAergic neurons, especially in the cortex, thalamus and nigrostriatal pathway. The other possibility is that PREP activity and expression are not dependent on the signals coming from the soma to the terminal area.

One explanation is that the activation of microglia in the lesioned areas is masking the changes in the PREP activity or expression in the terminal areas caused by the lesions. It has been shown that PREP is significantly upregulated upon microglial activation (Klegeris et al. 2008). After the 6-OHDA lesion of the MFB such activation lasts at least 15 days or even longer (Marinova-Mutafchieva et al. 2009). In our study, the activity and expression of PREP was studied two weeks post-lesion. Therefore it is possible that the upregulation of PREP due to microglial activation affects our results.

As a conclusion, in study II we showed that there was no change in the PREP activity or protein expression after devastating the long projection neurons that use ACh, 5-HT, noradrenaline or dopamine as transmitters. The results support the earlier findings showing that PREP is not present in the projection areas of major long aminergic or cholinergic projection neurons but rather in short GABAergic, glutamatergic and cholinergic interneurons.

6.3 The colocalization and functional interaction of PREP with neuropeptides and its receptor in the nigrostriatal and mesolimbic dopaminergic pathways

In study III, we were interested in the possible colocalization and interaction of PREP with neurotensin and NTS1 in the nigrostriatal and mesolimbic dopaminergic pathways. As previously mentioned in chapter 2.5, PREP has been thought to participate physiological processes through the cleavage of neuropeptides but not without criticism mostly due to the anatomical dissociation of intracellular PREP and neuropeptides, which are secreted to the extracellular space. Therefore PREP may not be able to cleave the neuropeptides in vivo if not secreted, a situation that has not been demonstrated. However, the location of PREP
in the perinuclear space, and the changes in secretion at different PREP levels, have suggested the role of intracellular PREP in protein maturation and secretion (Schulz et al. 2005). Indeed, PREP has been shown to be associated with intracellular membranes in neurons, such as the RER and the Golgi apparatus (Myöhänен et al. 2008b; Tenorio-Laranga et al. 2008). Previous studies have shown that PREP, neurotensin and NTS1 should be located in the same neurons in the nigrostriatal and mesolimbic dopaminergic pathways, where neurotensin is known to regulate dopaminergic signalling (Binder et al. 2001). The anatomical location and known and proposed functional relationships of PREP, NTS1 and D2 in nigrostriatal and mesolimbic pathways are summarized in Figure 6-1.

We found that PREP was highly colocalized with neurotensin and NTS1 in the VTA and moderately in the striatum and SN. However, in the NAcc, the colocalization was low. Therefore, at least in the VTA there is a possibility for PREP, neurotensin and NTS1 to interact intracellularly. Interestingly, the colocalization data was supported by the results of the i.c. injections of NTS1 agonist (JMV-449). Striataly injected JMV-449 tended to increase the levels of dopamine and its metabolites in the striatum and SN. The injection of JMV-449 to NAcc increased dopamine levels in the VTA and dopamine metabolite levels in the NAcc and VTA. NTS1 antagonist (SR142948) reversed the dopamine- and metabolite-increasing effects of JMV-449 in the SN and VTA.

Interestingly, KYP-2047 treatment 30 min before the i.c. injections decreased the dopamine levels in the striatum. In the mesolimbic pathway, KYP-2047 increased the levels of dopamine metabolites in the VTA. In the SN and VTA, NTS1 antagonist tended to reverse the mild increasing effects of PREP inhibitor (KYP-2047) on the dopamine and metabolite levels. These findings support the view about the putative role of PREP in intracellular signalling, e.g. in the vesicle transport. Since PREP has been earlier shown to be located close to microtubules, RER and the Golgi network, it seems possible that PREP may control the transportation of dopamine or neurotensin vesicles or the vesicles formed after the internalization of NTS1. The decrease of the dopamine levels in the terminal areas, like the striatum, could be caused by a malfunction in the vesicle transportation. A similar decrease of striatal dopamine levels was also found in the microdialysis study of Jalkanen et al. 2012 with two PREP inhibitors KYP-2047 and JTP-4819. Furthermore, the effects of KYP-2047 on dopamine and its metabolite levels in the SN and VTA may be mediated via NTS1.

These results indicate that there is an interaction between PREP and neurotensin system in the nigrostriatal and mesolimbic dopaminergic pathways resulting in changes in the dopaminergic signalling. Furthermore, the interaction seems to be different in these two dopaminergic pathways.
**Figure 6-1.** The location, known and proposed interactions of dopaminergic $D_2$ receptor ($D_2$), neurotensin (NT), neurotensin receptor 1 (N1; in the text NT$S_1$) and prolyl oligopeptidase (PREP) in nigrostriatal and mesolimbic dopaminergic pathways. The contents of the bordered and numbered grey boxes are represented in detail in the grey coloured boxes on the right. Summarized from Binder et al. 2001 and Myöhänen et al. 2009.

**Known interactions:**
1. In the nigrostriatal and mesolimbic dopaminergic pathway, NT$S_1$ and $D_2$ receptors are located presynaptically. NT$S_1$ inhibits the $D_2$ autoreceptor leading to an increase in the release of presynaptic dopamine.
2. In the mesolimbic dopaminergic pathway, NT$S_1$ and $D_2$ receptors are located also postsynaptically. Postsynaptic NT$S_1$ inhibits postsynaptic $D_2$ receptor and the net effect is decrease in the dopaminergic signalling.

**Proposed interactions:**
3. PREP is located in the same neurons with neurotensin and/or NT$S_1$. PREP might regulate dopaminergic neurotransmission via two pathways: 
   a) PREP could have an effect on NT$S_1$ after its internalization for example through an interaction with the vesicles. Interaction with NT$S_1$ could lead to a change in dopaminergic signalling.
   b) PREP could influence the processing of neurotensin or its precursor peptide or their vesicle trafficking.

DA, dopamine; GABA, gamma-aminobutyric acid; VTA, ventral tegmental area.
6.4 The effect of PREP inhibition on motor functions, memory and learning, and rotational behaviour in rats

The data about the role of PREP and PREP inhibitors in behaviour is not conclusive. In fact, the only kind of behavioural effects that have been extensively studied are the effects of PREP inhibitors on cognitive processes (reviewed in Männistö et al. 2007), and even those results are far from consistent. We studied the effects of a PREP inhibitor KYP-2047 on behaviour in three animal models: locomotor activity (IV), eight-arm radial maze (IV) and rotational behaviour (unpublished). The data about the behavioural effects (except the data from cognitive studies, which has been reviewed by Männistö et al. 2007 of PREP inhibitors from previous and our studies is listed in Table 6-1.

In the radial-arm maze study, KYP-2047 (5 mg/kg) did not improve the memory and learning of young or old rats in the radial-arm maze. There were clear differences in the performances of the young and old rats. Generally, old rats needed more time to complete the task than young rats, and they made more errors. Also the responses to scopolamine and KYP-2047 treatments were different in young and old rats. Old rats were more sensitive to the impairing effects of scopolamine than the young rats. Although KYP-2047 did not improve rats’ memory in the radial-arm maze, it increased the motility of young rats in the experiments by increasing the frequencies (arms visited per minute). Therefore, we tested the effects of KYP-2047 (3 and 5 mg/kg) on locomotor activity in a separate test and saw an increase in the locomotor activity. In the rotation experiments (unpublished), 3 mg/kg KYP-2047 had only a tendency to decrease the LD/CD-induced contralateral rotations in 120 min.

The results of the radial-arm maze study highlight the fact that the memory-improving effects of PREP inhibitors have not been attested. Although there are several studies supporting the role of PREP in improvement of memory and learning, the data is inconsistent and the underlying mechanisms have not been detected (Männistö et al. 2007). One of the best studied PREP inhibitor in the animal models of cognitive functions is JTP-4819 (Männistö et al. 2007). It has been shown to improve memory in many animal models, such as the Morris water maze (Shinoda et al. 1996; Toide et al. 1997), radial-arm maze (Miyazaki et al. 1998) and passive avoidance test (Toide et al. 1995a; Shinoda et al. 1996), but the effects have rarely been robust or dose-dependent. Another intensively studied PREP inhibitor S-17092 has even been in clinical studies (Morain et al. 2002). But obviously even S-17092 has not been promising enough, since the latest reports from the human studies date back to year 2007 (Morain et al. 2007).

A common theory to explain the role of PREP in memory and learning has been the increase in the levels of cognition-enhancing neuropeptides, such as SP, TRH and AVP, by PREP inhibition. However, the theory has been more like an assumption than a proven fact. The effects of PREP inhibitors on neuropeptide levels have been tested, but the results have varied with the PREP inhibitor used, the length of the treatment and the brain area studied (Männistö et al. 2007). Jalkanen et al. 2007 studied the levels of neurotensin and SP after acute and 10 days JTP-4819 and KYP-2047 treatment, and found no changes in the SP levels in the cortex, hippocampus and hypothalamus and in neurotensin levels in the hypothalamus. They concluded that there are many other enzymes participating in the
neuropeptide metabolism and blocking one enzyme may not be sufficient enough to increase the levels of neuropeptides. Recently, the same group used microdialysis, and saw that PREP inhibitors JTP-4819 and KYP-2047 do not increase the levels of neurotensin and SP in the rat striatum (Jalkanen et al. 2011). Therefore, it seems unlikely that PREP would participate in cognitive or other behavioural processes through neuropeptide metabolism.

All of our behavioural experiments were related to motor functions. We saw an increase in the frequencies in the radial-arm maze and in the locomotor activity in young rats. Interestingly, PREP is known to be present in the brain areas participating in motor functions, such as the nigrostriatal system, primary motor cortex and cerebellum (Myöhänen et al. 2007). However, there is no evidence about the functions of PREP in these brain areas. One suggestion is that PREP may regulate the activity of striatal GABAergic neurons, although that has never been measured. According to the results obtained in study III, the inhibition of PREP does not affect robustly the dopaminergic neurotransmission in the nigrostriatal system, at least not in a way that would change behaviour. Also further studies about the motility-increasing effects of PREP inhibition are still needed. The test of locomotor activity is quite rough, and does not reveal any mechanisms behind the increase in the motility. Also, small changes in the dopaminergic signalling are difficult to see with the rotational experiments.

In addition to our findings, also the other studies listed in Table 6-1 show that the effects of PREP inhibitors on behaviour are not clear. PREP inhibitor Z-321 did not have effects on locomotor activity, when tested with three different doses p.o. (Oosuka et al. 2000). In the same study, also the effect of Z-321 on sexual receptivity was tested, but it was only impaired with the highest dose (300 mg/kg). A couple of PREP inhibitors have been tested in the animal models of depression (Khlebnikova et al. 2009a; Khlebnikova et al. 2009b; Khlebnikova et al. 2012). In these tests, PREP inhibitors reversed some of the depressive symptoms caused by the dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or dipeptidyl peptidase IV inhibitor. However, even though the rats were characterized as depressed after the 14 days MPTP treatment, MPTP has also other kind of behavioural effects, such as the impairment of motor functions. Therefore it is difficult to separate the effects of PREP inhibitors on depressive symptoms and on MPTP-treated rats in general and whether that is valid in the human depression. However, these findings point to the fact that PREP inhibitors may exerts some kind of effects on neurotransmission via classical or other neurotransmitter molecules that in turn cause mild effects on behaviour.

The effects of PREP on behaviour have not been studied thoroughly enough to make any conclusions. In future, PREP knockout mice will provide more data about the role of PREP in behaviour. PREP knockout (Di Daniel et al. 2009) and knockdown mice (D’Agostino et al. 2012) have already been created but their behavioural phenotype has not been completely reported. They are more aggressive and hyperactive (Di Daniel et al. 2009) supporting our behavioural data. The behavioural changes caused by PREP inhibitors may be modest, or they are undetectable in the animal models previously used. They may also be varying with age, sex and species, which should be also taken into account when planning future studies.
**Table 6-1.** The behavioural effects of PREP inhibitors in animal models other than cognition.

<table>
<thead>
<tr>
<th>PREP INHIBITOR</th>
<th>TREATMENT</th>
<th>ANIMAL</th>
<th>BEHAVIOURAL TEST</th>
<th>FURTHER DETAILS</th>
<th>RESULT</th>
<th>REFERENCE</th>
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<tbody>
<tr>
<td><strong>ANIMAL MODELS OF PARKINSON’S DISEASE</strong></td>
<td></td>
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<tr>
<td>KYP-2047</td>
<td>3, 10, 30 mg/kg i.p. single dose</td>
<td>Male rat</td>
<td>LD/CD-induced rotational behaviour</td>
<td>Unilateral 6-OHDA lesion (10 µg) of the MFB</td>
<td>↔</td>
<td>Peltonen et al. unpublished</td>
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<tr>
<td><strong>ANIMAL MODELS OF MOTOR FUNCTIONS</strong></td>
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<tr>
<td>KYP-2047</td>
<td>3, 5 mg/kg i.p. single dose</td>
<td>Male rat</td>
<td>Locomotor activity 60 min</td>
<td>↑ 3 mg/kg ↔ 5 mg/kg</td>
<td>Study IV</td>
<td></td>
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<tr>
<td>Z-321</td>
<td>100, 200 or 300 mg/kg p.o. single dose</td>
<td>Female rats</td>
<td>Locomotor activity 180 min</td>
<td>↔ Estrogen- and progesterone-treated ovariectomized</td>
<td>Oosuka et al. 2000</td>
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<tr>
<td><strong>ANIMAL MODELS OF DEPRESSION</strong></td>
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<tr>
<td>Z-Ala-Pro-OH</td>
<td>3 mg/kg i.p. 7 days</td>
<td>Male rat</td>
<td>Several different tests for depressive symptoms</td>
<td>MPTP 20 mg/kg i.p. 14 days</td>
<td>↓ Depressive symptoms</td>
<td>Khlebnikova et al. 2009b</td>
</tr>
<tr>
<td>Benzylxycarbonyl-methionyl-2(S)-cyanopyrrolidine</td>
<td>1 mg/kg i.p. 14 days</td>
<td>Male rat</td>
<td>Several different tests for depressive symptoms</td>
<td>MPTP 20 mg/kg i.p. 14 days</td>
<td>↓ Behavioural despair, swimming behaviour ↔ Other depressive symptoms</td>
<td>Khlebnikova et al. 2009a</td>
</tr>
<tr>
<td>Benzylxycarbonyl-methionyl-2(S)-cyanopyrrolidine</td>
<td>2 mg/kg i.p. 10 days</td>
<td>Male rat</td>
<td>Forced swimming test</td>
<td>DPPIV-inhibitor during postnatal period (days 5-18)</td>
<td>↓ Depression-like behaviour ↑ Normalizing effect on PREP activity in the brain</td>
<td>Khlebnikova et al. 2012</td>
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<tr>
<td><strong>OTHER ANIMAL MODELS</strong></td>
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<tr>
<td>Z-321</td>
<td>100, 200 or 300 mg/kg p.o. single dose</td>
<td>Female rats</td>
<td>Sexual receptivity</td>
<td>Estrogen and progesterone-treated ovariectomized</td>
<td>↔ 100 and 200 mg/kg ↓ 300 mg/kg</td>
<td>Oosuka et al. 2000</td>
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</tbody>
</table>

CD, carbidopa; DPPIV, dipeptidyl peptidase IV; LD, L-dopa; MFB, medial forebrain bundle; MPTP, 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine; 6-OHDA, 6-hydroxydopamine

6.5 General discussion

The physiological role of PREP has been studied for several decades with the main focus on the cleavage of neuropeptides and the cognitive effects related to the putative physiological substrates. Several PREP inhibitors have been developed, but they have not provided enough evidence about the functions of PREP. Although these inhibitors have been extensively studied as memory-modulating molecules, only two have progressed into the clinical trials. There is still an obvious lack of studies concerning the overall effects of PREP.
inhibitors on *in vitro* as well as *in vivo* functions. For example, there is not much data about the pharmacodynamic, pharmacokinetic and toxicity profiles of the PREP inhibitors. In addition, PREP has been mostly studied in animal models related to cognitive functions, but the data about other behavioural studies is scarce. Therefore, the potential of PREP as a therapeutic target for the treatment of CNS diseases has remained undefined. Also, the newly discovered effects of PREP alternative to its hydrolytic activity, such as the protein-protein interactions (Di Daniel et al. 2009; Myöhänen et al. 2012), may offer new insights in studying the enzyme.

Our studies affirmed the view that PREP may not be as important in the hydrolysis of neuropeptides or in the regulation of memory and learning as it has been previously thought. We conducted several experiments related to PREP and neurotransmission, such as the lesions of the neurotransmitter nuclei in paper II and study about the colocalization and interaction of PREP and neurotensin in paper III. All of the behavioural experiments can be considered to also measure neurotransmission. Our results are not easily interpreted. Especially the interaction of PREP with dopaminergic systems needs to be considered carefully. First of all, the lesion study (paper II) showed that lesioning the MFB does not affect the activity or expression of PREP in the striatum. Also, after the similar lesion, PREP inhibitor KYP-2047 did not affect the behaviour of the rats in the rotational experiments. Both of these findings suggest that PREP is not connected to dopaminergic signalling in the nigrostriatal dopaminergic pathway. However, in study III, the single i.p. treatment with PREP inhibitor decreased the levels of dopamine in the striatum and increased the levels of dopamine metabolites in the VTA. Furthermore, KYP-2047 treatment also increased locomotor activity of young rats after acute treatment suggesting an involvement in the dopaminergic signalling, although locomotor activity can be also regulated via other mechanisms.

In conclusion, PREP may control dopaminergic signalling as well as other signalling cascades, and eventually behaviour, possibly through intracellular mechanisms that should be further confirmed. Neuropeptidergic processes can be involved, but the cleavage of neuropeptides extracellularly may not be the sole or most important mechanism of action for PREP. Furthermore, as discussed in the literature part of this thesis, the effects of neuropeptides on behaviour are complex and may vary depending on the brain area, species, sex and peptide fragment. Interpreting the effects of PREP may be even more difficult, since it may also possess specific effects in certain brain areas.
7. SUMMARY AND CONCLUSIONS

The present studies were done to discover novel and clarify reputed functions of PREP in the rodent brain. Our results support the view that PREP inhibitors may not be as important in the cognitive functions as previously thought. Instead, our findings suggest that PREP may be involved in dopaminergic signalling and motor functions. The following specific conclusions were drawn from the results:

I  PREP activity was inhibited by several psychoactive compounds. However, even though these compounds may inhibit PREP in the brain, the action usually occurred at concentrations that cannot be achieved in the therapy. Therefore PREP is probably not the molecular target for known psychopharmacological compounds.

II Lesioning the nuclei of the tracts using classical modulatory neurotransmitters did not affect the activity and expression of immunoreactive PREP in respective projection areas suggesting that there is no PREP in the terminal areas of cholinergic or aminergic long projection neurons. Instead, the present and previous findings suggest that PREP is located in short GABAergic, cholinergic and glutamatergic interneurons.

III There are different interactions between PREP and neurotensin or its receptor NTS1 in the nigrostriatal and mesolimbic dopaminergic pathways. Based on these findings, we hypothesize that PREP may control the transport of dopamine or neurotensin vesicles thereby affecting site-dependently dopaminergic signalling.

IV PREP inhibition increased locomotor activity in young rats but had no effect on the memory and learning in radial-arm maze or rotational behaviour after a unilateral 6-OHDA lesion. The increase in the locomotor activity suggests a role for PREP in motoric functions.
Figure 7-1. The schematic conclusions of the study. Proved interactions are marked with a solid line and putative interactions with a dash line.
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