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The Potential of Prolyl Oligopeptidase as a Drug Target

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ABSTRACT

Prolyl oligopeptidase (PREP, EC 3.4.21.26; also known as prolyl endopeptidase, POP, PE, PO or PEP) cleaves proline-containing small peptides. Despite the fact that it is an abundant peptidase in the mammalian brain and periphery, the physiological role of PREP remains still unknown. PREP has received attention since its inhibitors have been shown to improve learning and memory in various animal models. These effects have been associated with reduced breakdown and elevated brain levels of neuropeptide substrates of PREP, or with elevated brain levels of classical neurotransmitters such as acetylcholine (ACh). However, evidence of the neuropeptide and neurotransmitter modifications *in vivo* is inconsistent.

The general objective of this study was to characterize the pharmacokinetic (PK) and pharmacodynamic (PD) properties of two model PREP inhibitors, JTP-4819 and KYP-2047, in the rat, and to evaluate the potential of PREP as a central nervous system (CNS) drug target.

In the PK studies, differences were observed in the brain penetration properties between the model inhibitors. The analysis of brain drug levels and PREP inhibition both revealed that KYP-2047 could penetrate the brain and reach the intracellular PREP better than JTP-4819 after a single intraperitoneal dose of 50 μmol/kg. These differences may have been attributable to the poor cell membrane permeability properties of JTP-4819.

In the PD studies, KYP-2047 (15 μmol/kg i.p.) was shown to alleviate scopolamine-induced memory impairment in young but not in old rats in the Morris water maze. Potential mechanisms of actions were investigated in biochemical studies in rats using both KYP-2047 and JTP-4819. At pharmacologically active brain concentrations, these compounds had no effect on the levels of substance P, neurotensin, inositol-1,4,5-trisphosphate (IP_3) or dopamine (DA) in the rat brain. In contrast to earlier observations, a significant decrease in the extracellular striatal ACh levels was observed. The effect on ACh levels did not correlate with the degree of brain PREP inhibition, indicating that the decline may not be related to the catalytic activity of PREP. *In vitro* specificity screening could not identify any off-targets that could explain the observed effects on ACh levels, but instead confirmed that the present inhibitors have high selectivity towards PREP.

In conclusion, KYP-2047 had suitable PK characteristics to be used as a pharmacological tool to study the potential of PREP as a CNS drug target. This study confirmed that PREP inhibitors have some beneficial effects on memory and learning in rats, but the mechanism of action remains to be clarified. However, it seems that the cholinergic or DAergic systems or IP_3 are not involved in this process. Furthermore, it is not likely that PREP inhibitors could modulate extracellular neuropeptide levels in the brain due to the fact that PREP is located intracellularly and peptide cleavage occurs mainly extracellularly.

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TIIVISTELMÄ

Prolyylioligopeptidaasi (PREP, EC 3.4.21.26; myös POP, PE, PO tai PEP) pilkkoo proliinia sisältäviä pieniä bioaktiivisia peptidejä. Vaikka PREP:ä on runsaasti lähes kaikissa elimissä, sen fysiologinen merkitys on edelleen epäselvä. PREP on herättänyt kiinnostusta mahdollisena lääkevaikutuksen kohteena, koska selektiivisten PREP:n estäjien on havaittu parantavan muistia ja oppimista eri eläimalleissa. PREP:n eston on uskottu vähentävän PREP:n substraateina toimivien neuropeptidien hajoamista aivoissa. Tämän seurauksena joko näiden peptidien tai muiden muistiin ja oppimiseen liittyvien välittäjäaineiden, kuten asetyylikoliiniin (ACh), määrä lisääntyisi aivoissa. Tätä teoriaa ei ole kuitenkaan pystytty luotettavasti todistamaan *in vivo*.

Tämän tutkimuksen tavoitteena oli tutkia kahden malliyhdisteen (JTP-4819 ja KYP-2047) avulla PREP-estäjien farmakokineettisiä (PK) ja farmakodynaamisia (PD) ominaisuuksia rotalla sekä arvioida keskushermoston PREP:n merkitystä mahdollisena lääkevaikutuksen kohteena. PK-tutkimuksissa havaittiin eroja malliyhdisteiden aivopenetraatiossa. Rotan vatsaonteloon (intraperitoneaalisesti; i.p.) annetun kerta-annoksen (50 μmol/kg) jälkeen suurempi osuus KYP-2047:stä läpäisi veri-aivoesteen, minkä seurauksena aivojen PREP estyi tehokkaammin kuin JTP-4819:llä. JTP-4819:n pienempi veri-aivoesteen läpäisevyys johtunee siitä, että se läpäisee solukalvoja huomattavasti huonommin kuin KYP-2047.

Farmakodynaamisissa kokeissa KYP-2047:n (15 μmol/kg i.p.) havaittiin parantavan nuorten mutta ei vanhojen rottien tilamuistia Morrisin vesisokkelossa. Tämän vaikutuksen mahdollisia mekanismeja tutkittiin mittaamalla eri välittäjäaineiden aivopitoisuuksia JTP-4819- tai KYP-2047-käsittelyjen jälkeen. PREP:n esto ei vaikuttanut rotan aivojen substanssi P-, neurotensiini-, inositoli-1,4,5-trisfosfaatti- (IP_3) tai dopamiinipitoisuuksiin. Aikaisemmista havainnoista poiketen todettiin, että malliyhdisteet laskivat aivojuovion solunulkoisen ACh:n määrää. Lasku ei kuitenkaan korreloinut aivojen PREP-aktiivisuuden kanssa, joten on todennäköistä, että havaittu vaikutus ei liity PREP:n katalyyttisen aktiivisuuden estoon. ACh-vaikutuksen mahdollista mekanismia selvitettiin seulomalla, onko aineilla muita sitoutumispaikkoja kuin PREP. Näissä *in vitro*-kokeissa aineiden todettiin olevan erittäin selektiivisiä PREP:n estäjiä, joten ACh-vaikutuksen aiheuttama mekanismi jäi siis epäselväksi.

KYP-2047:n osoitettiin olevan käyttökelpoinen farmakologinen työkalu, jolla voidaan tutkia keskushermoston PREP:n merkitystä lääkevaikutuksen kohteena. Lisäksi vahvistettin aiempia havaintoja siitä, että PREP-estäjillä on muistia ja oppimista parantavaa vaikutusta. Tämän vaikutuksen mekanismi jäi edelleen epäselväksi, mutta tulosten perusteella se ei todennäköisesti liity kolinergiseen, dopaminergiseen tai IP_3-järjestelmään. Lisäksi on todennäköistä, että PREP ei osallistu proliinia sisältävien peptidien solunulkoiseen pilkkomiseen, koska PREP on solunsisäinen entsyymi.

Luokitus: WL 104, WL 200, QV 38, QU 136, QU 143
Yleinen suomalainen asiasanasto: keskushermosto; aivot; aivotutkimus; neurokemia; entsyymit; inhibiittorit; farmakokinetiikka; farmakodynamiikka; neuropeptidit; välittäjäaineet; kognitio; oppiminen; muisti; rotat

To Henna

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Kuopio, January 2012

Aaro Jalkanen

List of the original publications

This dissertation is based on the following original publications, referred to in the text by Roman numerals **I-V**:

I Männistö PT, Venäläinen J, Jalkanen AJ, Garcia-Horsman A. Prolyl oligopeptidase: A potential target for the treatment of cognitive disorders. *Drug News & Perspectives 20: 293–305, 2007.*

II Jalkanen AJ, Hakkarainen JJ, Lehtonen M, Venäläinen T, Kääriäinen TM, Jarho E, Suhonen M, Forsberg MM: Brain pharmacokinetics of two prolyl oligopeptidase inhibitors, JTP-4819 and KYP-2047, in the rat. *Basic & Clinical Pharmacology & Toxicology 109: 443-451, 2011.*

III Jalkanen AJ, Puttonen KA, Venäläinen JI, Sinervä V, Mannila A, Ruotsalainen S, Jarho EM, Wallén EAA, Männistö PT: Beneficial effect of prolyl oligopeptidase inhibition on spatial memory in young but not in old scopolamine-treated rats. *Basic & Clinical Pharmacology & Toxicology 100: 132–138, 2007.*

IV Jalkanen AJ, Savolainen K, Forsberg MM: Inhibition of prolyl oligopeptidase by KYP-2047 fails to increase the extracellular neurotensin and substance P levels in rat striatum. *Neuroscience Letters 502: 107-111, 2011.*

V Jalkanen AJ, Piepponen TP, Hakkarainen JJ, De Meester I, Lambeir AM, Forsberg MM: The effect of prolyl oligopeptidase inhibition on extracellular acetylcholine and dopamine levels in the rat striatum. *Neurochemistry International, doi:10.1016/j.neuint.2011.12.010. In press.*

Contents

Abbreviations

Aβ	Amyloid β peptide
ACE	Angiotensin-converting enzyme
ACh	Acetylcholine
AChE	Acetylcholine esterase
Ac-SDKP	N-acetyl-seryl-aspartyl-lysyl-proline
AD	Alzheimer's disease
APP	β-Amyloid precursor protein
AUC	Area under the concentration-time curve
AVP	Arginine-vasopressin
Bax	Bcl2-associated X protein
BBB	Blood-brain barrier
BBMEC	Bovine brain microvessel endothelial cell
b.i.d.	*Bis in die* (twice daily)
BSA	Bovine serum albumin
BSA-CF	Body surface are correction factor
CF	Cystic fibrosis
ChAT	Choline acetyltransferase
C_{max}	Maximal concentration
CNS	Central nervous system
COPD	Chronic obstructive pulmonary disease
CSF	Cerebrospinal fluid
DA	Dopamine
DAG	Diacylglycerol
DOPAC	3,4-dihydroxyphenylacetic acid
DPPIV	Dipeptidyl peptidase IV
EC_{50}	Drug concentration producing 50% of the maximum attainable response
ECF	Extracellular fluid
ELISA	Enzyme-linked immunosorbent assay
E_{max}	Maximum attainable inhibition
FST	Forced swimming test
GAP-43	Growth-associated protein 43
HED	Human equivalent dose
HPA	Hypothalamic-pituitary-adrenal
HVA	Homovanillic acid
IC_{50}	Inhibitor concentration producing 50% inhibition of enzyme activity
IMPase	Inositol monosphosphatase
IP_3	Inositol-1,4,5-trisphosphate
IPP	Inositol polyphosphate 1-phosphatase
K_i	Inhibitory constant
LC-MS	Liquid chromatography-mass spectrometry
LTD	Long-term depression
LTP	Long-term potentiation
MIPP	Multiple inositol polyphosphate polyphosphatase
MMP	Matrix metalloproteinase
mPREP	Membrane-bound prolyl oligopeptidase
MS	Multiple sclerosis
α-MSH	Melanocyte stimulating hormone
NT	Neurotensin
P_{app}	Apparent permeability
PD	Pharmacodynamic
PGP	Proline-glycine-proline
PIP_2	Phosphatidylinositol bisphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PREP	Prolyl oligopeptidase
RIA	Radioimmunoassay
RR-MS	Relapsing-remitting multiple sclerosis
SP	Substance P
$t_{½β}$	Elimination half-life
Tβ4	Thymosin β4
t_{max}	Time required to reach maximal concentration
TRH	Thyrotropin releasing hormone
ZIP	Z-L-prolyl-L-prolinal insensitive Z-Gly-Pro-7-amino-4-methylcoumarin hydrolyzing peptidase
ZPP	Z-Pro-prolinal

1 Introduction

The activity and lifespan of proteins or peptides in living organisms are highly dependent on their processing by proteases, *i.e.* proteolytic enzymes. These enzymes perform a number of tasks that are vital to the organism, including the regulation of peptide functions, their post-translational modifications, and digestion into smaller fragments that can be either inactive or active.

Most peptidases are unable to cleave peptide bonds formed by proline residues because of the unique cyclic structure of this amino acid. Proline residues are present in many biologically active peptides, and thus, proline can be considered to protect these peptides from degradation. There are, however, specific proteases that recognize the cyclic proline and are able to degrade proline containing bioactive peptides, which makes these proteases highly interesting drug targets.

One such proline specific protease is prolyl oligopeptidase (PREP; EC 3.4.21.26, also known as prolyl endopeptidase, POP, PE, PO or PEP). It was first discovered as an oxytocin-cleaving enzyme in uterus in the 1970's (Walter et al., 1971; Cunningham and O'Connor, 1997b). It is an 80 kDa cytosolic enzyme of ancient origin belonging to the PREP family of serine proteases (Venäläinen et al., 2004). Despite the fact that it is an abundant peptidase in the brain and periphery, the physiological function of PREP is still largely unknown. It has a rather unique substrate specificity towards proline-containing peptides, including a variety of bioactive peptides, for example substance P (SP), arginine-vasopressin (AVP), neurotensin (NT) and bradykinin (see Cunningham and O'Connor, 1997b). Indeed, PREP is able to cleave several naturally occurring proline containing small peptides in *in vitro* peptidase assays.

Several of these PREP substrates have been associated with regulation of learning and memory (see Huston and Hasenohrl, 1995; Cunningham and O'Connor, 1997b; de Wied, 1997), and the highest PREP activities in human brain have been found from those brain areas closely associated with learning and memory. Therefore, already in the late 1970's, it was hypothesized that by inhibiting the catalytic activity of PREP with specific inhibitors, the brain levels of these promnesic neuropeptides could be elevated.

This neuropeptide hypothesis triggered the search for specific PREP inhibitors. Subsequently, hundreds of PREP inhibitors have been synthesized by academic and industrial research groups with the principal aim to generate antiamnesic drugs. At least three of them have even entered clinical trials as potential cognition enhancers, but have not proceeded to phase III trials for unknown reasons. A compound called S-17092 was developed by a French pharmaceutical company, Servier, in the 1990's. S-17092 was tested in a number of preclinical studies and in clinical phase I and II trials as a potential drug for Alzheimer's disease. Despite generally positive results, it never proceeded to phase III efficacy studies with large patient groups. Another PREP inhibitor, JTP-4819, was generated for senile dementia by Japan Tobacco Inc. already at mid 1990's. It was in phase II clinical trials when the company decided to discontinue the development for unknown reasons in 2001. A Finnish pharmaceutical company Orion Pharma patented two series of PREP inhibitors with a principal aim to generate drugs for neurogenerative diseases. However, none of these compounds have entered clinical trials to date. The apparent failures in the clinical studies indicate that there is a need for more detailed research on the physiological role of PREP, and the pharmacodynamic (PD) and pharmacokinetic (PK) properties of current PREP inhibitors before they can progress in the drug development process. Nevertheless, the few clinical trials have shown that certain PREP inhibitors can be administered to humans without concerns of safety.

In the last few decades, basic research has provided a number of scenarios for the physiological relevance of PREP, but these theories have been difficult to prove. Early reports associated altered plasma/serum PREP activity with several pathological conditions, such as psychiatric disorders (Maes et al. 1994; Maes et al. 1995). More recently, the enzyme has been linked with α-synuclein aggregation (Brandt et al., 2008), regulation of synaptic plasticity (Di Daniel et al., 2009; Szeltner et al., 2010), and neural growth-cone development (Di Daniel et al., 2009). It has also been postulated that PREP is a modulator of the inflammatory response (Gaggar et al., 2008) and that it participates in angiogenesis (Myöhänen et al., 2011). Furthermore, novel functions have been proposed for PREP in intracellular trafficking, sorting and protein secretion (Schulz et al., 2005). PREP has been also linked to the regulation of the phosphoinositide pathway (Williams et al., 1999). Interestingly, some of these functions seem to be unrelated to the catalytic activity of the enzyme. It is not surprising that these novel findings have significantly increased interest in PREP as a drug target.

Currently, several research groups are attempting to unravel the physiological role of PREP and the role of oligopeptidase inhibitors in brain function and dysfunction. The following review of the literature aims to summarize the recent findings about the role of PREP in health and disease. The first part of the review concentrates on the proposed role of PREP in the regulation of normal cellular functions, and the second part discusses the association of PREP with several pathological conditions. In addition, the potential of PREP inhibitors as possible treatments for some of these illnesses is discussed where applicable. The experimental section of this thesis concentrates on the characterization of the PD and PK properties of two model PREP inhibitors in rats, and evaluates the potential of PREP as a central nervous system (CNS) drug target.

2 *Review of the literature*

2.1 PROLYL OLIGOPEPTIDASE IN THE REGULATION OF CELLULAR FUNCTIONS

2.1.1 Overview of prolyl oligopeptidase

Prolyl oligopeptidase (PREP; EC 3.4.21.26) is found nearly in all organisms; it is present from bacterial and archeal species to humans (see Cunningham and O'Connor, 1997b). PREP belongs to the prolyl oligopeptidase family of serine proteases (clan SC, family S9). Close relatives of PREP are dipeptidyl peptidase IV (DPPIV; EC 3.4.14.5), oligopeptidase B (EC 3.4.21.83) and acylaminoacyl peptidase (EC 3.4.19.1) (Venäläinen et al., 2004).

In mammals, PREP is a cylinder shaped 80 kDa soluble protein of 710 amino acids consisting of two domains. The α/β-hydroxylase domain contains the catalytic residues (Fülop et al., 1998). The central sequence (residues 73-427) is a 7-bladed β-propeller domain radially arranged around the central tunnel embedded within the cylinder, where the active site is located.

PREP cleaves peptides at the carboxyl side of an internal proline (-Pro-Xaa-; where Xaa is not Pro). PREP also cleaves Ala-X bond but at a much lower reaction rate (Polgar, 1992). Furthermore, PREP has been shown to cleave an apoptosis rescue peptide, humanin, at the carboxyl side of a cysteine (Bär et al., 2006).

The size limitation of its substrates is the main difference between PREP and most of the other serine proteases. Eukaryotic PREP is able to digest peptides up to approximately 30 amino acids, but does not cleave larger, more structured proteins. The substrate induces an opening at the interface of the α/β-hydroxylase and β-propeller domains while entering into the active site, and the relatively small size of the interface only permits access to small peptides (Fülop et al., 1998). Furthermore, concerted movements of the propeller and the peptidase domains are required for the substrate binding and the enzyme action (Szeltner et al., 2004).

Mammalian PREP is encoded by the *Prep* gene that contains 15 exons (for details, see **I**). Earlier, PREP was suspected to be a housekeeping enzyme, since mouse *Prep* gene promoter was characterized by the absence of regulatory elements (Kimura et al., 1999). The regulatory mechanism of the *Prep* gene expression is still poorly understood, but recent reports indicate that the gene is highly regulated across tissues, indicating that PREP is not a cytoplasmic housekeeping enzyme (Moreno-Baylach et al., 2008; Matsubara et al., 2010).

In addition to the genetic factors, the activity of PREP may be regulated by at least one endogenous PREP inhibitor (Yoshimoto et al., 1982; Salers, 1994; Tenorio-Laranga et al., 2010). This inhibitor is poorly characterized, but it suspected to be a cytosolic competitive inhibitor with a molecular weight of 6.5 kDa. It has been proposed to be a regulator of PREP enzyme activity in cells, tissues and fluids (see Garcia-Horsman et al., 2007a), but its physiological role is unknown.

In peripheral mammal tissues, PREP protein is widely distributed in many organs, including placenta (Goossens et al., 1996; Agirregoitia et al., 2005; Myöhänen et al., 2008b; Matsubara et al., 2011). Generally, the highest peripheral PREP activities have been found in the kidney, liver and lungs. PREP activities in peripheral tissues are lower than those found in the brain, but higher peripheral activities have been reported in some tissues in mice (Myöhänen et al., 2008b).

PREP activity has also been detected from human body fluids, although the activities are low compared to the tissue levels (Goossens et al., 1996). However, this raises the question

of how PREP has reached extracellular fluids, since the protein is intracellular. Therefore, the existence of extracellular PREP activity has been subjected to considerable criticism (see chapter 2.2.1). The physiological role of peripherally located PREP is poorly understood, but recent findings indicate that peripheral PREP may be involved in the inflammation process (in lungs) and angiogenesis (Cavasin et al., 2004; Gaggar et al., 2008; Myöhänen et al., 2011).

PREP is found in all regions of the brain except for corpus callosum. However, the distribution is not homogenous throughout the brain, and activity is associated with neuronal cells rather than glial cells, suggesting that PREP is involved in neuronal functions (see **I**). In the rat, the most enriched brain areas for PREP immunoreactivity are the cerebellum, substantia nigra, cerebral cortex and hippocampus (Myöhänen et al., 2007). In humans, the highest brain PREP activities are found in cerebral cortical areas, hippocampus and nigrostriatal pathway, which could point to a role for PREP in learning and memory and motor functions (Irazusta et al., 2002; Myöhänen et al., 2007).

PREP is mainly considered as a soluble cytosolic enzyme, although a membrane bound form (mPREP) has also been characterized (O'Leary and O'Connor, 1995; Tenorio-Laranga et al., 2008). It has been postulated that mPREP is actually a variant of PREP that has undergone post-translational modifications, such as addition of a hydrophobic membrane anchor, necessary for PREP to be associated with membranes (Tenorio-Laranga et al., 2008). mPREP is significantly less active than cytosolic PREP, and the activity is mainly found in synaptosomes and endoplasmic reticulum. The role of mPREP remains unknown, although it has been speculated that it would actually be the enzyme that is responsible for the extracellular degradation of PREP substrates if it could be extended into the extracellular space. However, the localization and polarization of mPREP in the membrane is unknown, and there is no actual evidence that mPREP reaches the synaptic cleft.

Subcellularly, PREP is primarily detected in the perinuclear space tightly associated with the microtubulin cytoskeleton (Schulz et al., 2005; Myöhänen et al., 2008b), in growth cones (Di Daniel et al., 2009; Szeltner et al., 2010) and in cell organelle membranes known to be involved in protein synthesis (Myöhänen et al., 2008b). This subcellular localization could reflect regulatory functions for PREP in protein secretion, trafficking and processing (Schulz et al., 2005; Morawski et al., 2011).

2.1.2 General aspects of intracellular peptide processing and signalling

Since PREP has been shown to be present mostly free in the cytosol (Dresdner et al., 1982; Schulz et al., 2005; Myöhänen et al., 2008a), it would be logical to think that the physiological functions of PREP could be related to intracellular events. There is increasing evidence pointing to a role of PREP in the regulation of normal cellular functions such as axonal transport, protein secretion and intracellular peptide processing.

Intracellular protein degradation is essential for eukaryote cell homeostasis. The degradation process yields peptides which can then be further degraded into amino acids and used in synthesizing new proteins (see Goldberg, 2003). PREP may be involved in this intracellular peptide cycling process through multiple pathways (Figure 1). Free peptides in the intracellular compartment regulate important cellular functions, such as gene expression, metabolism, cell signalling and protein targeting (see Joliot and Prochiantz, 2004). Thus, PREP may have importance as a regulator of these functions.

The cell membrane consists of a lipid bilayer into which proteins and glycoproteins are attached. The hydrophobic nature of the lipid bilayer makes it impossible for hydrophilic compounds (such as proteins) to cross the membrane without the assistance of specific transport mechanisms. Therefore, hydrophilic cargoes are normally carried through the membrane by transmembrane shuttle proteins. For larger proteins, internalization occurs by endocytosis, resulting in the uptake of proteins into endocytic vesicles. In addition, an expanding family of cell-permeable peptides with the ability to reach the cytoplasmic

compartment after internalization has been described (pathway 2 in Figure 1) (see Joliot and Prochiantz, 2004). Once translocated into the cytosol, these peptides are freely available for degradation by cytosolic peptidases such as PREP. Furthermore, free intracellular peptides may be derived from peptides degraded extracellularly and then actively transported into the cytosol (pathway 3 in Figure 1).

A large fraction of the newly synthesized proteins is rapidly degraded, within even minutes of synthesis (Schubert et al., 2000). The degradation takes place mainly in proteasomes, which are large intracellular protein complexes. The proteasome pathway (pathway 4 in Figure 1) is thought to be especially important in destructing proteins that are misfolded or otherwise damaged (see Goldberg, 2003). For example, the proteasome has been shown to be a key player in the degradation of α-synuclein, a major component of the Lewy bodies forming cytotoxic insoluble fibrils in the brain of Parkinson's disease patients (Bennett et al., 1999; Conway et al., 2000; Conway et al., 2001; Gosavi et al., 2002). Inhibition of the proteasome leads to accumulation of toxic α-synuclein aggregates. The peptides generated by the proteasome in the cytoplasm may serve as substrates for PREP, although this has not yet been experimentally demonstrated. Furthermore, peptides released by proteasomes may exert a variety of biological effects, including activation or inactivation of intracellular peptidases (pathway 5 in Figure 1).

Subcellularly, PREP is located in the perinuclear space tightly associated with the microtubulin cytoskeleton (Schulz et al., 2005; Myöhänen et al., 2008b), which has important functions in intracellular protein transport. Interestingly, an overall increase in protein/peptide release was observed after a human glioma U-343 cell line was treated with a PREP inhibitor for 24 h, suggesting intracellular functions for PREP *e.g.* in protein secretion (Schulz et al., 2005).

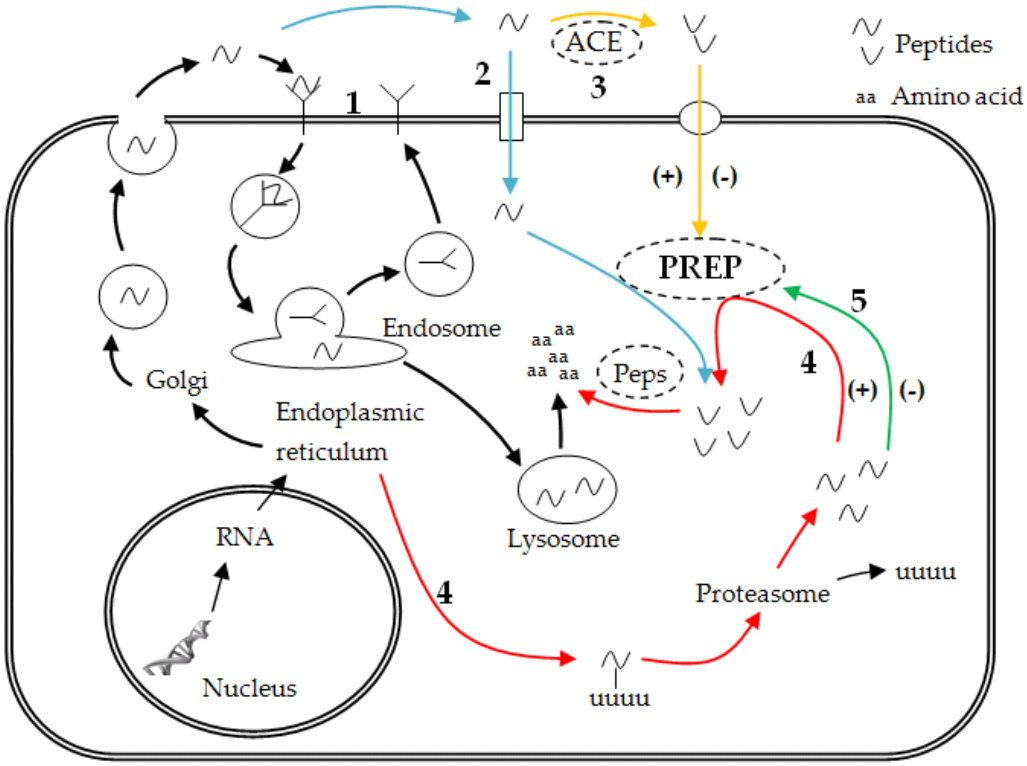

Figure 1. Some examples of general pathways of intracellular peptide formation and degradation. PREP may be involved in intracellular peptide processing through pathways 2-5. 1) Conventional pathway (black arrows): following the synthesis and release extracellularly acting peptides bind to cell surface receptors which mediate their biological effects. The peptide-receptor complexes are then internalized and transported by vesicles to the endosome where the complexes are dissociated. Peptides are then degraded in the lysosomes and the free amino acids are excreted into the cytosol. 2) Extracellular peptides can enter the cell via transmembrane shuttle proteins (blue arrows). In the cytosol, they may be degraded into amino acids e.g. by PREP and other peptidases (Peps) and join the cytosolic peptide pool. 3) Extracellular peptides may be degraded extracellularly (e.g. by angiotensin-converting enzyme; ACE) or on the cell surface (e.g. possibly by membrane-bound PREP) (yellow arrows). The resulting peptides are then transported into the cytosol, where they may activate or inhibit peptidases such as PREP. 4) Proteasome pathway (red arrows): Intracellularly acting peptides are derived from intracellular proteins tagged with polyubiquitin (uuuu). Ubiquitination targets proteins for cleavage by cytoplasmic proteasomes, and the resulting peptides can then serve as substrates for cytosolic peptidases such as PREP. 5) Intracellular peptides released by proteasomes (green arrow) may exert a variety of effects, including activation or inactivation of intracellular peptidases (e.g. PREP). Modified from Ferro et al. (2004).

2.1.3 Novel PREP substrates and products

The *in vivo* effects of PREP inhibitors on brain peptide levels have been characterized with two potent PREP inhibitors, JTP-4819 and S-17092, and changes in PREP substrate levels following PREP inhibition have been detected (Table 1; for details, see **I**). However, these changes have rarely been dose-dependent, and negative results have also been reported. It is notable that in particular chronic treatments have been ineffective, suggesting that other peptidases may compensate for PREP when it is inactivated. In summary, neuropeptide elevations after PREP inhibition are not invariably detected.

Table 1. The effect of PREP inhibition on neuropeptide levels in rat brain (for details, see **I**).

Peptide	Compound	Species	Treatment	Brain area/result	Reference
SP	JTP-4819	Young rat	Single dose 1-3 mg/kg p.o.	CTX ↑ HC↑	Toide et al., 1996
		Aged rat	Single dose 3 mg/kg p.o.	CTX ↑ HC ↔	Toide et al., 1995b
		Aged rat	Repeated 1 mg/kg p.o. × 21 days	CTX ↑ HC ↑	Toide et al., 1995b
		Young rat	Repeated 0.3-3 mg/kg p.o. × 15 days	CTX ↔ HC ↔	Shinoda et al., 1996
	S-17092	Young rat	Single dose 1-30 mg/kg p.o.	STR ↑	Morain et al., 2002
		Young rat	Repeated 1-30 mg/kg p.o. × 7 days	STR ↑	Morain et al., 2002
		Young rat	Single dose 10-30 mg/kg p.o.	CTX ↑ HT ↑	Bellemere et al., 2003
		Young rat	Repeated 10-30 mg/kg p.o. × 8 days	CTX ↔ HT ↔	Bellemere et al., 2003
TRH	JTP-4819	Young rat	Single dose 1-3 mg/kg p.o.	CTX ↔ HC ↑	Toide et al., 1996
		Young rat	Repeated 0.3-3 mg/kg p.o. × 15 days	CTX ↑ HC ↔	Shinoda et al., 1996
	S-17092	Young rat	Single dose 10-30 mg/kg p.o.	CTX ↑ AMG ↔	Bellemere et al., 2005
		Young rat	Repeated 10-30 mg/kg p.o. × 8 days	CTX ↑	Bellemere et al., 2005
AVP	JTP-4819	Young rat	Single dose 1-3 mg/kg p.o.	CTX ↑ HC↑	Toide et al., 1996
		Aged rat	Single dose 1 mg/kg p.o.	CTX ↔ HC ↔	Toide et al., 1995b
		Aged rat	Repeated 1 mg/kg p.o. × 21 days	CTX ↔ HC ↔	Toide et al., 1995b
		Young rat	Repeated 0.3-3 mg/kg p.o. × 15 days)	CTX ↔ HC ↔	Shinoda et al., 1996
	S-17092	Young rat	Single dose 10-30 mg/kg p.o.	HC ↑ CTX ↔	Bellemere et al., 2005
		Young rat	Repeated 10-30 mg/kg p.o. × 8 days	HC ↔ CTX ↔	Bellemere et al., 2005
α-MSH	S-17092	Young rat	Single dose 10-30 mg/kg p.o.	CTX ↑ HT ↑	Bellemere et al., 2003
		Young rat	Repeated 10-30 mg/kg p.o. × 8 days	CTX ↔ HT ↔	Bellemere et al., 2003

↑ elevated peptide levels; ↔ no change in peptide levels. Abbreviations: α-MSH, melanocyte stimulating hormone; AMG, amygdala; AVP, arginine-vasopressin; CTX, cortex; HC, hippocampus; HT, hypothalamus; SP, substance P; STR, striatum; TRH, thryrotropin releasing hormone.

Traditionally, assessments of brain peptide levels have been carried out by treating animals with a PREP inhibitor and then analyzing the levels of a potential substrate from crude tissue preparations using antibody based assays such as ELISA or radioimmunoassay (RIA). However, antibody based assays have to be targeted against a known substrate and thus, they can be used to test a hypothesis but they cannot detect unknown substrates.

In recent years, efforts to identify possible enzyme substrates have increasingly relied on the use of mass spectrometry based peptidomics assays that identify changes in the peptidome associated with changes in the activity of an enzyme (Che et al., 2005; Pan et al., 2005; Zhang et al., 2008). By using this approach, it is possible to detect multiple neuropeptides without any prior knowledge of their identities. To date, potential PREP substrates and cleavage products have been sought in at least three peptidomics studies (Brandt et al., 2005; Nolte et al., 2009; Tenorio-Laranga et al., 2009). In these experiments, most of the identified PREP substrates contained an internal proline, suggesting that they may be potential PREP substrates *in vivo*.

The first attempt to use the peptidomics approach to identify new potential PREP substrates was made by Brandt and co-workers (2005). They sought substrates in porcine brain by incubating brain peptide extracts with recombinant PREP. The peptidomics analysis revealed 269 masses disappearing and 261 masses appearing in the peptide extract after incubation with PREP, indicative of potential PREP substrates and products, respectively. The most interesting peptides produced by recombinant PREP included fragments of myelin basic protein (a structural protein of myelin) and a small part of α-synuclein protein. They also detected a fragment of phospholipase C (PLC), a key enzyme in the phosphoinositol cycle, disappearing from the extract, suggesting that it had been a PREP substrate. Theoretically, this could provide a novel but yet unidentified route for PREP to affect inositol-1,4,5-trisphosphate (IP_3) turnover.

However, the experimental set-up and the PREP digestion were nonphysiological and unavoidable unspecific *post-mortem* proteolysis was not sufficiently prevented in the study by Brandt et al. (2005). Therefore, Tenorio-Laranga et al. (2009) collected the brain samples from PREP inhibitor treated rats (ZPP 30 mg/kg i.p. 4 h after treatment) and made also several improvements to the sample preparation and mass spectrometry conditions and then approximately 200 significantly altered peptides were identified. The peptides were classified as PREP substrates if their levels were found to increase under PREP inhibition and as PREP products if the levels were decreased under the same conditions. The observed PREP products were fragments of large intracellular enzyme complexes or proteins or precursors of secreted peptides. This may point to a role for PREP in intracellular processing of peptides. It is notable that none of the PREP products were derived from peptides identified as PREP substrates (=substrate-product pairs), indicative of the involvement of other proteases in the degradation processes. They also found modest evidence for the involvement of PREP in the cleavage of classical PREP substrates, namely SP, TRH, AVP and α-MSH.

The latest attempt to use the peptidomics approach to identify PREP regulated peptides was carried out by Nolte and co-workers (2009). They treated mice with a PREP inhibitor (S-17092, 30 mg/kg i.p. 1 or 4 h before the sample collection) and analyzed changes in the brain and spinal cord peptide levels using a label-free peptidomics platform. They were able to detect changes in the levels of 23 distinct peptides, including SP, in the brain 1 h after the PREP inhibitor treatment. Fifteen peptides were classified as PREP substrates and 8 as products. Surprisingly, there were only few substrate overlaps with the earlier peptidomics assays, probably because these studies tend to vary extensively in their experimental conditions (*i.e.* the animal model, inhibitors, time of inhibition and mass spectrometry technique).

In contrast to the study of Tenorio-Laranga et al. (2009), Nolte et al. (2009) were able to identify few substrate-product pairs, indicative that the substrates truly were cleaved by

PREP *in vivo*. Among the identified PREP substrates perhaps the most interesting were thymosin β4 (Tβ4) derived peptides Tmsb4x(8-22) and Tmsb4x(8-25), which are precursors of a pro-angiogenic peptide *N*-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP; see chapter 2.1.7 for detailed discussion). Furthermore, several PREP regulated peptides were identified as proline rich peptides with two or more prolines in their amino acid sequence. These proline-rich domains are typically found in proteins associated with protein-protein interactions, *i.e.* a possible role for PREP in the regulation of these interactions.

Although the peptidomics studies have identified a number of interesting novel substrate candidates for PREP, it should be kept in mind that peptidomics methodology is still relatively new and the validation of the reported results will continue in the future. For example, it is not known whether the observed changes in the peptidome are a result of PREP catalytic activity or alterations in gene expression.

Humanin, an interesting novel PREP substrate, was found by Bär and co-workers (2006). Humanin is a cellular rescue factor and protects neurons from apoptotic cell death by preventing the translocation of Bax (Bcl2-associated X protein, an apoptosis-inducing factor) from cytosol to mitochondria (Guo et al., 2003). PREP was able to cleave humanin in a cell extract of a human glial cell line U-343, and this could be completely prevented by addition of a PREP inhibitor CNfmoc (Bär et al., 2006). Interestingly, the PREP catalyzed hydrolysis was found to occur at the carboxy terminal of cysteine, not proline, as would have been expected. The authors concluded that PREP has limited post-cysteine cleaving specificity, and PREP inhibition might represent a new target for apoptosis prevention. Earlier, PREP has been associated with neuronal apoptosis in various studies (see **I**). For example, a PREP inhibitor, ONO-1603, prevented age-induced apoptosis in cultivated neurons (Katsube et al., 1999). On the basis of these results, it would be interesting to confirm the cleavage of humanin by PREP *in vivo* as well.

Recently, a novel PREP product proline-glycine-proline (PGP) was described (Gaggar et al., 2008). The role of PREP in PGP release and the significance of PGP in neutrophil inflammation cascade are discussed in chapter 2.2.5.

2.1.4 Cell proliferation and differentiation

PREP has been suggested to have a role in cell proliferation and differentiation since changes in PREP mRNA levels as well as effects of PREP inhibitors on cell regeneration and tissue differentiation have been reported (see **I**). It was shown already in early animal studies that treatment of rats with a synthetic PREP inhibitor, Z-Gly-Pro-CHN_2, resulted in the suppression of liver regeneration, suggesting that PREP plays a role in the growth and differentiation of liver cells (Yamakawa et al., 1994). Moreover, studies with the flesh fly *Sarcophaga peregrina* revealed that PREP is involved in the differentiation of *Sarcophaga* imaginal discs, since a PREP inhibitor ZTTA inhibited their differentiation from the eversion to the elongation stage, and that the *Sarcophaga* PREP gene was activated at the eversion stage of differentiation (Ohtsuki et al., 1994; Ohtsuki et al., 1997). Furthermore, elevated PREP activities have been reported in several neoplastic diseases (Sedo et al., 1991; Goossens et al., 1996; Larrinaga et al., 2010).

Localization data suggest a role for PREP in the nuclear machinery and cell proliferation. PREP has been found to be present in the nucleus of peripheral cells (Myöhänen et al., 2008b), and at an early developmental phase, significant PREP activity has been found in neuronal nuclei as well (Moreno-Baylach et al., 2008; Moreno-Baylach et al., 2011). The role of PREP in cell proliferation is also supported by the fact that in peripheral tissues PREP was partially colocalized with nuclear proliferation marker Ki-67 (Myöhänen et al., 2008b).

Recently, the subcellular distribution of PREP in human neuroblastoma cells under proliferating conditions and under differentiation induced by retinoic acid has been studied (Moreno-Baylach et al., 2011). After the induction of cell differentiation, PREP activity was reduced in the nucleus but elevated in the cytoplasm and treatment with PREP inhibitors

could retard the onset of the differentiation. Moreover, in a primary culture of rat cerebellar neurons, cytoplasmic PREP activity was found to increase by 10-fold during the neuronal differentiation, and the peak expression coincided with the moment of complete differentiation into granular neurons (Moreno-Baylach et al., 2008).

The expression of PREP mRNA in the rat brain and spinal cord from embryo to adult stages was recently investigated (Agirregoitia et al., 2010). PREP mRNA levels were found to be more abundant during the perinatal stages, coinciding with a period of neuronal migration and differentiation. From that time point on, PREP mRNA expression declined, reaching its nadir levels at adulthood. Based on this data, it was suggested that PREP mediates specific functions related to neurodevelopment.

In a recent study, a PREP inhibitor SUAM-14746 was shown to suppress the growth of human neuroblastoma cells in a dose dependent manner without causing cell death (Sakaguchi et al., 2011). The growth inhibition was associated with pronounced G_0/G_1 arrest and a transient inhibition of the *S* and G_2/M phase progression, indicating a role for PREP in cell cycle regulation.

The localization of PREP in mouse placenta has been reported to change during development, suggesting that PREP plays a role in the differentiation of some placental cells or in the establishment of fetal-maternal connection (Matsubara et al., 2011). PREP was also found to be located in the nucleus of early developing mouse placenta, *i.e.* involvement in the regulation of cell proliferation and differentiation.

As a summary, the current evidence suggests that PREP plays a role in the cell proliferation and differentiation. However, no specific mechanisms for this action have been described. For example, it is not known whether these actions are dependent on the catalytic activity of PREP. Nevertheless, the recent observation that a PREP inhibitor can suppress the growth of malignant tumor cells (Sakaguchi et al., 2011) opens an interesting field for future PREP studies.

2.1.5 Regulation of inositol cycle

Stimulation of a large variety of neurotransmitter receptor subtypes (*e.g.* muscarinic M_1, M_3 and M_5, noradrenergic α_1 and serotonergic 5-HT_2) induces the hydrolysis of membrane phospholipids. The binding of an agonist to these kinds of receptors activates G proteins which stimulate PLC. PLC hydrolyses phosphatidylinositol bisphosphate (PIP_2) into two second messengers, diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3). IP_3 stimulates the mobilization of intracellular calcium (Ca^{2+}) from endoplasmic reticulum and DAG activates protein kinase C (PKC) (Figure 2). Intracellular Ca^{2+} has many vital functions *e.g.* in signal transduction and in muscle cell contraction.

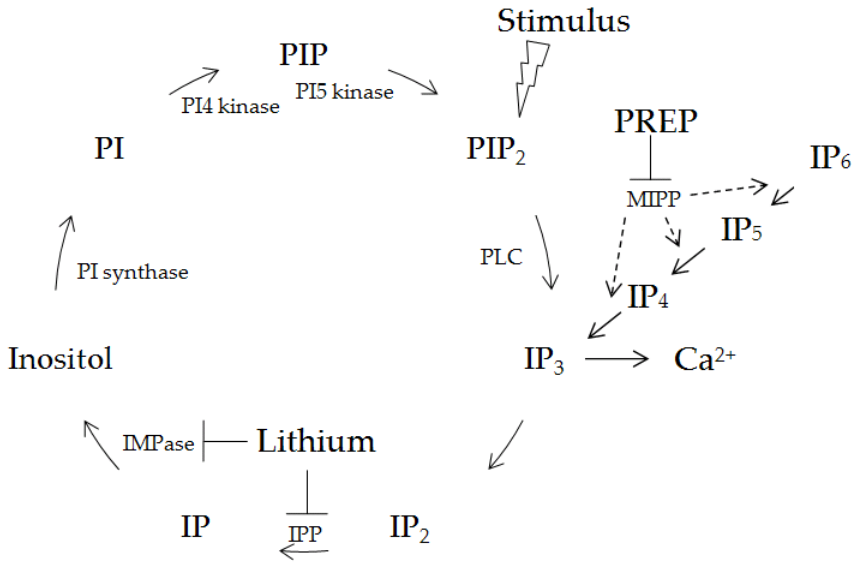

Figure 2. The role of PREP in the inositol cycle. Upon cell stimulation phospholipase C (PLC) converts phosphatidylinositol bisphosphate (PIP_2) into inositol-1,4,5-trisphosphate (IP_3), which triggers calcium (Ca^{2+}) release from intracellular storages. Another route to produce IP_3 is to degrade high order inositol phosphates (IP_6, IP_5 and IP_4), which is catalyzed by multiple inositol polyphosphate polyphosphatase (MIPP). PREP regulates MIPP activity, and reduced PREP activity (by inhibition or mutation) leads to enhanced degradation of high order phosphates and increased IP_3 levels. IP_3 is further degraded by inositol polyphosphate 1-phosphatase (IPP) and inositol monophosphatase (IMPase) to inositol. Inositol is incorporated into PIP_2 by PI synthase, PI4 kinase and PI5 kinase. Lithium inhibits IPP and IMPase, which in turn causes inositol depletion and slows down the inositol cycle. (The diacylglycerol (DAG) hydrolyzation route of PIP_2 was excluded from the illustration for clarity reasons). (Williams and Harwood, 2000; Harwood, 2011).

The link between PREP and IP_3 was first established when it was discovered that the mutated social amoeba *Dictyostelium discoideum* missing the *Prep* gene (DpoA mutant) was resistant to the well-documented inositol and IP_3 depleting effect of lithium (Williams et al., 1999). Lithium inhibits inositol monophosphatase (IMPase) and inositol polyphosphate 1-phosphatase (IPP), which are key enzymes in the production of inositol. The concentration of the second messenger IP_3 is dependent on the amount of available inositol, which is depleted during lithium treatment, resulting in reduced IP_3 levels (Berridge et al., 1989). Reduced PREP activity can counteract this and increase the amount of IP_3 during lithium treatment (Williams et al., 1999). The same effect has been observed in wild-type cells treated with a PREP inhibitor, suggesting that reduced PREP activity can enhance the turnover of phosphoinositides and increase the cellular concentration of IP_3, probably by regulating the multiple inositol polyphosphate polyphosphatase (MIPP), the enzyme which produces IP_3 by dephosphorylating IP_6, IP_5 and IP_4 (Williams et al., 1999; Schulz et al., 2002).

The IP_3 elevating effect of PREP inhibition may not arise from direct elevation of IP_3 production, but is due to changes in genes encoding inositol synthesis and recycling (King et al., 2010). These include IMPase (*impA1*), inositol synthase (*ino1*), IPP and IP_3 5'phosphatase genes. MIPP and PREP are key components in the gene regulatory network that controls inositol synthetic genes, however, the mechanism leading to gene expression changes remains unknown and requires clarification.

A number of studies have linked PREP and inositolphosphate signalling in mammalian cells as well. There is an inverse correlation between the IP_3 concentration and PREP expression in mammalian neuronal cells (Schulz et al., 2002). In primary neurons, lithium-mediated growth cone spreading is reversed by PREP inhibition (Williams et al., 2002) (see also chapter 2.1.6.) Furthermore, PREP and IP_3 type 1 receptor (IP_3R1) are highly colocalized in distinct areas of rat brain, hinting at a regulatory relationship between PREP and IP_3 (Myöhänen et al., 2008c; Myöhänen et al., 2009).

A simple scenario to explain the proposal that PREP is involved in the inositol cycle would be that PREP regulates the activity of crucial intracellular peptides involved in the phosphorylation/dephosphorylation cascades of the inositol cycle. Despite several attempts using the peptidomics approach, however, no IP_3 regulating peptides have been found. Furthermore, the connection between IP_3 and PREP has never been observed in any of the *in vivo* models.

2.1.6 Regulation of neuronal growth

The growth cone is a specialized area at the tip of the extending axon that is responsible for sensing the local environment and moving towards the neuron's synaptic target (see Kalil and Dent, 2005). The growth cones are hand-shaped with several long finger-like filopodia reaching out to touch or sense the environment. Growth cones are key elements in axon guidance, directing the initial wiring of the nervous system during development and they are also important in axonal regeneration following an injury.

There are recent findings for a role for PREP in the regulation of neuronal growth cone dynamics. Three mood stabilizing drugs, lithium, carbamazepine and valproate, cause similar changes in the growth cone dynamics of rat sensory neurons *in vitro* (Williams et al., 2002). These changes, *i.e.* the decrease in number of collapsed growth cones and the increase in the growth cone area, can be reversed by addition of myo-inositol indicating that these drugs act *via* inhibition of the inositol cycle. Interestingly, PREP inhibitors can reverse the effects of the three mood stabilizers on the growth cone dynamics thus mimicking the action of inositol.

Di Daniel and co-workers (2009) studied the growth cone response of mood stabilizing drugs in PREP null-mutant ($PREP^{-/-}$) mouse neurons and found out that untreated $PREP^{-/-}$ neurons differed from wild-type $PREP^{+/+}$ neurons in terms of growth cone area and collapse. However, the $PREP^{-/-}$ phenotype could be completely reversed with the transduction of either native or a catalytically inactive PREP. This is a clear indication that PREP regulates the growth cone dynamics *via* cytosolic protein-protein interactions rather than *via* its peptidase activity.

Potential intracellular PREP protein binding partners that could mediate the growth cone response were sought using yeast-two-hybrid assays (Di Daniel et al., 2009). For example, growth-associated protein 43 (GAP-43) was identified as a possible PREP interactor in yeast. GAP-43 has been shown to be involved in several cellular functions, such as growth cone formation and axon guidance, cellular signalling, synaptic plasticity, neurodegeneration and apoptosis (see Aigner and Caroni, 1993; Benowitz and Routtenberg, 1997; Denny, 2006). It is a particularly interesting PREP binding partner, since it has been shown to be predominantly expressed in the brain areas involved in cognitive processes (Neve et al., 1988; Benowitz et al., 1989). Furthermore, PREP has been shown to partially co-localize and bind to GAP-43 in mammalian cells (Di Daniel et al., 2009; Szeltner et al., 2010).

It can be hypothesized that a protein-protein interaction between PREP and GAP-43 would affect many processes in which GAP-43 is involved, and thus could also explain the altered growth cone dynamics of $PREP^{-/-}$ mouse neurons. However, Szeltner and co-workers (2010) could not find any strong physical interaction between these proteins. Instead of a direct physical interaction, PREP could affect the functions of GAP-43 via an indirect pathway. For example, PREP has been associated with tubulin, the main structural

component of the microtubulin cytoskeleton (Schulz et al., 2005). Microtubules play a key role in intracellular trafficking and axonal transport. Since GAP-43 is axonally transported into the growth cones, PREP could interfere with this transportation system and thus affect the GAP-43 mediated cellular functions.

If the effects of PREP on the growth cone dynamics are mediated through protein-protein interactions and not through the catalytic activity of the enzyme, this raises the question how PREP inhibitors can modulate these non-catalytic actions. This could be explained by the fact that the access of substrates to the catalytic site of PREP involves the movement of a flexible loop, which evokes conformational changes in the tertiary structure of the enzyme (Fuxreiter et al., 2005). Since most PREP inhibitors are substrate-like compounds, it is likely that the binding of the inhibitor to the enzyme causes similar conformational changes, which in turn could perturb the protein-protein interactions.

2.1.7 Regulation of angiogenesis

A recent intriguing finding in PREP biology relates to its association with Tβ4, the most abundant β-thymosin in mammals (see Hannappel, 2010). Tβ4 is a 43 amino acid peptide which has been proposed to possess cardioprotective properties. For example, intracardiac or intraperitoneal Tβ4 administration in a mouse model of myocardial infarction improved cardiac function and enhanced myocyte survival (Bock-Marquette et al., 2004). Furthermore, exogenously administered Tβ4 affects inflammatory events and wound healing (Young et al., 1999; Bock-Marquette et al., 2004; Sosne et al., 2007). Tβ4 has also been linked to angiogenesis (*i.e.* the formation of new blood vessels) in a number of *in vitro* and *in vivo* studies (Grant et al., 1995; Malinda et al., 1997; Koutrafouri et al., 2001; Myöhänen et al., 2011). Indirect evidence of the involvement of Tβ4 in angiogenesis emerges from the observation that it is overexpressed in a number of human tumors and the overexpression correlates to malignant progression (Conlon et al., 1988; Hall, 1991).

Ac-SDKP is the N-terminal amino acid sequence (Tβ4 2-5) of Tβ4 (see Hannappel, 2010). Ac-SDKP is present in the blood, and it has been shown to decrease cardial fibrosis, prevent collagen synthesis *in vitro*, and have anti-inflammatory properties in rats (Peng et al., 2001; Rhaleb et al., 2001a; Rhaleb et al., 2001b; Yang et al., 2004; Sharma et al., 2008). Ac-SDKP is hydrolyzed in plasma by ACE, and it has been suggested that some of the cardioprotective effects of ACE inhibitors may be mediated through elevated plasma levels of Ac-SDKP (Junot et al., 2001; Wang et al., 2004). It has been shown *in vitro* that PREP is the main enzyme responsible for the cleavage of Ac-SDKP from Tβ4 (Cavasin et al., 2004). It was shown using rat kidney cortex homogenates that Ac-SDKP was generated from exogenous Tβ4, and this generation was significantly reduced by addition of several PREP inhibitors. Furthermore, *in vivo* studies in rats have demonstrated that long term administration of the PREP inhibitor, S-17092, significantly decreased the plasma levels of Ac-SDKP, and prevented the Ac-SDKP increase induced by an ACE inhibitor, captopril, in the plasma, urine, heart and kidney (Cavasin et al., 2004).

Since PREP is not known to be able to cleave peptides larger than 30 amino acids, it is unlikely that the 43 amino acid long Tβ4 would be a PREP substrate. Therefore, a preceding cleavage step would be necessary to produce suitable PREP substrates from Tβ4. Indeed, it has been demonstrated that PREP does not cleave whole Tβ4, but is a second step enzyme in the release of Ac-SDKP from Tβ4 (Myöhänen et al., 2011). Furthermore, a PREP inhibitor, KYP-2047, can block the release of Ac-SDKP from Tβ4 in rat kidney homogenate. The first step in Tβ4 cleavage is attributable to unidentified protease(s), and interestingly, PREP was shown to inhibit these first step proteases, thus autoregulating the release of Ac-SDKP. This effect could be independent on the hydrolytic activity of PREP, or alternatively, PREP could generate peptide products that inhibit the proteases responsible for the initial cleavage of whole Tβ4. Since Tβ4 is located intracellularly, the Ac-SDKP release is probably an intracellular event, which is also appropriate in view of the intracellular location of PREP.

Ac-SDKP promotes angiogenesis both *in vitro* and *in vivo* (Liu et al., 2003; Wang et al., 2004). Pathological angiogenesis is a hallmark of cancer as well as various inflammatory and ischemic diseases (Carmeliet and Jain, 2000). The plasma and bone marrow Ac-SDKP levels have been shown to be elevated in patients with leukemia, and overexpression of Ac-SDKP has been associated with cancer (Liu et al., 2010). Since PREP may play a role in the release of Ac-SDKP, it has become interesting to study the association of PREP with angiogenesis (Myöhänen et al., 2011). PREP was found to increase and the PREP inhibitor, KYP-2047, decreased angiogenesis *in vitro* and *in vivo*. The observed decrease in angiogenesis opens new possibilities for future research examining PREP inhibitors in tumor models.

Tβ4 is also expressed in the brain. Nolte and co-workers (2009) studied the peptidomics of PREP in mice brain, and found evidence for PREP involvement in the cleavage of Tβ4 in the CNS as well. Two Tβ4 derived Ac-SDKP precursors, Tmsb4x(8-22) and Tmsb4x(8-25), were found to be elevated in mouse brain after a single dose treatment with S-17092, indicating that Tβ4 cleavage is dependent on PREP activity in the brain as well. The role of Ac-SDKP in the CNS is unknown, but this study indicates that the Tβ4-Ac-SDKP pathway could represent important new functions for PREP in the CNS.

2.2 ROLE OF PREP AND PREP INHIBITORS IN DISEASE PROCESSES

2.2.1 Problems associated with plasma PREP activity measurements

Changes in human plasma/serum PREP activities have been described in several pathological conditions (Table 2). However, no specific mechanisms have been established to account for these altered activities. Furthermore, the relevance of the plasma PREP activity studies was questioned when it was discovered that the reported plasma enzyme activity consisted of two separate enzymes, namely PREP and Z-L-prolyl-L-prolinal insensitive Z-Gly-Pro-7-amino-4-methylcoumarin hydrolyzing peptidase (ZIP) (Cunningham and O'Connor, 1997a; Birney and O'Connor, 2001). Later ZIP was characterized and identified as seprase or fibroblast activation protein α (FAP) (Collins et al., 2004). When Z-Gly-Pro-AMC is used as the substrate in activity assays, ZIP/FAP accounts for about 60% of the plasma activity previously attributed to PREP, but its activity in other tissues than blood has not been described. Therefore, the observed alterations in the plasma PREP activity in different diseases may reflect the enzymatic activity of ZIP/FAP rather than PREP.

ZIP/FAP and PREP have several common proline-containing substrates, but ZIP/FAP is completely resistant towards ZPP inhibition (Cunningham and O'Connor, 1997a; Birney and O'Connor, 2001). Furthermore, in humans, ZIP/FAP activity seems to be restricted to plasma, whereas blood PREP activity can be detected in the lymphocytes and plasma (Breen et al., 2004). Since ZIP/FAP has been shown to be a substantial component of plasma based PREP assays, clinical studies using plasma samples need to be re-examined in order to eliminate the possible contaminating role of ZIP/FAP. On the other hand, an interesting question is how PREP and ZIP/FAP activities are even found in plasma, since plasma is the extracellular component of blood, whereas PREP protein is known to be cytosolic (Myöhänen et al., 2008a) and FAP a membrane bound glycoprotein (O'Brien and O'Connor, 2008). Furthermore, it is questionable to what extent the plasma enzyme activity reflects the brain enzyme activity in disease states.

Table 2. Observed changes in plasma/serum PREP activity in various pathological conditions.

Disease	Change in plasma PREP activity	References
Alcoholism	↓	Maes et al., 1999a
Anorexia and bulimia nervosa	↓	Maes et al., 2001
Autistic spectrum disorder (ASD)	↑ (+ much higher variation in plasma PREP activity of ASD patients	Momeni et al., 2005
Bipolar disorder		
- Lithium-treated patients	↓	Breen et al., 2004
- untreated patients	↑	Maes et al., 1995
Depression	↓	Maes et al., 1994 & 1995
Mania	↑	Maes et al., 1995
Post-traumatic stress disorder	↑	Maes et al., 1999b
Schizophrenia	↑	Maes et al., 1995
Stress induced anxiety	↑	Maes et al., 1998

↑, increased plasma PREP activity;↓, decreased plasma PREP activity

2.2.2 PREP in depression

In clinical studies, plasma PREP activity has been found to be significantly lower in patients with depression in comparison to the activity in healthy individuals (Maes et al., 1994; Maes et al., 1995) (Table 2). The activity also correlated with the severity of the symptoms; the individuals with the most severe depression had the lowest plasma PREP activities. Interestingly, patients treated with antidepressants such as fluoxetine displayed significantly higher plasma PREP activity than untreated patients (Maes et al., 1995). However, these data need to be interpreted with caution because of the complexity of plasma PREP activity measurements, especially in view of the poor relevance of extracellular PREP (see chapter 2.2.1). Furthermore, although statistically significant, the observed changes were rather small, typically 10% less than the healthy controls.

There is increasing evidence indicating that various stress-responsive neuropeptide systems play important pathogenic roles in depressive conditions as well as in other stress-related disorders (see Brain and Cox, 2006; Alldredge, 2010). Neuropeptides are important in governing the hypothalamic-pituitary-adrenal (HPA) axis, and perturbations in the HPA axis are common markers of depression and anxiety.

Since PREP has been closely associated with the metabolism of neuropeptides and alterations in plasma PREP activities have been described in patients with major depression, the effects of two novel PREP inhibitors in two animal models of depression were recently studied (Krupina et al. 2011). The first of these experimental depression syndromes was induced in rats by administering the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) leading to a DA deficient state. In the second model, the animals were exposed to an irreversible DPPIV protease inhibitor (Met-Prd-N) during the early postnatal period. Both models lead to depression-like behavioral changes, and increased PREP activity in various brain areas. Subchronic (10 days) administration of the PREP inhibitors, Z-Met-Prd-N (2 mg/kg i.p.) and Z-Ala-Pro-OH (3 mg/kg i.p.) (K_i values against rat cortical PREP 1.4 nM and 90 μM, respectively), were found to suppress the depression-like behavior in Porsolt's forced swimming test (FST), *i.e.* antidepressant-like effects. However, the K_i value of Z-Ala-Pro-OH against PREP is poor, and it is not plausible that the effect seen in FST would be dependent solely on brain PREP inhibition.

There is not much other experimental data linking PREP inhibitors to antidepressant action. A variety of recognized psychopharmacological compounds were tested for their inhibitory action against PREP *in vitro*, but only weak if any inhibition was observed (Peltonen and Männistö, 2011), and it is doubtful if such minor inhibition would be therapeutically important. One unpublished study in rats claimed dose-dependent antidepressant-like effects for ORM-12139 (1-3 mg/kg s.c.; compound also known as KYP-2153) in FST (personal communication, Johanna Holappa, Orion Pharma). In an attempt to elucidate the possible mechanism of the antidepressant action of ORM-12139 (3 mg/kg s.c.), the activation of the brain derived neurotrophin factor (BDNF) receptor TrkB in rat prefrontal cortex was measured, but no significant effect on this marker of neuronal plasticity and antidepressant drug action (Castren and Rantamäki, 2010) was observed (Jalkanen et al., unpublished data).

PREP has been primarily associated with neuropeptide metabolism, but it is difficult to explain the few observed antidepressant actions of PREP inhibitors through elevations in brain neuropeptide levels. In fact, several PREP substrates, such as SP and AVP, have been shown to hyperactivate the HPA axis, and thus, induce stress related behavior and they seem to possess anxiogenic and depressive properties in rodents (Ebner et al., 2004; Dinan and Scott, 2005; Ebner and Singewald, 2006; Ebner and Singewald, 2007; Ebner et al., 2008). On the other hand, the PREP substrate, oxytocin, has been suggested to have protective properties against stress and anxiety (Campbell, 2008). Moreover, it is possible that the antidepressant actions are mediated by the inhibition of the non-catalytic actions of PREP. Taken together, the available experimental data does not support a major role for PREP inhibitors in the treatment of depression or anxiety.

2.2.3 Neurodegenerative diseases

Post mortem brain PREP activity has been reported to be altered in several neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease, Huntington's disease and multiple sclerosis (MS) (Mantle et al., 1996). Earlier, the link between PREP and neurodegeneration was based on the possible role of PREP in neuronal apoptosis (see **I**). Recently, some new mechanisms through which PREP could be involved in neurodegeneration have been proposed. These mechanisms and the current knowledge of the role of PREP in the neurodegenerative diseases are discussed below.

Neuroinflammation

There is a substantial amount of evidence pointing to a role for neuroinflammation in the pathogenesis of neurodegenerative diseases such as AD and Parkinson's disease (see Hirsch and Hunot, 2009; Lee et al., 2010; Philips and Robberecht, 2011). There is some data associating PREP with the inflammatory process (see chapter 2.2.5). Since brain PREP activity has been reported to be altered in several neurodegenerative diseases (see below), it is tempting to draw a connection between PREP, neuroinflammation and neurodegeneration (Penttinen et al., 2011).

Neuroinflammation is distinct from the inflammatory conditions occurring in other organs. Neuroinflammation is characterized by microglia activation in the primary inflammatory state. Leukocyte invasion, when it occurs, is a secondary phenomenon as a consequence of blood-brain barrier disruption (see McGeer and McGeer, 2004). Microglia activation results in a flooding of surrounding tissue with inflammatory mediators, oxidizing free radicals, pro-apoptotic factors, matrix-degrading proteases and chemoattractant molecules. In the lungs, PREP is believed to play a role in the release of one such chemoattractant, PGP, (Gaggar et al., 2008; O'Reilly et al., 2009), but there are no reports linking PGP and neuroinflammation. Moreover, PREP has been considered to be the main enzyme in the release of an anti-inflammatory tetrapeptide Ac-SDKP from Tβ4 in

the brain (Yang et al., 2004; Sharma et al., 2008; Nolte et al., 2009). In summary, neuroinflammation seems to be one of the most intriguing targets for future PREP research.

Alzheimer's disease

AD is the most common neurodegenerative disease and a major cause of dementia in the elderly, with an estimated prevalence of approximately 4% of individuals over 60 years of age (see Ballard et al., 2011). The early symptoms are seen as inability to acquire new memories. As the disease advances, symptoms expand to involve long-term memory loss, aggression and mood swings, etc., and ultimately AD leads to loss of bodily functions and death of the subject.

Senile plaques spreading over the cortical brain areas are typical neuropathological hallmarks of AD (see Ballard et al., 2011). The main protein component of these plaques is amyloid β-peptide (Aβ). Deposition of Aβ triggers neuronal dysfunction and death in the brain. This peptide derives from the β-amyloid precursor protein (APP). Under normal conditions, APP is cleaved by α-secretase to generate soluble APPα which precludes Aβ generation. The presence of APPα has been suggested to enhance neuronal survival, synaptic plasticity, learning and memory (Han et al., 2005; Zhang et al., 2011).

The α-secretase pathway of APP processing can be stimulated by PLC-linked receptor activation. These receptors include metabotropic glutamate (Lee et al., 1995), muscarinic ACh (Nitsch et al., 1992) and serotonin 5-HT_{2a} and 5-HT_{2c} receptors (Nitsch et al., 1996). The activation of PLC results in the generation of two second messengers, DAG and IP_3, which subsequently release calcium from intracellular storages (Figure 2). Interestingly, PREP inhibition increases intracellular IP_3 levels, which may contribute to the stimulation of APPα production, which would in turn decrease Aβ generation. However, this theory has not been experimentally tested.

APP can also be cleaved sequentially by β-secretase and γ-secretase to release extracellular peptide fragments called $A\beta_{1\text{-}40/1\text{-}42}$. These neurotoxic fragments can oligomerize and at later stages form insoluble aggregates ultimately leading to the formation of senile plaques (Ballard et al., 2011; Zhang et al., 2011). Since the aggregation of Aβ in the brain seems to be a primary cause of AD, the inhibition of Aβ generation has become a topic of much current research. Thus, the levels of several enzyme activities that may be involved in the generation of Aβ, including PREP, have been measured from *post-mortem* AD brains. Both increased and decreased levels of PREP activity have been assayed in brains of AD patients (Aoyagi et al., 1990; Ichai et al., 1994; Mantle et al., 1996; Terwel et al., 1998). The decreased levels have been attributed to the oxidative stress induced by *antemortem* hypoxia (Garcia-Horsman et al., 2007a; Terwel et al., 1998). Furthermore, Rossner et al. (2005) found less PREP immunoreactive neurons in brain structures of AD patients affected by Aβ plaques.

It is unlikely that the PREP hydrolyzing activity contributes to the production of Aβ. Purified human PREP does not degrade synthetic $A\beta_{1\text{-}40/1\text{-}42}$ *in vitro*, which is not surprising given that $A\beta_{1\text{-}40/1\text{-}42}$ do not contain internal proline and are too large to be PREP substrates.

The Aβ precursor protein APP does have internal prolines in its amino acid sequence, but it is a large protein (~700 amino acids) and does not meet the size criterion of a PREP substrate. Theoretically, PREP could be a second step enzyme in APP cleavage, but here too the substrate size seems to be a limiting factor. Indeed, two PREP inhibitors, ZPP and S-17092, had no effect on $A\beta_{1\text{-}40/1\text{-}42}$ production in a human cell line (Petit et al., 2000) supporting the belief that PREP does not participate in APP cleavage. On the other hand, infusion of soluble $A\beta_{25\text{-}35}$ (a shorter, biologically active fragment of Aβ) into rat hippocampus or frontal cortex resulted in increased PREP activities in those brain areas (Arif et al., 2009), but the significance of this change is not clear. Moreover, correlations between PREP activity in brain regions with Aβ –like immunoreactivity in human and

senescence accelerated mice have been conflicting (Fukunari et al., 1994; Kato et al., 1997; Rossner et al., 2005).

Taken together, the data linking PREP activity to the pathological changes in AD are not consistent, and it seems more likely that the observed changes in brain PREP activity in AD are associated with neuronal damage rather than Aβ accumulation (Laitinen et al., 2001). Thus, it is unlikely that one could prevent Aβ generation and achieve a reduction of senile plaque formation in AD with PREP inhibitors.

Multiple sclerosis

MS is a progressive neurodegenerative disease of the CNS affecting more than two million people all around the world (see Dutta and Trapp, 2011). The underlying cause of the disease is still unknown, but the pathological hallmarks of the disease are the presence of multifocal inflammatory demyelinated plaques distributed over time and space within the CNS. Other typical pathological findings are the disruption of the blood-brain barrier, oligodendrocyte loss, reactive gliosis and axonal degeneration (see Dutta and Trapp, 2007; Trapp and Nave, 2008), the latter probably being the major cause of the progressive neurological disability. The primary phase of the disease is termed relapsing-remitting MS (RR-MS), and during this phase patients experience alternating episodes of neuronal disability and recovery (Ebers et al., 2008). However, within 25 years, 90% of patients transform into a secondary-progressive disease course with a steady neurological disability.

PREP has been associated with several factors that might be relevant to MS. For instance, PREP is involved in the regulation of the inflammatory response (Gaggar et al., 2008) and microglia toxicity (Klegeris et al., 2008). Indeed, a recent report established a direct connection between PREP and MS; the plasma PREP activities of patients with RR-MS were significantly reduced (Tenorio-Laranga et al., 2010). Interestingly, the reduction correlated with the severity of disease symptoms, but not with patient age. Instead, an inverse correlation between PREP activity and age was observed in healthy controls, and in elderly controls the levels were comparable to those found in MS patients.

The reduction in the plasma PREP activity of MS patients could partly be explained by the substantial increase in the levels of an endogenous PREP inhibitor (see chapter 2.1.1). Another explanation could be that PREP activity is reduced because of the oxidative stress induced by the disease (Koch et al., 2006; Koch et al., 2007). It does seem that the reduced plasma PREP activity correlates with the symptoms of the disease. It is worth noting that the study by Tenorio-Laranga et al. (2010) was more reliable than many others investigating plasma PREP activity, since ZIP/FAP activity in plasma was separated from PREP activity.

Parkinson's disease

Parkinson's disease is a progressive neurodegenerative disorder affecting 1% of individuals over 60 years old (see Samii et al., 2004). The motor symptoms of the disease include resting tremor, rigidity, akinesia and bradykinesia, and these occur when approximately 60-80% loss of DAergic neurons projecting from the substantia nigra pars compacta to the striatum.

Reduced PREP activity has been reported in brain tissue of Parkinson's disease patients (Mantle et al., 1996). Furthermore, the PREP activity in the cerebrospinal fluid (CSF) of patients with Parkinson's disease was significantly lower than the PREP activity of control patients, while activity in plasma did not change significantly (Hagihara and Nagatsu, 1987). However, it is not known whether the CSF PREP activity is a reliable measure of brain PREP activity, since CSF is an extracellular fluid and PREP is mainly located in the cytosol of neurons. Moreover, the reported plasma activity may have been attributable to peptidases other than PREP (see chapter 2.2.1).

The neuropathological hallmark of Parkinson's disease is the progressive degeneration of melanised DAergic neurons in substantia nigra pars compacta together with intracellular

inclusions known as Lewy bodies. A major component of the Lewy bodies is a 140 amino acid protein, α-synuclein. This is a protein enriched in the presynaptic terminals of neurons, but its function in the neuron is largely unknown (Spillantini et al., 1997; Fellner et al., 2011). α-Synuclein is not only found in the brains of Parkinson's disease patients, but it also has a role in other neurodegenerative diseases such as dementia with Lewy bodies, the Lewy body variant of AD, multiple system atrophy, and neurodegeneration with brain iron accumulation type I (Uversky, 2007).

Under normal conditions, α-synuclein is degraded mainly by the high-capacity intracellular ubiquitin/proteasome pathway (pathway 4 in Figure 1). There are factors known to decrease the activity and clearance capacity of the proteasome, *e.g.* age and oxidative stress (Cuervo et al., 2010). Under certain conditions, α-synuclein monomers interact to form prefibrillar aggregates or protofibrils, which can create cytotoxic insoluble fibrils (Conway et al., 2000; Conway et al., 2001; Gosavi et al., 2002). These fibrils cannot be degraded by the proteasome, and they impair the function of this intracellular proteolytic system. This leads to an accumulation of α-synuclein protofibrils (and other proteins that are degraded by the proteasome) in the cytosol (Bennett et al., 2005) and as a consequence, α-synuclein protofibrils are increased in brains of Parkinson's disease patients. These fibrils have been associated with neurotoxicity in α-synuclein overexpressing cells and mouse models (Masliah et al., 2000; Gosavi et al., 2002).

Abnormal accumulation of misfolded α-synuclein may lead to mitochondrial changes which can promote oxidative stress and evoke cell death (Hsu et al., 2000). Furthermore, three point mutations (A53T, A30P or E46K) in the α-synuclein gene are known to be involved in the pathogenesis of familial form of Parkinson's disease (Polymeropoulos et al., 1997; Zarranz et al., 2004).

However, although α-synuclein seems to be a major contributor to Parkinson's disease, it is still not clear which form of the α-synuclein aggregates induce cell death in neurons (Xie et al., 2010). It has also been postulated that certain fibrillar α-synuclein aggregates could even represent a cytoprotective mechanism (Caughey and Lansbury, 2003; Kaplan et al., 2003). For example, in neuronal cell lines, overexpression of wild type α-synuclein offered protection against the oxidative stress through some unknown mechanism, but the mutated (A53T and A30P) forms resulted in increased oxidative stress damage (Lee et al., 2001).

The link between PREP and α-synuclein was first established by Brandt and co-workers (2005) when they were searching for endogenous PREP substrates from a porcine brain homogenate using mass-spectrometry based peptidomics analysis and recombinant PREP. Several potential PREP substrates and products were identified; among them a hexapeptide YQDYEP, which was found to be identical with porcine α-synuclein residues 133-138, suggesting that PREP had been involved in the cleavage of α-synuclein downstream to the primary cleavage site.

In a subsequent study, it was shown that PREP did not cleave intact purified recombinant α-synuclein (Brandt et al., 2008). This is understandable in view of the fact that PREP cleaves peptides only shorter than 30 amino acids and α-synuclein (140 amino acids) does not meet this criterion. Therefore, it is likely that the putative PREP product YQDYEP was not cleaved by PREP from full-length α-synuclein but from a truncated form. It has been shown that α-synuclein is mainly degraded by the intracellular ubiquitin-proteasome pathway (Bennett et al., 1999), and intracellular peptidases are known to further degrade the peptides generated by the proteasome. Thus, PREP might play a role in the final stages of α-synuclein cleavage in the brain.

Furthermore, it was shown *in vitro* that the aggregation rate of α-synuclein was enhanced when the protein was incubated with a clone of wild-type porcine PREP, and this enhancement depended upon the PREP concentration (Brandt et al., 2008). Moreover, a mutated variant without PREP activity (S544A) did not accelerate the aggregation rate. Enhanced aggregation could also be prevented by the addition of PREP inhibitors,

suggesting that the effect was dependent on the PREP enzymatic activity. Thus, PREP possibly binds to the monomeric or aggregated α-synuclein with its catalytic centre, since the accelerating effect on aggregation can be reversed by mutation and inhibitors.

A protein-protein interaction between PREP and α-synuclein was reported in yeast (Di Daniel et al., 2009). Recent evidence has suggested that PREP inhibitors can block the increased α-synuclein aggregation induced by oxidative stress in human α-synuclein over-expressing neuroblastoma SH-SY5Y cells (Lambeir, 2011). PREP colocalizes with α-synuclein in SH-SY5Y cells, and this colocalization disappears after incubation with PREP inhibitors, pointing to an interaction between PREP and α-synuclein.

The association between PREP and α-synuclein may have interesting clinical implications in the future. Theoretically, inhibition of brain PREP activity could prevent α-synuclein aggregation and thus, prevent the formation of the cytotoxic protofibrils present in the Lewy bodies. Therefore, PREP inhibitors could potentially have therapeutical value in the treatment of neurodegenerative disorders where accelerated α-synuclein aggregation has been described. However, before such a claim can be made, thorough studies on the role of PREP and PREP inhibitors in α-synuclein aggregation *in vivo* will need to be conducted *e.g.* using transgenic mouse models exhibiting fibrillar α-synuclein inclusions (Giasson et al., 2002; Lee et al., 2002).

2.2.4 Learning and memory

Originally, PREP inhibitors were developed with the principal aim of generating anti-amnesic drugs (see **I**). The effects of several PREP inhibitors in various cognitive tasks have been characterized, and there is some kind of consensus that PREP inhibitors have positive effects on learning and memory (Table 3). However, the effects in rodents have been relatively weak, rarely dose-dependent, and have been difficult to replicate. This may explain the fact that only a few studies investigating the cognitive effects of PREP inhibitors have been published in the recent 4-5 years. Overall, it seems that the belief that PREP inhibitors could be potential cognition enhancers has dissipated.

The ability of KYP-2047 (5 mg/kg i.p.) to prevent scopolamine-induced (0.4. mg/kg i.p.) amnesia in a radial-arm maze was examined in young and old rats (Peltonen et al., 2010). KYP-2047 had no effect on the memory of either age group, but when given without scopolamine, it slightly increased the maze motility of young rats and decreased the motility of old rats through some unknown mechanism.

The effect of subchronic administration of rosmarinic acid, a non-competitive PREP inhibitor (with a relatively high IC_{50} value of 63.7 μM), was tested in the Morris water maze in rats, and an enhancement in spatial memory was reported (Park et al., 2010). However, the brain pharmacokinetics and penetration of rosmarinic acid have not been characterized. It does seem unlikely that one could achieve sufficient brain penetration to reach pharmacologically active brain concentrations of rosmarinic acid since the compound possesses 13 hydrogen bond acceptors and donors and has a high polar surface area (145 $Å^2$). Once the total number of donors and acceptors exceeds 8-10 and polar surface area 100 $Å^2$, the transport of a drug across the blood-brain barrier (BBB) is expected to be minimal (Pardridge, 2005).

Table 3. Effects of PREP inhibitors on behavioral parameters.

PREP inhibitor	Treatment (mg/kg)	Animal age	Behavioral test(s)	Memory impairment	Result (dose)	Reference
JTP-4819	1, 3, 10 p.o. single	♂ Rat NA	Passive avoidance	Scopolamine 0.5 mg/kg s.c.	↑ 3, 10 retention ↑ 1, 3 acquisition	Toide et al., 1995a
JTP-4819	0.01, 0.1, 1 p.o. 7 days	♂ Rat 2,5 mo	Passive avoidance	Middle cerebral artery occlusion	↑ 0.1, 1 ↔ 0.01	Shinoda et al., 1996
JTP-4819	0.1, 0.3, 1, 3 p.o. 7 days	♂ Rat 2,5 mo	Morris water maze	Middle cerebral artery occlusion	↑ 1 ↔ 0.1,0.3,3	Shinoda et al., 1996
JTP-4819	0.3, 1, 3 p.o. 20 days	♂ Rat 3 mo vs. 24 mo	Morris water maze	Age	↑ 1 ↔ 0.1, 3	Toide et al., 1997
JTP-4819	0.3, 1, 30 p.o. 34-41 days	♂ Rat NA	Eight-arm radial maze	Dorsal hippocampal lesion	↑ 3 ↔ 0.3, 1	Miyazaki et al., 1998
JTP-4819	0.3, 1, 3, 10 p.o. 15 days	♂ Rat 2,5 mo	Morris water maze	Ibotenate lesion of Nucleus basalis magnocellularis	↑ 1, 3 ↔ 0.3, 3	Shinoda et al., 1999
KYP-2047	5 i.p. single	♂ Rat 3 or 8 mo	8-arm radial maze	Scopolamine 0.4 mg/kg i.p.	↔	Peltonen et al., 2010
S 17092	10 p.o. single	Aged mice	2-choice discrimination task	Age	↑	Marighetto et al., 2000
S 17092	0.01-30 i.p. single	Rat NA	Passive avoidance	Scopolamine 0.3 mg/kg s.c.	↑ 3-30	Morain et al., 2002
S 17092	10 p.o. 7 days b.i.d	♂ Mouse 3-5 mo	Elevated Y-maze	Scopolamine 0.3 mg/kg s.c.	↑	Morain et al., 2002
S 17092	1, 3, 10, 30 p.o. single or 7 days	Rat NA	Social recognition test		↔ single dose ↑ 7 days 10 mg/kg	Morain et al., 2002
S 17092	1, 3, 10 p.o. 7 days	♂ Monkey adult	Delayed response, alternation, matching-to-sample, visual discrimination	Chronic low dose (0.075 mg/kg i.v.) MPTP	↑ 3 ↔ 1, 10	Schneider et al., 2002
ZTTA	6 p.o. single	♂ Rat 2 mo	Three-panel runway task	Cerebral ischemia	↑	Shishido et al., 1996
ZTTA	10 p.o.4 days	♂ Rat 2 mo	Passive avoidance	Basal forebrain lesion	↑	Shishido et al., 1998

↑ = improved performance; ↔ = no effect; mo = month-old; NA = not applicable

2.2.5 Peripheral diseases

Chronic airway inflammatory diseases

Chronic obstructive pulmonary disease (COPD) is a term referring to two separate chronic lung disorders: chronic obstructive bronchitis with obstruction of small airways, and emphysema with enlargement of air spaces and destruction of lung parenchyma, loss of lung elasticity, and closure of small airways (see Barnes, 2000). It is one of the most prevalent and fatal diseases in the world, and the World Health Organization (WHO) predicts that COPD will rise from the 6th most common cause of death to 3rd place by 2020 (Lopez and Murray, 1998). Furthermore, this prediction may well be an underestimate, since COPD most likely contributes to other common causes of death. In the developed countries, cigarette smoking is the leading cause for COPD, but in the less developed countries also other environmental pollutants, such as wood smoke, are important sources (Dennis et al., 1996). It is also claimed that genetic factors are important in the development of COPD (Silverman et al., 1998).

The pathogenesis of COPD is complex, but one key feature is a chronic neutrophilic airway inflammation occurring in the peripheral airways (bronchioles) and lung parenchyma (see Barnes, 2000). Cigarette smoke and other irritants initiate an inflammatory immune response where airway epithelial cells and macrophages release inflammatory mediators and chemoattractants such as tumour necrosis factor (TNFα), interleukin-8 (IL-8) and a tripeptide PGP. These chemoattractants trigger the migration of neutrophils to the site of inflammation. Activated neutrophils and macrophages release multiple proteinases such as matrix metalloproteinases 8 and 9 (MMP-8 and MMP-9) that break down connective tissue (*e.g.* collagen) in the lung parenchyma, which in turn leads to tissue destruction, emphysema and mucus hypersecretion.

PREP may be involved in the inflammation process that is a characteristic of COPD, and also in other chronic airway inflammatory diseases such as cystic fibrosis (CF) (Gaggar et al., 2008; Braber et al., 2011). Cigarette smoke or other noxious stimuli can act on airway epithelial cells such that lung collagen is cleaved by MMP-8 and MMP-9 into smaller peptide fractions, which can act as substrates for PREP (Gaggar et al., 2008). PREP cleaves these fractions into PGP, which has been shown to be an important neutrophil chemoattractant both *in vitro* and *in vivo* (Pfister et al., 1998; Weathington et al., 2006). Interestingly, PREP seems to be the only protease that can cleave PGP from the collagen fragments (an often repeated PPGP sequence) generated by MMP-8 and MMP-9 (Gaggar et al., 2008). The release of PGP leads to continuous recruitment of neutrophils to the inflammation area, which subsequently releases proteases and again, PGP (Figure 3). Earlier, PREP had been associated with inflammatory processes in only a few studies. PREP activity was found to be increased in the knee joint synovial membrane of patients with rheumatoid arthritis (Kamori et al., 1991). The mechanism of this pro-inflammatory role has been unknown, but on the basis of the above recent findings, it may have been attributable to PGP and neutrophil mediated inflammation.

Since PREP seems to play an important role in the release of PGP and thus, in the chronic neutrophil mediated inflammatory response, PREP inhibitors may represent a novel therapeutic approach to the treatment of COPD, CF or other chronic inflammatory disorders. Theoretically, PREP inhibitors might be able to interrupt the inflammatory cascade by preventing the formation of PGP from collagen. Indeed, the production of PGP from collagen in the sputum of CF patients has been prevented with the PREP inhibitor ZPP *in vitro* (Gaggar et al., 2008). Moreover, PREP activity and PGP production were found to be elevated in the sputum samples of CF patients, implicating increased PREP activity with PGP generation *in vivo*.

Furthermore, it has been shown that PREP activity and PGP formation were elevated in mice lung homogenates after chronic exposure to cigarette smoke *in vivo* (Braber et al., 2011), and that intratracheal administration of a combination of MMP-8, MMP-9 and PREP generated PGP in mice lungs *in vivo* (Gaggar et al., 2008). Human blood neutrophils are known to contain PREP, MMP-8 and MMP-9, and they can generate PGP from collagen in the presence of an inflammatory stimulus (O'Reilly et al., 2009).

An interesting observation was made very recently when it was discovered that ZPP could antagonize chemokine receptors CXCR1 and 2 (which mediate the proinflammatory effects of IL-8) and prevents neutrophils migration to IL-8 and PGP, thus blocking two pro-inflammatory mechanisms in the same pathological pathway (Hardison et al., 2011).

PREP has been considered to be the main enzyme in the release of a tetrapeptide Ac-SDKP from Tβ4 (see chapter 2.1.7). There is increasing evidence that Ac-SDKP possesses anti-inflammatory properties (Yang et al., 2004; Sharma et al., 2008). PREP inhibitors can block Ac-SDKP release *in vivo* (Cavasin et al., 2004), but it is not known whether this is relevant in terms of inflammation.

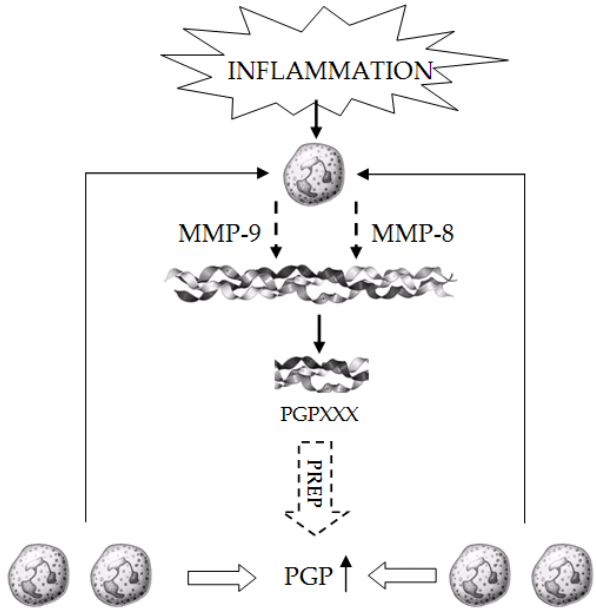

Figure 3. A schematic illustration of the involvement of PREP in the chronic neutrophilic inflammation cascade. Activated neutrophils release MMP-8 and MMP-9, which cleave collagen into smaller (<30 amino acids) fragments (PGPXXX). These fragments are then cleaved by PREP into PGP, which then acts as a neutrophil chemoattractant. This triggers a vicious cycle of increased neutrophil influx, which induces oxidant injury, releases more proteolytic enzymes and finally leads to chronic airway inflammation and organ dysfunction. Modified from Gaggar et al. (2008).

Cancer

Elevated PREP activity has been found in a number of human lung tumors, and in malignant prostate and sigmoid carcinomas (Sedo et al., 1991; Goossens et al., 1996). Furthermore, increased PREP activity has been found in clear cell renal cell carcinoma, urothelial carcinoma of the renal pelvis and head and neck squamous cell carcinoma (Larrinaga et al., 2010). PREP has also been associated with cell proliferation by a yet unidentified mechanism, and PREP inhibitors have been shown suppress the growth of malignant tumor cells (see chapter 2.1.4 for discussion and references).

Since cancer progression is dependent on tumor neovascularization, pro-angiogenic agents are of importance in neoplastic diseases. The levels of the pro-angiogenic tetrapeptide Ac-SDKP and PREP are elevated in a number of malignant tissues, perhaps indicative of a contribution for Ac-SDKP and PREP in the pathogenesis of cancer (Liu et al., 2003; Liu et al., 2010). Interestingly, angiogenesis can be significantly reduced by PREP inhibition (Myöhänen et al., 2011), suggesting that PREP inhibitors could have beneficial effects in anti-tumor therapy.

PREP has been shown to play a key role in the release of an extracellular neutrophil chemoattractant, PGP (see above). PGP attracts activated neutrophils, which have been shown to have an important role during cancer progression (Noel et al., 2008). Activated neutrophils subsequently release multiple proteinases, such as MMPs, that degrade components (*e.g.* collagen) of the extracellular matrix. MMPs are central regulators in the complex tumor ecosystem composed of cancer cells, blood or lymphatic endothelial cells, pericytes, smooth muscle cells, fibroblasts, adipocytes, immune and inflammatory cells (Albini and Sporn, 2007), and they facilitate angiogenesis, tumor cell invasion, and metastasis (Noel et al., 2008; Roy et al., 2009). The activated PGP-MMPs –pathway in tumor ecosystem may be associated with the increased PREP activity observed in malignant tumors. Furthermore, PREP inhibitors can block PGP formation at least *in vitro* (Gaggar et al., 2008). Thus, PREP inhibition might offer a novel therapeutic approach for cancer treatment by blocking the PGP-MMPs –pathway.

Celiac disease

Celiac disease (also known as celiac sprue) is a heritable disorder of the small intestine. It has a high prevalence of 1:100-1:50, but even this may be an underestimate because many patients have atypical symptoms or none at all (see Farrell and Kelly, 2002). The disease is characterized by an inflammation response to ingested wheat gluten and to similar rye and barley proteins, leading to destruction of the intestinal villi and a greatly reduced ability to absorb nutritients. Currently, the only effective treatment for celiac disease is consumption of a gluten free diet.

Wheat gluten is a mixture of proline-rich gliadin and glutenin polypeptides. When dietary gluten is proteolyzed by pepsin and pancreatic enzymes, a mixture of thousands of peptides is released (see Farrell and Kelly, 2002). One peptide generated from α-gliadin, a 33-mer peptide, is an exceptionally pro-inflammatory agent and is recognized by mucosal T cells in the small intestine of celiac disease patients. This peptide could be detoxified in *in vitro* and *in vivo* assays by exposure to a bacterial PREP, suggesting a potential strategy for oral peptidase supplement therapy for celiac disease (Shan et al., 2002). Indeed, it has been demonstrated *in vitro* and *in vivo* in rats that a two-enzyme cocktail comprising of a glutamine-specific cysteine protease (EP-B2) that functions under gastric conditions and bacterial PREP, which acts in concert with pancreatic proteases under duodenal conditions could accelerate the breakdown of a gluten-rich solid meal. This combination has been proposed as representing a potent candidate for celiac disease therapy (Gass et al., 2006; Siegel et al., 2006; Gass et al., 2007), and it has recently entered phase II clinical trials.

However, the 33-mer peptide is actually a relatively potent mammalian PREP inhibitor and is highly resistant to human intestinal preparations despite their significant endogenous PREP activity (Garcia-Horsman et al., 2007b). Moreover, a causative role of PREP in the pathogenesis of celiac disease has been ruled out. It has been suggested that a mixture of other peptidases are needed to eliminate gliadin-derived toxic peptides in the human intestine. Interestingly, proteases (including PREP) from germinating wheat can degrade gliadin into small peptide fragments and reduce its epithelial toxicity *in vitro* and *ex vivo* (Stenman et al., 2009). Thus, PREP may act in concert with other proteases and have important role in the detoxification of gliadin.

2.3 SUMMARY OF THE REVIEW OF THE LITERATURE

In recent years, PREP has been associated with a number of physiological and pathological functions, but the main physiological role of PREP remains unknown. In particular, current understanding on the role of PREP *in vivo* is restricted. Interestingly, some of the proposed novel functions may even be unrelated to PREPs peptidase activity. Hence, the potential of PREP as a drug target remains elusive.

Since the enzyme was discovered in the 1970's, the strongest *in vitro* evidence has linked PREP with the cleavage of proline containing small peptides. However, despite numerous attempts using various techniques the *in vivo* evidence of this role is inconsistent and needs further investigation. Novel peptidomics assays have identified several interesting substrate candidates for PREP *in vivo*, but validation of the reported results will continue in the future. Furthermore, it seems that the most promising sources for future *in vivo* research involve clarifying the roles of PREP in neutrophil mediated inflammation, in α-synuclein aggregation and in cell proliferation and differentiation.

Several PREP inhibitors have entered clinical trials as potential cognition enhancers, but these projects have failed for unknown reasons. The mechanism of action of PREP inhibitors on cognition has not been established. Apparently, the PK and PD properties of current PREP inhibitors *in vivo* and *e.g.* their specificities towards PREP are not well enough characterized. For example, it has not been reliably shown that PREP inhibitors can penetrate the BBB and reach the intracellular target site in brain. On the other hand, the effects of PREP inhibitors on neurotransmitter turnover are not known. A well-characterized pharmacological tool would help the researchers to unravel the physiological functions of PREP and also to design new potent and safe PREP inhibitors.

3 *Aims of the study*

The general objective of this study was to characterize the PK and PD properties of model PREP inhibitors in the rat, and to evaluate the potential of PREP as a CNS drug target. The specific aims of this pharmacological study were:

1. To critically review previously reported effects of PREP inhibitors on cognition and on putative PREP substrates in the brain (**I**) in order to provide a comprehensive background for the subsequent studies (**III-V**).
2. To assess the ability of two model PREP inhibitors, JTP-4819 and KYP-2047, to penetrate the brain, to reach and to inhibit brain PREP after a single dose treatment (**II**).
3. To confirm the positive cognitive effects of PREP inhibitors by evaluating the effect of KYP-2047 on scopolamine-induced spatial memory impairment in Morris water maze in young and in old rats (**III**).
4. To examine the effects of PREP inhibitors on some putative PREP substrates in the rat brain *in vivo.* A special emphasis was placed on the extracellular substrate levels, since the effect of PREP inhibition on the peptide levels in their main site of action is not known (**III, IV**).
5. To find a proof of mechanism for the positive cognitive effects observed with various PREP inhibitors by measuring the effects of PREP inhibitors on the levels of several neurotransmitters in rat brain (**III, IV, V**).

4 Materials and methods

4.1 ANIMALS

Male Han/Wistar rats, supplied by the National Laboratory Animal Centre (Kuopio, Finland) were housed in stainless steel cages and kept on a 12-h light/12-h dark cycle at an ambient temperature of 22 ± 1°C. Both young (2-3-month old, n=351) (**II-V**) and old (8- to 9-month old, n=38) (**III**) rats were used in the studies. Animals had free access to pelleted food (Lactamin R36; Lactamin AB, Södertälje, Sweden) and fresh tap water (Kuopion Vesi, Kuopio, Finland). Before the experiments, the rats were housed in groups. After installation of microdialysis guides, the animals were housed in individual cages (**II, IV, V**). All experiments were performed during the lights-on time of the day (between 7.00 am and 7.00 pm).

All procedures with the animals were performed according to the European Community Guidelines and reviewed by the Animal Ethics Committee at the University of Kuopio/University of Eastern Finland, and approved by the local provincial government or by the national Animal Experiment Board.

4.2 TREATMENTS

PREP inhibitors JTP-4819 {(*S*)-2-[[(*S*)-2-(hydroxyacetyl)-1-pyrrolidinyl]carbonyl]-N(phenylmethyl)-1-pyrrolidinecarboxamide} and KYP-2047 (4-phenylbutanoyl-L-prolyl-2(*S*)-cyanopyrrolidine) (Figure 4) (**II-V**) were synthesized by Dr. Elina Jarho (University of Eastern Finland, Kuopio, Finland) as previously described (Venäläinen et al., 2002; Jarho et al., 2004). The dosing regimens used in the *in vivo* studies (**II-V**) are summarized in Table 4.

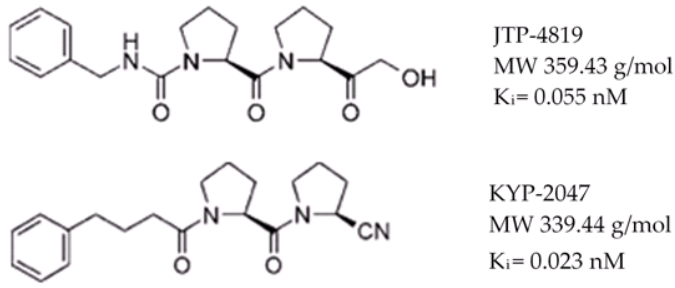

Figure 4. The chemical structures, molecular weights and inhibitory constants (K_i against pig PREP) of JTP-4819 and KYP-2047 (Toide et al., 1995a; Venäläinen et al., 2006).

JTP-4819 was dissolved in saline (**II, III, V**). KYP-2047 was dissolved in saline containing 5% of Tween® 80 due to its low water solubility (**II-V**). Control animals received the same amount of vehicle as the drug treated animals. Drugs were given intraperitoneally in a volume of 5 or 10 ml/kg.

Chloral hydrate (350 mg/kg i.p., 3.5% solution in saline, Sigma Chemical Co, St. Louis, USA) was used as an anesthetic (**II, IV, V**).

The acetylcholine (ACh) muscarine receptor antagonist, scopolamine hydrobromide (Sigma Chemical Co), was dissolved in saline and was given in a volume of 10 ml/kg i.p. (**III**). In order to elevate the levels of ACh in the dialysates, neostigmine bromide (Sigma Chemical Co) was used as an additive in the perfusate (final concentration 100 nM) (**V**). Bacitracin and bovine serum albumin (BSA) (both from Sigma Chemical Co) were used as additives in the perfusate (**IV**) in order to prevent non-specific peptide degradation in the perfusate and binding to the apparatus, respectively.

Table 4. The treatment groups and doses used in the *in vivo* experiments (**II-V**).

Study	Assay	Route	JTP-4819[1]	KYP-2047[2]	Additional drugs
II	PK	i.p.	**50** µmol/kg - Single dose - Microdialysis (n=5-7/tissue) - Tissue conc.: (n=4/time point)	**50** µmol/kg - Single dose - Microdialysis (n=5-7/tissue) - Tissue conc.: (n=4/time point)	
III	MWM	i.p.	NA	**15** µmol/kg (old rats) (n=12) **3 & 15** µmol/kg (young rats) - Single dose (n=14-16)	Scopolamine 0.4 mg/kg i.p.
	NT SP	i.p.	**9 & 27** µmol/kg - Single dose (n=6) - 10 days (b.i.d.) (n=5)	**9 & 27** µmol/kg - Single dose (n=6) - 10 days (b.i.d.) (n=5)	
	IP_3	i.p.	**9** µmol/kg - 2,5 days b.i.d. (n=8)	NA	
IV	NT SP	i.p.	NA	**50** µmol/kg - Single dose (NT: n=5) (SP: n=4)	Bacitracin (0.03%) BSA (0.2%) in perfusate
V	ACh	i.p.	**50** µmol/kg - Single dose (n=9)	**50** µmol/kg - Single dose (n=8)	Neostigmine (100 nM) in perfusate
		Retro-dial.	In perfusate **12.5** µM (n=5) **37.5** µM (n=5) **125** µM (n=3) (× 60min×1.5µl/min)	In perfusate **12.5** µM (n=4) **37.5** µM (n=5) **125** µM (n=3) (×60 min×1.5µl/min)	
	DA	i.p.	**15** µmol/kg - Single dose (n=3) **50** µmol/kg - Single dose (n=6)	**15** µmol/kg - Single dose (n=4) **50** µmol/kg - Single dose (n=7)	

[1]to convert into mg/kg multiply by 0.36; [2]to convert into mg/kg multiply by 0.34.

Abbreviations: MWM, Morris water maze; i.p., intraperitoneal; NA, not applicable; NT, neurotensin; SP, substance P; b.i.d., bis in die (twice daily); ACh, acetylcholine; DA, dopamine, PK, pharmacokinetics; BSA, bovine serum albumin.

4.3 PHARMACOKINETIC STUDIES

4.3.1 *In vitro* permeability assay (II)

A primary bovine brain microvessel endothelial cell (BBMEC) model was used to assess the *in vitro* brain permeability of the two studied PREP inhibitors. BBMECs were isolated based on the method described earlier (Audus and Borchardt, 1987; Audus et al., 1996). The cells were cultured and the permeability assays were conducted as described earlier (Hakkarainen et al., 2010). Drugs were dissolved in Ringer's solution, and the initial drug concentration in the donor chamber was 20 μM. JTP-4819 and KYP-2047 sample concentrations were measured using an LC-MS method (see chapter 4.5.2). The radiotracer samples (low permeability reference compound [^{14}C]sucrose and high permeability reference compound [^{14}C]diazepam) were analyzed by liquid scintillation counting (1450 MicroBeta Trilux, Wallac, Finland) after the addition of 500 μl scintillation cocktail Optiphase, Wallac (Milton Keynes, UK).

4.3.2 Pharmacokinetic microdialysis in anaesthetized rats (II)

The microdialysis guide cannulas (MAB 6.10.IC, AgnTho's AB, Lidingö, Sweden) were implanted stereotaxically into the frontal cortex (coordinates from bregma: AP 3.2 mm; L +0.8 mm; DV -2.5 mm), hippocampus (AP -6.0 mm; L -5.5 mm; DV -3.8 mm) and striatum (AP +0.5 mm; L -3.0 mm; DV -3.8 mm) (Paxinos and Watson 2007) under anaesthesia. After a one week recovery period, the rats were again anaesthetized and microdialysis probes (MAB 9.10.3/4; 3 mm exposed membrane in the cortex and hippocampus, 4 mm exposed membrane in the striatum, 6 kDa cut-off, AgnTho's AB) were inserted into each brain area. An intravenous microdialysis probe (MAB 11.20.10; 10 mm exposed membrane, 6 kDa cut-off, AgnTho's AB) was inserted into the left femoral vein, and all probes were perfused with Krebs Ringer solution (consisting of 138 mM NaCl, 1 mM $CaCl_2$, 5 mM KCl, 1 mM $MgCl_2 \cdot 6H_2O$, 11 mM $NaHCO_3$, 1 mM $NaH_2PO_4 \cdot H_2O$, and 11 mM D-Glucose, pH 7.4) (Benveniste and Hüttemeier, 1990) at a flow rate of 2 μl/min. The probes were washed out for 80 min before the drug administration, and the dialysate was collected in twenty-minute fractions for 6 hours into polypropylene vials (AgnTho's AB). The samples were stored at -80 °C until analyzed. After the termination of each microdialysis experiment, the correct probe placement in the brain was assured (Figure 5).

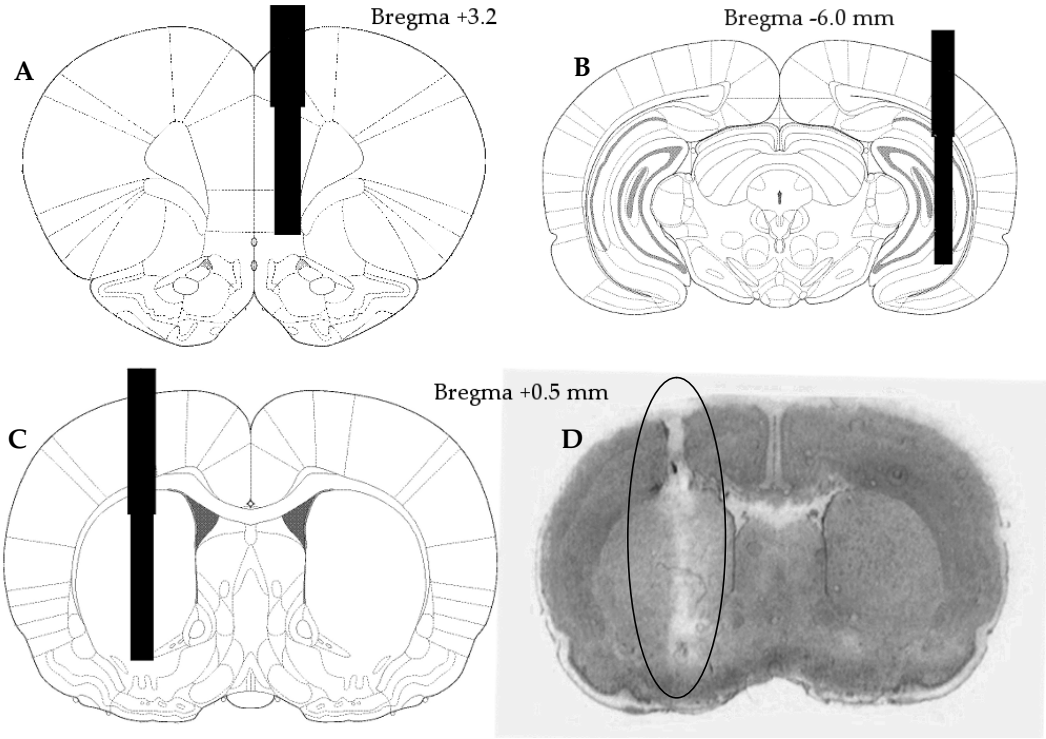

Figure 5. A schematic illustration of the microdialysis guide cannula and probe placement sites as measured relative to the bregma (**II, IV, V)**. The placement sites in the frontal cortex, hippocampus and striatum are shown in A, B, and C, respectively. The stained section demonstrating the tissue damage caused by the probe implantation (circled) in striatum is shown in D. After the termination of each microdialysis experiment, the rats were decapitated and the brains were rapidly removed and frozen in cooled isopentane. The brains from randomly selected rats were cut into 30 µm slices with a cryostat (Bright Instruments, Huntingdon, England), and the sections were stained with 0.5% cresyl violet as described by Paxinos and Watson (2007). The sizes of the guides and probes are in a true relation to the brain size. Modified from Paxinos and Watson (2007) with permission from Elsevier Ltd.

4.3.3 Determination of plasma and brain tissue levels of JTP-4819 and KYP-2047 (II)

The rats were anaesthetized and treated with either KYP-2047 or JTP-4819. At pre-determined time points (10, 30, 60, 120, 180 and 300 min), the brains were made rather bloodless by a 3-min transcardial perfusion with 0.1 M phosphate buffered saline. Samples from out flowing blood were collected into K_3EDTA tubes (Vacuette, Greiner Bio-one, Frickenhausen, Germany) and the plasma was separated by centrifugation (1500 × *g*, 10 min, +4 °C). Finally, the rats were decapitated and the brains were cut sagittally into two halves; one half being used for the determination of PREP inhibitor concentrations, and the other used for the PREP activity assay (see chapter 4.5.1).

The brain tissue samples were homogenized on ice in 1:4 w/v of ultrapure water containing a structural analogue of JTP-4819 and KYP-2047, PREP inhibitor KYP-2153 {(*R*)-N-benzyl-5-((*S*)-2-cyanopyrrolidine-1-carbonyl)cyclopent-1-enecarboxamide}, as an internal standard. Then, acetonitrile (1:11 w/v) was added to precipitate proteins and the mixture was centrifuged (16000 × *g*, 10 min, +4 °C). The PREP inhibitor concentration in the supernatant was analyzed by an LC-MS method (see chapter 4.5.2).

JTP-4819 and KYP-2047 were extracted from the plasma samples using protein precipitation. An aliquot (100 µl) of each plasma sample was mixed with 10 µl of perchloric acid (30 %) and 50 µl of internal standard solution (KYP-2153). Then, 200 µl of ice-cold acetonitrile was added and carefully mixed. After centrifugation (11000 × *g*, 10 min, +10 °C), the PREP inhibitor concentration in the supernatant was analyzed by an LC-MS method (see chapter 4.5.2).

4.4 PHARMACODYNAMIC STUDIES

4.4.1 Morris water maze (III)

The effect of PREP inhibition on long-term spatial memory was tested in the Morris water maze (Stewart and Morris, 1993) with the modification described by Pitkänen et al. (1995). A circular fiberglass tank, 160 cm in diameter, was filled with tap water (20 ± 2 °C). The platform (10 cm in diameter) was placed in a permanent location in one quadrant of the pool, and the water surface was set at 1.5 cm above the platform. Thirty min after the administration of a PREP inhibitor (or vehicle) and scopolamine, the rat was placed into the water, and the swimming path was recorded with a video camera. The trial lasted until the rat found the platform or 70 s had elapsed. The rat was placed onto the platform for 10 s, unless it was not already on it. The animal was transferred to a cage for 30 s to recover. The trial was repeated 3 times in sequence. In each trial, the starting point varied. However, for each rat, the procedure was the same during each day. One test series consisted of 3 trials/animal/day over 5 days. The path length, the swimming time and the speed were calculated.

4.4.2 Microdialysis of endogenous compounds in conscious rats (IV, V)

The rats were anaesthetized and an intracerebral guide cannula (CMA12, CMA Microdialysis, Solna, Sweden) (**IV**) or MAB 6.10.IC, AgnTho's AB) (**V**) was implanted stereotaxically into the striatum (AP +0.5 mm; L -3.0 mm; DV -3.8 mm from bregma) (Paxinos and Watson 2007). The animals were allowed to recover from the surgery for 5-6 days.

One h (**IV**) or 12 h (**V**) before the baseline collection, the animals were moved to the microdialysis bowls (CMA 120, CMA Microdialysis), and the microdialysis probes CMA12 Elite (4 mm exposed membrane, 20 kDa cut-off) (**IV**) or MAB 9.10.4 (4 mm exposed membrane, 6 kDa cut-off, AgnTho's AB) (**V**) were inserted into the striatum. The probes were perfused with calcium enriched Ringer's solution (138 mM NaCl, 1.3 mM $CaCl_2$, 5 mM KCl, 1 mM $MgCl_2 \cdot 6H_2O$, 11 mM $NaHCO_3$, 1 mM $NaH_2PO_4 \cdot H_2O$, and 11 mM *D*-glucose, pH 7.4) (Benveniste and Hüttemeier, 1990) at a flow rate of 5 (**IV**) or 1.5 (**V**) µl/min. 0.2% (m/v) bovine serum albumin and 0.03% (v/v) bacitracin were added to the perfusate (**IV**).

In the ACh study, the perfusate was changed to Ringer's solution containing 100 nM of neostigmine after the 12 h wash out period and nine 20 min baseline samples were collected (**V**). In the DA study (**V**), four 20 min baseline samples, and in the neuropeptide studies (**IV**), three 20 min baseline samples, were collected.

After a stable baseline had been attained, the appropriate PREP inhibitor was administered either intraperitoneally (**IV, V**) or by retrodialysis (**V**) *via* the microdialysis probe (see Table 4 for dosing details). The dialysate was collected for 4 (**IV**) or 5 h (**V**) as 20-min fractions.

In the DA study (**V**), 5 µl of antioxidant solution (containing 1mM of oxalic acid, 0.1 M of acetic acid and 3.0 mM of cysteine in water) were added to the sample tubes to prevent DA oxidation.

All dialysates were immediately frozen (-20 °C) and stored at -80 °C until analyzed. After each PD microdialysis experiment, the correct probe placement in the brain was assured (Figure 5).

4.4.3 Determination of the total tissue levels of NT, SP and IP_3 (III)

At appropriate time points after the drug administration, the rats were sacrificed by decapitation and the brains were rapidly removed and dissected (frontal cortex, hippocampus and hypothalamus) and frozen in liquid nitrogen. Each brain region was homogenized in +90 °C 1 M acetic acid (1:40 w/v) and incubated in +90 °C for 5 min. After cooling, the homogenates were centrifuged at 12000 × *g* and +4 °C for 20 min. Frozen supernatants were freeze-dried and the remaining sediments were dissolved in the assay buffers delivered with the ELISA kits (see chapter 4.5.3).

For the IP_3 assay, rats were decapitated 1 h after the last dose of JTP-4819, and the hippocampus and cortex were dissected and frozen until analyzed. Then, the samples were homogenized in ice cold perchloric acid (20%) (1:20 w/v) and centrifuged (2000 × *g*, +4 °C for 15 min). The supernatants were titrated to pH 7.5 (±0.1) with 1.5 KOH (in 60 mM HEPES), and again centrifuged (2000 × *g*, +4 °C for 5 min) to remove precipitated $KClO_4$. The IP_3 contents of the supernatants were assayed as described in chapter 4.5.4.

4.4.4 *In vitro* specificity screening (V)

The *in vitro* binding of JTP-4819 and KYP-2047 to 70 pharmacological targets was assayed commercially (GenSEPII profile, Caliper Life Sciences, Hanover, USA). The binding assays were carried out in duplicate at a concentration of 10 μM.

The inhibitory effects of JTP-4819 and KYP-2047 against several serine proteases and proline-specific proteases were assessed with colorimetric or fluorometric standard methods (see **V;** Table 2).

4.5 ANALYTICAL PROCEDURES

4.5.1 PREP activity assay (II)

The brain PREP activity in the perfused brain samples was measured as described earlier using Suc-Gly-Pro-AMC as the PREP substrate (Venäläinen et al., 2006). The formation of AMC was measured using a Victor2 fluorescence plate reader (PerkinElmer Inc., Waltham, USA) at the excitation and emission wavelengths of 360 and 460 nm. The protein concentrations were determined with a Bio-Rad protein assay kit. The PREP activities were calculated as pmol AMC × min^{-1} × mg^{-1} protein and expressed as a percent of the activity in vehicle treated control animals.

4.5.2 Assay of JTP-4819 and KYP-2047 levels (II)

KYP-2047 and JTP-4819 levels in the *in vitro* permeability samples, brain and blood microdialysates, and tissue samples were quantified by a liquid chromatography-mass spectrometry (LC-MS) method. The LC-MS system consisted of an Agilent 1200 Series Rapid Resolution LC System (Agilent Technologies, Waldbronn, Germany) coupled with an atmospheric pressure chemical ionization (Finnigan Ion Max APCI) linear ion trap mass spectrometer (Finnigan LTQ, Thermo Electron, San Jose, USA). The sample was injected onto an HPLC column (Zorbax SB-Phenyl Narrow Bore RR column 2.1 × 100 mm, 3.5 μm) (Agilent Technologies, Palo Alto, USA) using a gradient elution at a flow rate of 0.2 ml/min. The mobile phase consisted of formic acid (0.1 %, v/v) in water (A) and formic acid (0.1 %, v/v) in methanol (90 %) (B). Selected reaction monitoring (SRM) was used and the following transitions were monitored: *m*/*z* 340 → 244 for KYP-2047, *m*/*z* 360 → 231 for JTP-4819, and

m/z 324 → 228 for the internal standard KYP-2153. The divert valve was programmed to allow flow into the mass spectrometer from 1.5 to 8 min of each run.

4.5.3 Neuropeptide immunoassays (III, IV)

The whole tissue concentrations of NT and SP in the frontal cortex, hippocampus and hypothalamus were assayed with commercial ELISA kits, distributed by R&D Systems Inc. (Minneapolis, USA) and Phoenix Pharmaceuticals (Belmont, USA), respectively (**III**).

In order to measure NT and SP levels in the brain microdialysates, highly sensitive RIAs were set up (**IV**). In each assay, ten-point standard curves were constructed for either NT or SP by using known amounts of synthetic peptide (all RIA reagents from Phoenix Pharmaceuticals, unless otherwise stated). Samples were incubated with NT- or SP-antiserum, ^{125}I labeled NT or SP and RIA buffer for 72 h at 4 °C. Antibody-bound radioligand was separated from unbound radioligand by addition of goat anti-rabbit IgG serum and normal rabbit serum. After 2 h incubation in room temperature and centrifugation (2000 × *g* for 20 min at room temperature), the supernatant was carefully aspirated. Scintillation fluid (Optiphase HiSafe2, Perkin Elmer Inc.) was added and the bound radioactivity was counted in a liquid scintillation counter (1450 MicroBeta Trilux, Wallac). Samples either without standards or without antibodies were incubated simultaneously to measure maximal tracer binding and non-specific binding, respectively.

The acceptability of each assay run was controlled by using quality control (QC) samples at low, medium and high analyte concentration levels. The assay was accepted when 2/3 of the QC samples were within 15% of their respective nominal values. QC samples were prepared and analyzed on the basis of the FDA Bioanalytical Guidance for Industry (2001). In addition, a precision profile was determined for both analytes in order to assess the optimal concentration working ranges for the immunoassays (Figure 6) (Lee et al., 2006). In the working range of the assay the intra-assay coefficient of variation was less than 10%. The detection limits for both RIAs were 1.2 fmol per 100 μl sample.

According to the manufacturer, the NT antibody displays no cross-reactivity with SP, kinetensin or bombesin. Similarly, the SP antibody is claimed to exhibit no cross-reactivity with neurokinin B, somatostatin and physalaemin, but 100% cross-reactivity with SP fractions 2-11, 3-11, 4-11, and 5-11, a 5% cross-reactivity with fraction 6-11, and minor cross-reactivity (<0.5%) with fraction 7-11, neurokinin A and endothelin-1. The NT and SP antibodies exhibited no appreciable cross-reactivities to any of the components of the Ringer's solution used in the study.

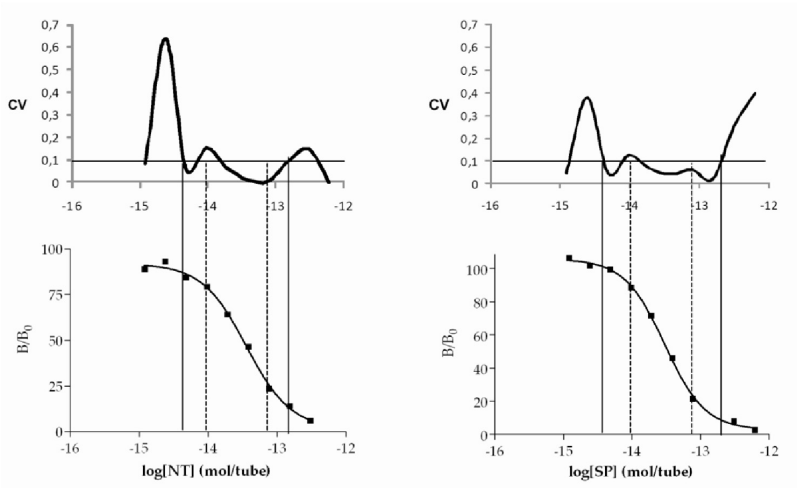

Figure 6. The precision profiles and sample calibration curves of NT (left column) and SP (right column) radioimmunoassays. The precision profile represents the plot of the coefficient of the variation (CV) of the back calculated calibrator concentration vs. the nominal calibrator concentration. The optimal working range of an assay is between the solid lines where the intra-assay CV<10%. The linear ranges of the standard curves are marked with dotted lines. The precision profile shows whether an assay is capable of measuring the analyte of interest in a predetermined concentration range. It also illustrates that the linear portion of the calibration curve does not always define the optimal working range of an assay.

4.5.4 IP_3 receptor binding assay (III)

Concentrations of IP_3 in hippocampus and cortex were assayed with a commercial *D-myo*-inositol-1,4,5-trisphosphate [^{3}H] radioligand binding assay kit (TRK 1000, Amersham Biosciences, Buckinghamshire, UK).

4.5.5 Assay of ACh and DA levels (V)

ACh levels in the microdialysates were measured with a highly sensitive liquid chromatography/tandem mass spectrometry (LC/MS/MS) method described by Keski-Rahkonen et al. (2007). DA, HVA and DOPAC levels in microdialysates were measured with high performance liquid chromatography coupled with electrochemical detection (HPLC-ECD) as described by Piepponen et al. (2002).

4.6 DATA ANALYSIS AND STATISTICS (II-V)

4.6.1 Microdialysis experiments (II, IV, V)

The concentrations of JTP-4819 and KYP-2047 in blood and brain microdialysates were corrected with *in vivo* recovery values using the retrodialysis by drug approach (**II**) (Bouw and Hammarlund-Udenaes, 1998). The probes (n=4/probe type/drug) were perfused with Ringer's solution containing JTP-4819 or KYP-2047 (C_{in}= 1 μM). Eighty min after the probe insertion the retrodialysis was initiated, and after a 20 min stabilization period, five consecutive samples in fractions of 20 min were collected. The *in vivo* recovery was calculated from the last three samples with the Equation 1

$$R = \frac{C_{in} - C_{out}}{C_{in}} \qquad \text{Eq. 1}$$

where R is the *in vivo* recovery, C_{in} is the concentration of JTP-4819 or KYP-2047 in the perfusate and C_{out} is the concentration in the dialysate. The unbound drug concentrations in the brain extracellular fluid (ECF) and blood were then calculated with the Equation 2

$$C = \frac{C_{out}}{R} \qquad \text{Eq. 2}$$

where C is the *in vivo* recovery corrected concentration in the tissue, C_{out} is the measured concentration in the dialysate and R is the *in vivo* recovery value for the corresponding probe type and tissue. The values of the area under the time-concentration curve ($AUC_{0\text{-}300min}$; min × μM) were calculated from the individual data with the trapezoid rule using GraphPad Prism 4.03 software (GraphPad Software, San Diego, CA, USA). The pharmacokinetic parameters (C_{max}, t_{max}, $t_{½\beta}$) were calculated from the individual time-concentration vs. time data sets based on a one-compartment model using the WinNonlin Professional v5.0.1. software (Pharsight Corporation, Mountain View, CA, USA) (**II**).

In the ACh, DA, NT and SP microdialysis studies, the mean of the last three (**IV**) or four (**V**) baseline samples was calculated for each animal as a baseline value (=100%) and the changes in the extracellular transmitter levels after PREP inhibitor or vehicle treatment were expressed as a percentage of this value. The transmitter levels were not corrected for recovery.

The AUC (min × % of baseline) for each animal was calculated with the trapezoid rule from the time of the drug administration to the end of the dialysis (0-240 min) using GraphPad Prism 4.03 software (GraphPad Software) (**IV**). Peaks above the baseline (100%) were regarded as positive areas, and peaks below the baseline were regarded as negative areas, and the reported AUC values were calculated as the net area between positive and negative peaks.

4.6.2 Pharmacokinetic-pharmacodynamic relationship

The relationship between the total blood concentration or total brain tissue concentration (C) of JTP-4819 or KYP-2047 and brain PREP activity (E) was determined after a single intraperitoneal dose of 50 μmol/kg i.p. with the Equation 3

$$E = E_0 - \frac{E_{max} \times C}{EC_{50} + C} \qquad \text{Eq. 3}$$

(Holford and Sheiner, 1981).The brain PREP activities (pmol × min^{-1} × mg^{-1}) were plotted against the corresponding blood or brain concentration values (nM). The baseline PREP activity (E_0), maximum attainable PREP inhibition (E_{max}) and the concentration producing 50% of the maximum attainable PREP inhibition (EC_{50}) were fitted as free parameters using GraphPad Prism 5.03 software.

4.6.3 Statistical analyses (II-V)

A two-tailed Student's t-test was used to test the statistical significance of differences between two groups in the PK studies (apparent permeability [P_{app}]values in the BBMEC model and the $AUC_{0\text{-}300min}$, C_{max}, t_{max} and $t_{½\beta}$ values in microdialysates) (**II**), and in the NT and SP microdialysis studies to compare the effect of KYP-2047 vs. control on $AUC_{0\text{-}240min}$ values (**IV**). Tests were performed using GraphPad Prism 4.03 software.

The water maze data were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni *post hoc* multiple group comparison test (**III**).

A mixed model was constructed to assess the difference between groups in ACh and DA levels at different time points (time, group and their interaction as fixed effects and rat number as random effect) using SPSS for Windows 14.0.1 software (SPSS Inc., Chicago, USA) (**V**).

The differences with p-values <0.05 were considered as statistically significant.

5 Results

5.1 PHARMACOKINETICS OF JTP-4819 AND KYP-2047 (II)

5.1.1 *In vitro* permeability

Both JTP-4819 and KYP-2047 permeated through the cell monolayer in the BBMEC model, but the P_{app} of KYP-2047 was 8.5-fold higher than the corresponding value of JTP-4819 (Table 5) ($p<0.0001$).

The P_{app} value for the low permeability reference compound sucrose was $30.9 \pm 4.4 \times 10^{-6}$ cm/s (n=7). For the high permeability reference diazepam it was $391.0 \pm 24.3 \times 10^{-6}$ cm/s (n=7), both of which are in agreement with previously reported values (Eddy et al., 1997; Hakkarainen et al., 2010).

5.1.2 *In vivo* pharmacokinetics

The PK parameters of JTP-4819 and KYP-2047 after a single dose of 50 µmol/kg i.p. are summarized in Table 5. The microdialysis data revealed that both JTP-4819 and KYP-2047 penetrated the brain ECF (Figure 7). In the blood, the apparent unbound $AUC_{0\text{-}300min}$ values were almost 7-fold higher for JTP-4819 ($p<0.001$). In the brain ECF, the unbound $AUC_{0\text{-}300min}$ values of JTP-4819 were 4-6-fold higher ($p<0.05$) than those of KYP-2047 depending on the brain area. However, the unbound brain/blood ratio (*i.e.* the fraction penetrated into the brain) was maximally 56% higher for KYP-2047 (in the hippocampus), indicating better BBB penetration of KYP-2047 after single-dose administration.

KYP-2047 showed similar blood-to-brain distribution patterns in the cortex, hippocampus and striatum as measured by the unbound brain/blood $AUC_{0\text{-}300min}$ ratios (Table 5). In contrast, there was a trend (ns) towards differences in the drug concentrations between the brain areas in JTP-4819 treated animals; highest concentrations were measured from the cortex (Figure 7).

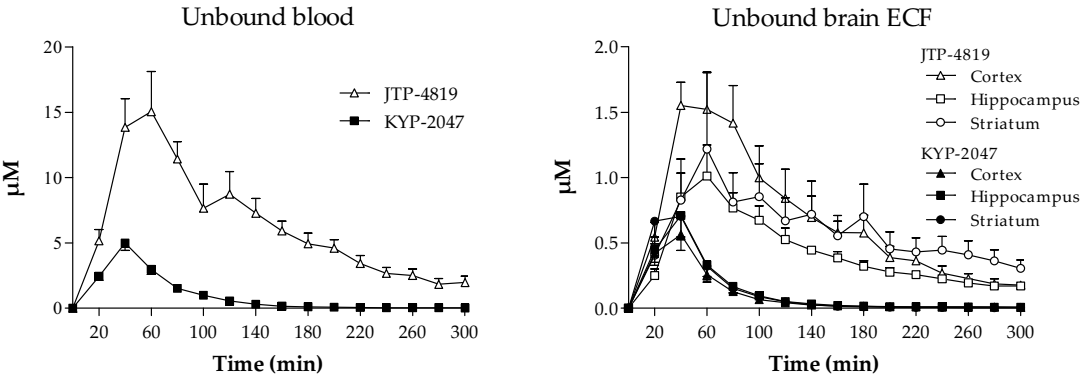

Figure 7. The unbound concentrations of JTP-4819 and KYP-2047 in the rat blood, cortex, striatum and hippocampus after a single intraperitoneal dose of 50 µmol/kg (n=5-7/tissue/compound).

The unbound concentration of KYP-2047 in the brain ECF correlated with its unbound blood concentration (correlation coefficient 0.89, $p<0.0001$ in each brain area), evidence of a homogenous blood-to-brain distribution pattern to all brain areas regardless of the amount of free drug in the blood (Figure 4 in **II**). In contrast, the unbound concentrations of JTP-4819 in the brain ECF were dependent on the corresponding unbound blood concentration only in the cortex and hippocampus (correlation coefficients 0.83, 0.73, respectively, $p<0.0001$), but not in the striatum (correlation coefficient 0.17, $p=0.13$). This is evidence of non-homogeneity in the regional distribution of JTP-4819 after a single intraperitoneal dose.

Table 5. The PK parameters (mean ± S.E.M.) of JTP-4819 and KYP-2047 calculated from the microdialysis, total tissue and BBMEC data.

	JTP-4819			**KYP-2047**		
	$AUC_{0\text{-}300min}$[1] (min × μM)	*C_{max}[2] (μM)*	*Brain/ blood ratio[3]*	*$AUC_{0\text{-}300min}$[1] (min × μM)*	*C_{max}[2] (μM)*	*Brain/ blood ratio[3]*
Unbound extracellular concentration						
Blood[4]	1947 ±287	16.7 ± 3.1		285 ± 30	5.01 ± 0.57	
Cortex[4]	206 ± 41	1.78 ± 0.29	0.13 ± 0.02	32 ± 6	0.57 ± 0.12	0.13 ± 0.01
Hippocampus[4]	128 ± 17	1.05 ± 0.23	0.09 ± 0.01	38 ± 9	0.71 ± 0.19	0.14 ± 0.02
Striatum[4]	178 ± 53	1.26 ± 0.58	0.10 ± 0.04	42 ± 11	0.72 ± 0.16	0.13 ± 0.03
Total tissue concentration						
Plasma	4230	78.6 ± 14.4		1350	37.0 ± 11.0	
Brain	145	1.47 ± 0.47	0.034	60	3.01 ± 1.38	0.045
In vitro permeability	*P_{app} (× 10^6 cm/s)* [5]			*P_{app}(× 10^6 cm/s)* [5]		
BBMEC	25.4 ± 4.3			213.8 ± 9.0		

[1]AUC, area under time-concentration curve; [2]maximal concentration; [3]AUC_{brain}/AUC_{blood} (calculated from the individual time-concentration curves for the unbound concentrations and from the group data for the total tissue concentrations); [4]apparent values; [5]apparent permeability coefficient.

KYP-2047 reached the total maximal plasma concentration early (10 min), whereas the total C_{max} of JTP-4819 was reached 30 min after the treatment (Figure 8). The total plasma levels of JTP-4819 were substantially higher; the AUC of JTP-4819 was over 3-fold higher than that of KYP-2047 (Table 5). However, the total C_{max} in the perfused brain was 2-fold higher for KYP-2047 (Figure 8). KYP-2047 achieved the maximal brain concentration faster than JTP-4819 (brain t_{max} 10 min vs. 30 min for KYP-2047 and JTP-4819, respectively.

The brain/blood $AUC_{0\text{-}300min}$ ratios calculated from the total tissue concentrations were approximately 30% higher for KYP-2047, which is about the same as the unbound brain/blood $AUC_{0\text{-}300min}$ ratios calculated from the microdialysis data (Table 5).

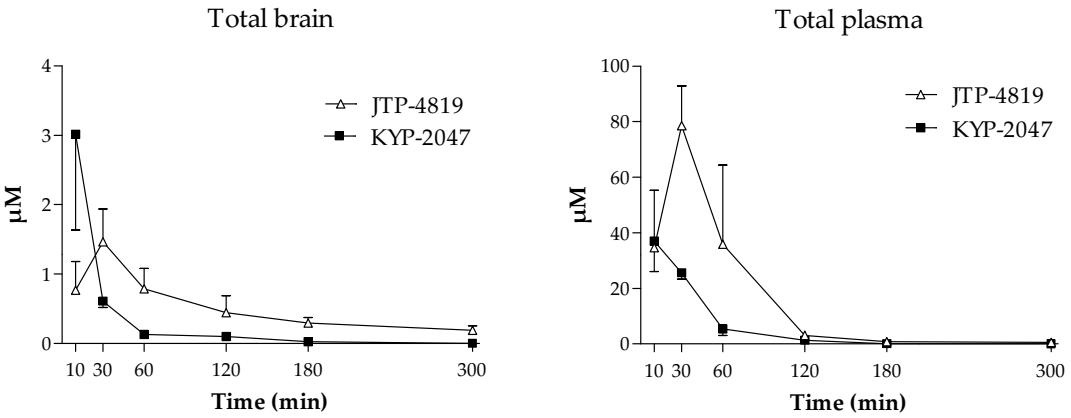

Figure 8. The total brain and plasma levels of JTP-4819 and KYP-2047 after a single dose of 50 µmol/kg i.p. (n=4/time point for both inhibitors).

5.2 PHARMACODYNAMICS OF JTP-4819 AND KYP-2047 (II-V)

5.2.1 Animal weight monitoring

During the 10-day treatment (**III**), the body weights of the animals were monitored daily (Figure 9) to control possible adverse effects of the compounds or vehicle. No signs of abnormal weight gain, behavior or toxicity were observed during the animal studies (**II**-**V**).

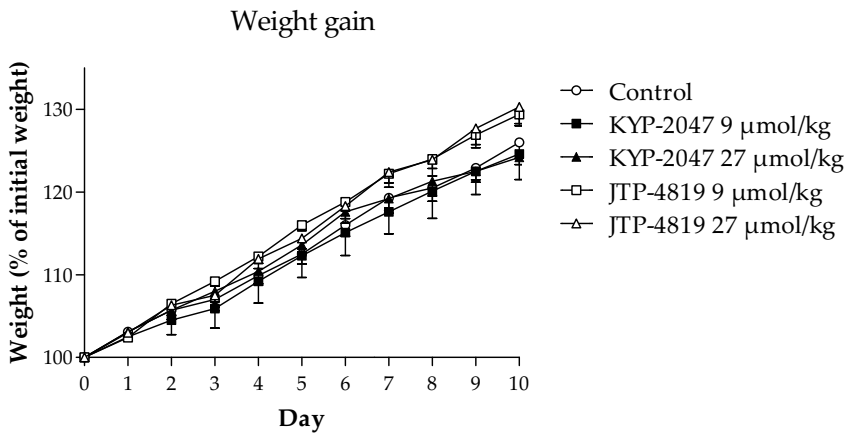

Figure 9. The body weight gain (% of initial weight) of the rats during the chronic 10-day b.i.d. administration of JTP-4819 or KYP-2047 (9 or 27 µmol/kg i.p., n=5/group) (III). Controls received saline containing 5% of Tween® 80.

5.2.2 Inhibition of brain PREP (II)

The basal PREP activity in perfused rat brain was 1223 ± 86 pmol × min^{-1} × mg^{-1} (n=8). KYP-2047 produced a higher degree of brain PREP inhibition than JTP-4819 (Figure 10). In the KYP-2047 group, the brain PREP activity was fully inhibited 10-60 min after drug administration. At 300 min, the PREP activity had recovered to a level of 35%.

JTP-4819 did not produce full inhibition of brain PREP activity at any time point. Moreover, the interindividual variation was much higher than in KYP-2047-treated animals. JTP-4819 appeared to have a longer duration of inhibitory action than KYP-2047, as the degree of inhibition remained unchanged between 180-300 min.

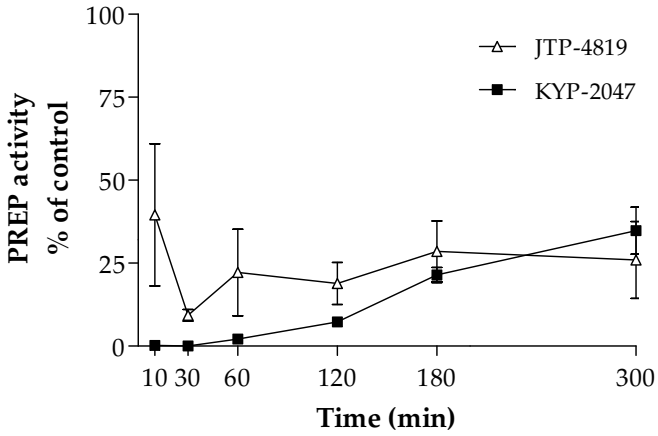

Figure 10. PREP activity as a function of time in the perfused rat brain tissue after a single dose (50 μmol/kg i.p.) of JTP-4819 or KYP-2047 (n=4/time point for both inhibitors).

5.2.3 Effect on spatial memory in rats (III)

In the Morris water maze, scopolamine was used to create a learning deficit in the rats. In old rats, scopolamine-treatment increased the escape path length significantly only on days 1 and 2 as compared to the vehicle-treated animals ($p<0.05$). In young rats, scopolamine impaired spatial learning significantly on days 1, 2, 3 and 4 as measured by increased path length (Figure 11).

In young rats, a low dose (3 μmol/kg i.p.) of KYP-2047 had no effect on the scopolamine-induced memory impairment on any testing day. However, a higher dose (15 μmol/kg i.p.) significantly shortened the swimming path length as compared to the scopolamine-treated animals on days 2 and 4 ($p<0.05$). Scopolamine-treatment significantly ($p<0.05$) increased the swimming time only on day 2 in comparison to the control animals. On the other testing days, scopolamine increased the swimming times by 10-15 sec/trial, and KYP-2047 (15 μmol/kg) restored this impaired performance to the level of the control animals.

Since the disrupting effects of scopolamine on the water maze performance in the old rats were seen only on days 1 and 2, the assessment of the anti-amnesic effects of KYP-2047 was limited only to those days. KYP-2047 (15 μmol/kg i.p.) could not alleviate the disrupting effect of scopolamine on the swimming path length on either day.

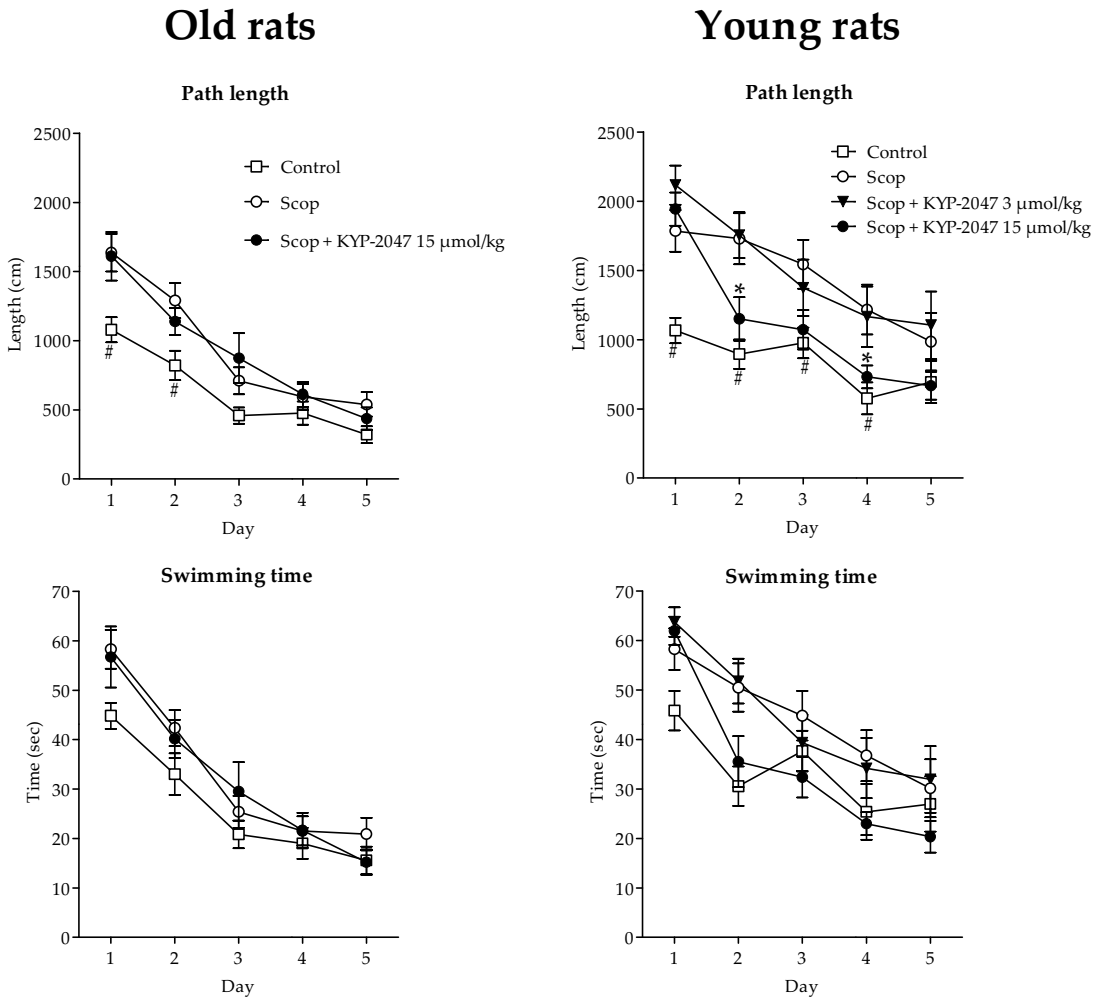

Figure 11. The effect of KYP-2047 on water maze performance in old (left column) and in young (right column) rats. The path length and swimming time represent the group mean (±S.E.M.) of three daily trials. The drugs were administered intraperitoneally 30 min before the first daily trial. Old rats: Control n=13; Scop n=13; Scop + KYP-2047 15 µmol/kg n=12. Young rats: Control n=14; Scop n=15; Scop + KYP-2047 3 µmol/kg n=14; Scop + KYP-2047 15 µmol/kg n=16.
** p<0.05 Scop-KYP-2047 15 µmol/kg vs. Scop; # p<0.05 Control vs. Scop.*

5.2.4 Effect on NT, SP and IP_3 levels in rat brain (III-IV)

The mean total concentration of NT in the hypothalamus of control animals was 41 ± 7 ng/g wet tissue (n=17) (**III**). JTP-4819 or KYP-2047 had no significant effect on the NT levels either after the single or with repeated administration (Figure 4 in **III**).

In the microdialysis study (**IV**), the mean baseline value for extracellular NT in the striatum was 14.4 ± 2.6 fmol/100 µl sample (n=10) (Figure 12). After the i.p. administration, there was a trend towards decreased NT levels in the control and KYP-2047 groups. A 40-50% reduction in NT levels was observed 40 min after the intraperitoneal injection of KYP-2047 (n=5) and vehicle (n=5). In both groups, the AUC net area was clearly negative.

The mean concentration of the total tissue SP in control animals varied between 7 ± 1 (hippocampus) and 133 ± 13 ng/g wet tissue (hypothalamus) (n=17). A single dose of KYP-2047 or JTP-4819 had no effect on the total tissue SP levels in any of the brain areas

investigated at either time point (1 and 3 h) (Figure 3 in **III**). Subhronic 10-day treatment did not differ from the situation seen after single dosing.

In the microdialysis study (**IV**), the mean baseline value for extracellular SP in the striatum was 1100 ± 80 fmol/100 µl sample (n=8) (Figure 12). The SP levels tended to increase slightly after intraperitoneal injection with either KYP-2047 (n=4) or vehicle (n=4). However, this increase was transient, and SP immunoreactivity reverted back to the baseline values in the next fractions. No significant differences in the net AUC values between the groups were found, *i.e.* KYP-2047 did not have any effect.

A subchronic 2.5-day JTP-4819 treatment (9 µmol/kg i.p. b.i.d, n=8) had no effect on the total tissue IP_3 levels in the cortex or hippocampus compared to the vehicle treated animals (Figure 5 in **III**).

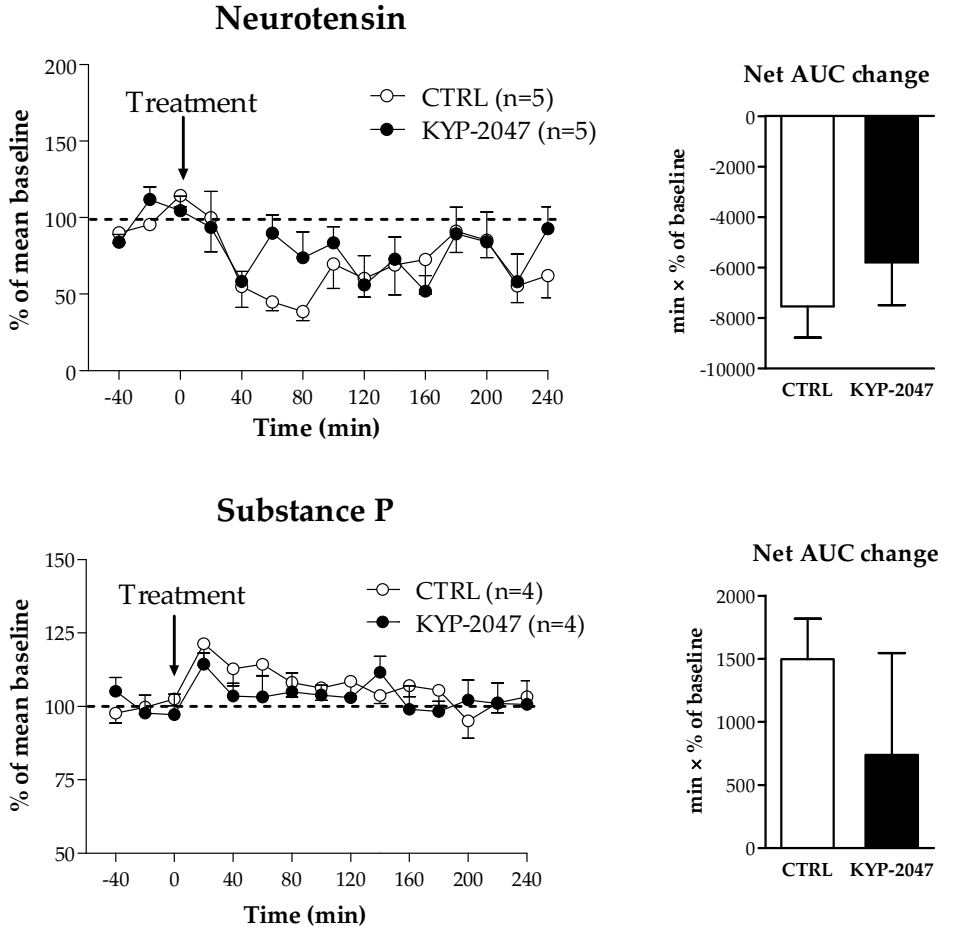

Figure 12. The extracellular neurotensin and substance P levels (% of mean baseline ± S.E.M.) in the rat striatum after KYP-2047 treatment (50 µmol/kg i.p.) as measured by the microdialysis method. The net AUC change (min × % of baseline ± S.E.M.) was calculated as the net area between the areas of positive (above baseline) and negative (below baseline) peaks 0-240 min after the treatment.

5.2.5 Effect on ACh and DA levels in rat brain (V)

The mean baseline extracellular ACh level in the striatum of the control animals was 12 ± 2 nM (n=8). KYP-2047 had no effect on the ACh levels after its systemic administration (Figure 13). When administered directly into the brain by retrodialysis, KYP-2047 decreased the ACh levels by 40-50% as compared to the control group during the 60 min drug infusion at the two higher concentrations (37.5 and 125 μM), but the difference reached statistical significance only in the 37.5 μM concentration group ($p<0.05$). In addition, the lowest drug concentration (12.5 μM) tended to decrease ACh levels (by ~35%), although more slowly. The ACh levels recovered slowly after the treatment; 240 min after the termination of the drug infusion, the ACh levels were still 30% below the baseline.

JTP-4819 decreased ACh levels maximally by 25% after the systemic administration ($p<0.05$ at 300 min, and $p=0.053$ at 280 min) (Figure 13). In the retrodialysis, all JTP-4819 concentrations decreased ACh levels by 30-50%. Due to the small group sizes, the difference in treated vs. control group was statistically significant only at 20 min (12.5 μM) and 100 min (125 μM). The maximum effect (40-50% decrease) was observed 80-100 min after the onset of the retrodialysis.

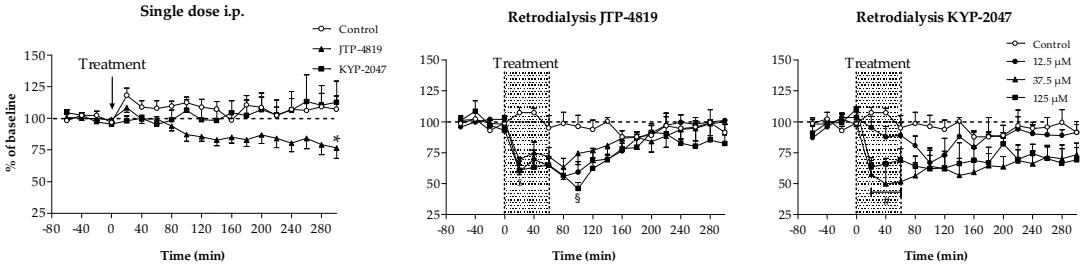

Figure 13. The effects of JTP-4819 and KYP-2047 on the extracellular ACh levels in rat striatum after a single intraperitoneal dose (50 μmol/kg i.p.; n=8-9) or retrodialysis administration for 60 min (gray area; n=3-5/dose). * *p<0.05 vs. Control; $ p<0.05 12.5 μM vs. Control; § p<0.05 125 μM vs. Control; # p<0.05 37.5 μM vs. Control.*

The mean baseline extracellular DA level in the striatum of control animals was 2.7 ± 0.3 nM (n=10). After the lower dose (15 μmol/kg i.p.; n=3-4), the DA levels tended (ns) to decrease in JTP-4819 treated rats but not in KYP-2047 treated animals, but with extensive interindividual variations (Figure 14). Maximally this decrease was almost 40% at 300 min after JTP-4819 administration. After the higher dose (50 μmol/kg i.p.; n=6-7), there was also a trend (ns) towards decreased DA levels, and it was observed in both treatment groups; the maximum decrease of approximately 30% was observed at 140 and 240 min after the administration of KYP-2047 and JTP-4819, respectively. However, DA levels were slightly decreased (by 10-15%) in control animals as well, indicating that the DA decrease in PREP inhibitor treated animals was not very significant. There were no differences in DOPAC and HVA levels between groups after the PREP inhibitor administration at either doses, and only the normal fluctuation associated with the circadian rhythm was observed (Castaneda et al., 2004) (Figure 14).

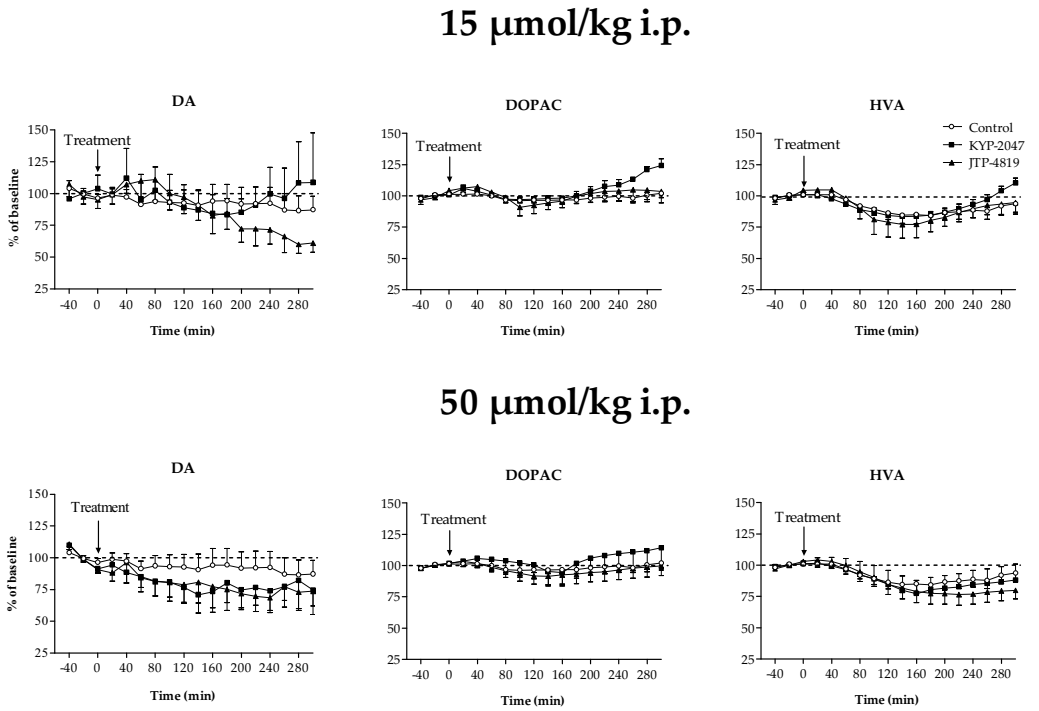

Figure 14. Extracellular DA, DOPAC and HVA levels in the rat striatum after a single intraperitoneal dose (15 or 50 μmol/kg i.p.) of JTP-4819 (triangle), KYP-2047 (square) or vehicle (circle).

5.2.6 Specificity of JTP-4819 and KYP-2047 *in vitro* (V)

The GenSEPII screening profile did not reveal any significant binding of KYP-2047 or JTP-4819 to the pharmacological targets tested (**V**; Table 1), *i.e.* according to the preset criteria of the GenSEPII profile (the compound should show inhibition of specific radioligand binding by >50%), no off-targets were found.

At 10 μM, JTP-4819 or KYP-2047 did not exert any significant inhibitory effect against several serine proteases and proline-specific proteases (**V**; Table 2). About 20 % inhibition of lysosomal prolyl carboxypeptidase activity was observed at 10 μM and 50% at 100 μM. Plasmin was inhibited by about 30% by the 100 μM concentration of KYP-2047.

5.3 PHARMACOKINETIC-PHARMACODYNAMIC RELATIONSHIP

The relationship between the total blood concentration of JTP-4819 or KYP-2047 and the brain PREP activity is presented in Figure 15. The data fitted the model well (R^2=0.89 and 0.93 for JTP-4819 and KYP-2047, respectively). The calculated E_0 estimates (1239 and 1219 pmol × min^{-1} × mg^{-1} for JTP-4819 and KYP-2047, respectively) were close to the actual value (1223 ± 86 pmol × min^{-1} × mg^{-1}) for both compounds. The EC_{50} value of KYP-2047 was much lower than that of JTP-4819 (27.3 nM vs. 461 nM, respectively).

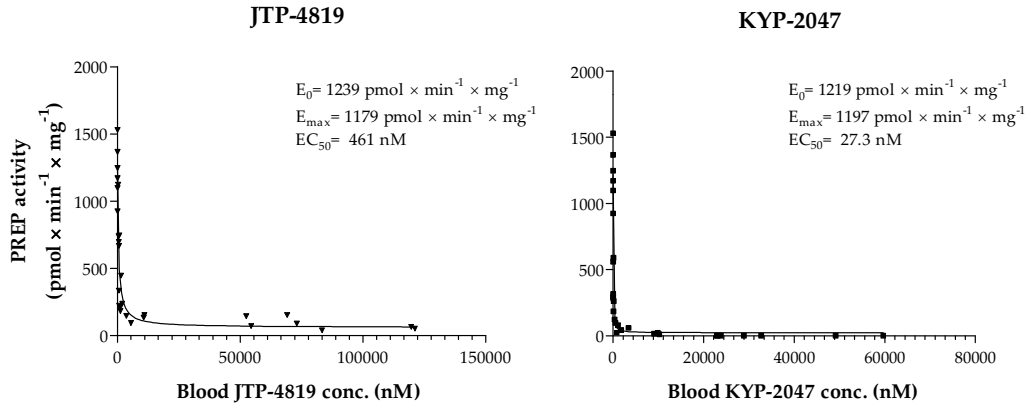

Figure 15. The relationship between the brain PREP activity and the total blood concentration of JTP-4819 or KYP-2047 after a single dose of 50 µmol/kg i.p. The inhibitory E_{max} model [$E= E_0-(E_{max}\times C)/(EC_{50} + C)$] was fitted to the data. Each observation represents a single animal.

The relationship between the total brain tissue concentration of JTP-4819 or KYP-2047 and the brain PREP activity is presented in Figure 16. The data fitted the model well (R^2=0.86 and 0.94 for JTP-4819 and KYP-2047, respectively). The calculated E_0 estimates (1233 and 1220 pmol × min^{-1} × mg^{-1} for JTP-4819 and KYP-2047, respectively) were close to the actual value (1223 ± 86 pmol × min^{-1} × mg^{-1}). The EC_{50} value of KYP-2047 was lower than that of JTP-4819 (1.55 nM vs. 70.4 nM, respectively).

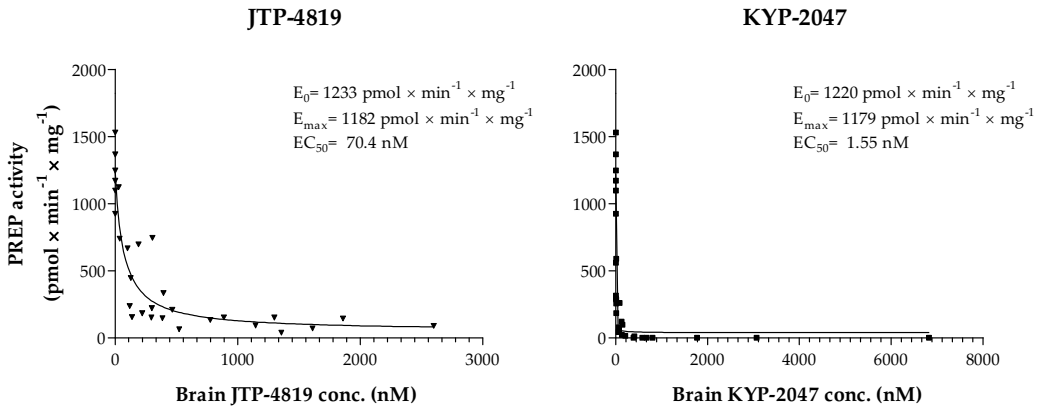

Figure 16. The relationship between the brain PREP activity and the total brain tissue concentration of JTP-4819 or KYP-2047 after a single dose of 50 µmol/kg i.p. The inhibitory E_{max} model [$E= E_0-(E_{max}\times C)/(EC_{50} + C)$] was fitted to the data. Each observation represents a single animal.

6 Discussion

6.1 PHARMACOKINETIC STUDIES

6.1.1 Assessment of drug brain penetration in CNS drug discovery

The brain is protected from xenobiotics by a specialized structural and functional obstacle called the blood-brain barrier (BBB) (Pardridge, 2005). The BBB also efficiently prevents the passage of many therapeutic agents into the brain. In fact, it has been estimated that 98% of all small drug molecules do not cross the BBB, and that the transport of small molecules across the BBB is an exception rather than a rule.

In the development of drugs for CNS diseases, it is obvious that one should select lead molecules that readily penetrate the BBB. However, it is not obvious which parameters best describe the brain penetration potential of a drug molecule. In general, the brain pharmacokinetics of a drug can be described by the extent and time (rate) required to reach brain equilibrium (Hammarlund-Udenaes et al., 2008; Liu et al., 2008). From a PD point of view, the rate of drug transport is a less crucial parameter than its extent, which better describes the target site concentration and thus, is the key feature determining the *in vivo* potency of a centrally acting drug. In the pharmaceutical industry, the extent of drug transport across the BBB has been traditionally described by the total brain-to-blood ratio (often referred to as K_P in the literature). However, the use of this parameter has been criticized, since it strongly depends upon the drug binding in plasma and especially the non-specific binding in brain. Thus, K_P often leads to overestimation of CNS drug exposure (Pardridge, 2003; Pardridge, 2004; Liu et al., 2008) and it is difficult to reliably assess the brain penetration on the basis of K_P alone (Doran et al., 2005).

On the basis of the classical free-drug hypothesis, it is the unbound fraction of a drug that exerts the pharmacological effects. Therefore, a better parameter than K_P to describe the extent of brain penetration is the unbound brain-to-blood ratio which can be measured with *in vivo* microdialysis (**II**) (Hammarlund-Udenaes et al., 2008). However, not even the unbound brain-to-blood ratio can estimate the target site drug concentrations if the drug target is located intracellularly, as is the case with PREP. Currently, there is no established method available to measure the unbound drug concentrations directly in the intracellular space, although an indirect experimental technique has been proposed (Friden et al., 2007).

In the present study, the brain penetration of PREP inhibitors was assessed by combining the total tissue (=K_P) and unbound extracellular measurements. By using this approach, it was possible to evaluate the distribution of the compounds between the extra/intracellular compartments in the brain, which is of importance because the target site is intracellular. Finally, the PK data was interpreted side-by-side with the primary PD response of the compounds (*i.e.* degree of brain PREP inhibition) in order to compare the true *in vivo* potency of the two model drugs.

6.1.2 Challenges in PREP inhibitor brain penetration

Since the targets for the potential therapeutic effects of PREP inhibitors are located in the CNS, the development of novel PREP inhibitors has focused on molecules that best inhibit brain PREP. The PREP enzyme concentration in the mammalian brain is high (in pig brain homogenate approximately 30 nM according to Venäläinen et al., 2002), and therefore high target-site inhibitor concentrations are also required to achieve full brain PREP inhibition. Therefore, a successful brain targeted PREP inhibitor should have the ability to cross the BBB with ease, a K_i value preferably in the nanomolar range and an appropriate duration of

action in the brain. The latter two of these properties are usually not a problem since most known potent PREP inhibitors, such as JTP-4819 and KYP-2047, are tight-binding inhibitors with slow dissociation rates from the active site of the enzyme (Venäläinen et al., 2002; Venäläinen et al., 2006). In contrast, adequate brain penetration may be difficult to achieve since most known PREP inhibitors are substrate-like short pseudopeptides that have a poor potential for crossing the BBB, mostly due to their low lipophilicity (Lawandi et al., 2010).

Earlier, the brain penetration of PREP inhibitors has been evaluated on the basis of the degree of PREP inhibition in animal brain extracts, but no reports on the actual drug concentrations in the brain or in the target site have been published. However, the fact that these molecules can inhibit brain PREP *ex vivo* does not prove that they were actually transported into the brain; inhibitors may have been trapped into the cerebral blood, and they may have reached the target enzyme only after brain homogenization. Alternatively they may have passed through the BBB to some extent (if the driving force of the unbound drug in blood is high), but are unable to pass through the neuronal cell membranes and accumulate within the brain ECF (Figure 17). Since the target protein PREP is located intracellularly, it is crucial to estimate the distribution of the inhibitors between blood, the ECF and brain parenchyma.

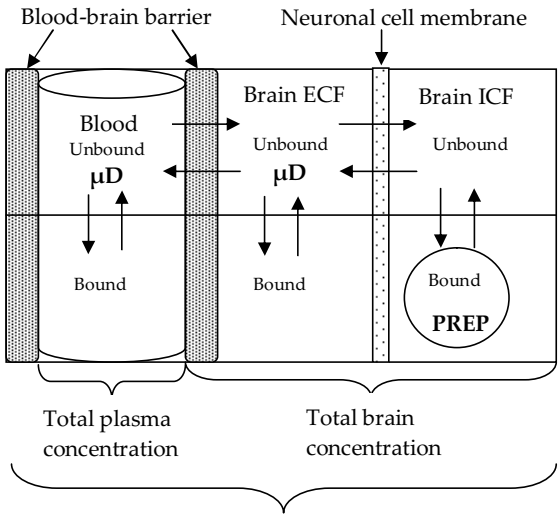

Figure 17. A schematic illustration of the blood-brain barrier (BBB) and the brain compartments. In order to reach and inhibit PREP in the intracellular fluid (ICF) of a neuron, an inhibitor has to pass through the BBB and the neuronal cell membrane. In the present study, microdialysis (μD) was used to monitor the unbound extracellular PREP inhibitor concentrations in the blood and in the brain ECF. The total tissue concentrations take into account both the unbound and bound inhibitor fractions in the blood and in the total brain tissue. If the residual cerebral blood is not removed by perfusion before brain sampling, the brain samples will contain the drug in plasma as well. This can lead to inaccurate estimations of the drug levels that have actually penetrated the brain.

6.1.3 Pharmacokinetics of PREP inhibitors

Considering the fact that PREP inhibitors have gained reasonable pharmaceutical interest during the last few decades and three of them (S-17092, Z-321 and JTP-4819) have entered clinical trials (Umemura et al., 1997; Umemura et al., 1999; Morain et al., 2002), it is

surprising that there are no previous published reports about their brain penetration characteristics. The available PK data is from dog, rat or human plasma.

S-17092 was reported to achieve peak plasma concentration rapidly after oral administration in rats and dogs (Morain et al., 2002), but the actual drug concentrations were not presented in the literature. Several peak concentrations were observed after high oral doses, indicating slow absorption or saturated active transport. The absolute oral bioavailability was 15% in rats and 4% in dogs, and it tended to increase with higher doses, an indication of saturated active efflux transport in the intestine, or alternatively, saturated first pass metabolism. Notably, no reports on the brain tissue levels of S-17092 have been published.

In humans, the pharmacokinetics of S-17092 was assessed after a single oral dose and after subchronic 7-day administration (100-1200 mg/day) (Morain et al., 2000). The drug was well-tolerated, but high interindividual variability in PK parameters was observed. S-17092 was claimed to be a centrally active substance as it induced statistically significant modifications in the electroencephalographs (EEG) of the S-17092 treated patients compared to those treated with placebo, but this cannot be taken as direct evidence of brain penetration of the compound. Moreover, the EEG modifications were not dose-dependent.

Z-321 was also reported to be well-tolerated in humans (Umemura et al., 1999). After a single dose of 30-120 mg, the plasma C_{max} values varied between 64 and 543 ng/ml (= 0.2-1.9 μM), respectively, and the elimination half life was 1.8 h. With repeated administration (60 mg b.i.d for 7 days), the C_{max} values were approximately 100 ng/ml (= 0.3 μM) after each dose. There are also no preclinical reports on the brain concentrations of Z-321.

Orally administered JTP-4819 (single dose of 30-120 mg or 60 mg three times daily for 7 days) was found to be well-tolerated in healthy male volunteers (Umemura et al., 1997). The plasma C_{max} values of JTP-4819 after 30 and 120 mg were 474-1649 ng/ml (= 1.3-4.6 μM), and the $AUC_{0-\infty}$ values 240-1000 min × μM, respectively. The elimination half life ($t_{½\beta}$) was about 2 h regardless of the dosage, which parallels the plasma elimination half life observed in the present study in rats (130 min). The assumption that JTP-4819 penetrates the brain has been based on the concept that the compound inhibits rat brain PREP *ex vivo* (Toide et al., 1995a).

In the present study, the BBB penetration characteristics of JTP-4819 and KYP-2047 were assessed using *in vitro*, *in vivo* and *ex vivo* methods. The *in vitro* data showed that the passive endothelial cell permeability properties of KYP-2047 were remarkably more favorable than those of JTP-4819. The measured P_{app} value of KYP-2047 was similar to that reported for several brain penetrating drugs such as ondansetron, midazolam and metoprolol (Hakkarainen et al., 2010). In contrast, the P_{app} value of JTP-4819 was even lower than that of the low-permeating reference compound, sucrose. This may be a reflection of the different physicochemical properties of the two compounds; KYP-2047 has a substantially higher logP (1.6 vs. 0.2), smaller polar surface area (64 vs. 90 $Å^2$), fewer hydrogen bond donors and acceptors (3 vs. 6) and a slightly lower molecular weight (339 vs. 359 g/mol) than JTP-4819, and all these factors are known to increase passive transport across the BBB (Pajouhesh and Lenz, 2005; Pardridge, 2005).

These poor cell membrane penetration properties of JTP-4819 were also witnessed in the *in vivo* and *ex vivo* studies. The unbound extracellular levels of JTP-4819 were found to be almost identical with the total tissue concentrations as estimated by the similar $AUC_{0-300min}$ values of JTP-4819 in the brain ECF and total brain tissue (Table 5). These results indicate that the unbound JTP-4819 may have been trapped within the brain extracellular compartment because of its poor membrane penetration. Thus, JTP-4819 seems to have a lower potential to reach the intracellular PREP than KYP-2047. This was supported by the lower degree of brain PREP inhibition obtained with JTP-4819 despite the 4-6-fold greater extracellular brain levels than those achieved with KYP-2047.

To compare the true *in vivo* potency of JTP-4819 and KYP-2047, the relationship between the total tissue drug concentrations and brain PREP activity was studied. The EC_{50} value of KYP-2047 (1.55 nM) was much lower than that of JTP-4819 (70.4 nM) (Figure 16). This difference in the *in vivo* potency cannot solely be explained by the slightly different *in vitro* potencies of the compounds, since these inhibitors have similar K_i values against PREP (0.023 nM and 0.055 nM for KYP-2047 and JTP-4819, respectively) (Toide et al., 1995a; Venäläinen et al., 2006). Therefore, the most likely explanation for the different EC_{50} values is the poor ability of JTP-4819 to reach its intracellular target site.

The unbound brain/blood ratio (*i.e.* the fraction penetrating into the brain) of KYP-2047 was at its greatest 56% higher than that of JTP-4819 (Table 5). This was considered to reflect the better BBB penetration of KYP-2047 after single dose administration. However, one could have predicted that the difference in the brain/blood ratio would have been even greater due to the poor cell membrane penetration properties of JTP-4819. The smaller than expected difference between the compounds may be explained by the high dose used in this study; the high dose led to a high unbound JTP-4819 concentration in the plasma, which created a massive gradient force driving the drug through the BBB. In general, however, it is not desirable to increase systemic drug exposure excessively to gain pharmacologically active brain concentrations. In that sense, KYP-2047 may possess better safety potential than JTP-4819.

The surprisingly small fraction of KYP-2047 penetrating the brain could also be explained by the function of the efflux proteins present in the BBB. Efflux transporters, such as the P-glycoprotein (P-gp), restrict the transport of their substrates from blood into the brain. This lowers the brain AUC values of a compound, and subsequently the brain/blood AUC ratio declines. At the moment, it is not known whether KYP-2047 is an efflux protein substrate. A preliminary calcein-AM study showed that KYP-2047 is not an inhibitor of the most common BBB efflux transporters, *e.g.* the P-gp and multidrug resistance protein 1 (MRP1) (Hakkarainen et al., unpublished data). However, a compound may be a substrate even if it does not inhibit the transporters. Thus, the role of efflux proteins in KYP-2047 brain transport should be studied more thoroughly by using for example bidirectional transport assays *in vitro*.

Because the peripheral microdialysis probe was inserted into the femoral vein, it was convenient to assess the PK characteristics of the model inhibitors in anaesthetized rats. Instead, the PD studies were conducted in conscious animals to avoid the disturbing effects of anaesthetics on transmitter release. Anaesthesia may impair *e.g.* the hemodynamics of the animals especially at the end of the experiment when the anaesthesia has been applied for several hours. This can subsequently alter drug distribution, excretion and metabolism. However, both inhibitors showed linear elimination throughout the experiments (Figures 7 and 8), indicating that the long-lasting anaesthesia had not been altered the elimination rate of the compounds.

The predicted clinical dosing of JTP-4819 has been reported to be 1 mg/kg (2.8 μmol/kg), and based on this value the clinical PK studies were conducted using a dose range of 30-120 mg (corresponding about 1.3-5 μmol/kg) (Umemura et al., 1997). As the current study is the first report on the brain levels of JTP-4819 in any species, this data offers the first chance to speculate on the brain levels and the degree of brain PREP inhibition obtained in the human studies. In the rat, the fraction of JTP-4819 absorbed into the brain after a single dose was 0.034 (calculated from the AUC ratios of total brain tissue and plasma levels). Should the fraction be the same in humans, the brain $AUC_{0-\infty}$ of JTP-4819 achieved after a single dose of 30-120 mg in humans would have been in the range 8-34 nM × min, respectively (reported $AUC_{0-\infty}$ in plasma 240-1000 nM × min). These levels are approximately 4-16 times lower than those calculated in the present PK study in the rat. Despite the much higher drug levels in the rat, it was not possible to achieve more than 85% PREP inhibition in the rat brain.

The above calculation raises the question to what degree the human brain PREP would be inhibited with the predicted clinical dose of 1 mg/kg. To evaluate this problem, it was decided to study the relationship between the plasma drug concentration and brain PREP activity in rats using an inhibitory E_{max} PK-PD model (Figure 15). The EC_{50} value of JTP-4819 in the rat was 461 nM. Thus, the peak plasma concentration of JTP-4819 after a dose of about 1 mg/kg (=60 mg single dose) in the humans was about five times (2500 nM) higher than the estimated EC_{50} value in the rat. This calculation suggests that pharmacologically active brain concentrations of JTP-4819 could have been achieved already at a dose of 1 mg/kg in humans, despite the limited brain penetration of this drug. This is also evidence of the high *in vivo* potency of current PREP inhibitors.

Additionally, there is a US Food and Drug Administration (FDA) guideline for calculating the human equivalent dose (HED) based on the body-surface area conversion factor (BSA-CF) between species (Guidance for Industry for estimating the maximum safe starting dose in initial clinical trials, 2005). From rat to human the BSA-CF is 6.2, *i.e.* the dose used in the preclinical rat study should be divided by 6.2 to obtain HED that produces the same degree of effect as that observed in the animals at a given dose. In the present study with JTP-4819, HED would have been 17/6.2 mg/kg = 2.7 mg/kg. This value is close to the highest dose used in the human studies (120 mg single dose/65 kg = 1.8 mg/kg), indicating that the dose range in the clinical study (Umemura et al., 1997) has been well justified. However, based on the above calculations, the highest degree of brain PREP inhibition by JTP-4819 in the human studies has most likely been lower than the 85% that was observed in the current preclinical study.

The translation of *in vivo* data from rats to humans is always fraught with difficulties because of the differences between the species. For example, there are marked differences in the structure of the BBB in rats and humans. These species-related characteristics can significantly affect the fraction that has been absorbed into the brain. In addition, nothing is known about the mechanism of transport of JTP-4819 across the BBB, *i.e.* it is not known whether it is transported passively, or is it a substrate of an active transport protein. If it is a transporter substrate, the translation from rat to human is further complicated, because the transporters have different characteristics across species (Syvänen et al., 2009).

There is also another limitation with the current PK-PD model. It is based on a single high-dose drug administration and this may overestimate the EC_{50} value due to missing low brain and plasma drug concentrations and the corresponding brain activity data. To confirm the EC_{50} values of JTP-4819 and KYP-2047, the study should be repeated with lower doses and longer follow-up times.

As a summary of the brain PK properties of JTP-4819 and KYP-2047, neither of the model compounds could readily penetrate the brain, as indicated by the unbound brain/blood AUC ratios between 0.09 and 0.14, and the total brain/blood ratios of 0.034 and 0.045, respectively. The unbound brain/blood ratios of JTP-4819 and KYP-2047 were in the same range as previously reported for low brain penetrating drugs such as atenolol and entacapone (Hakkarainen et al. 2010). The result is in line with the physicochemical properties of the PREP inhibitors that do not support extensive brain penetration. However, even though the fractions absorbed into the brain were relatively small, pharmacologically active brain concentrations were achieved with both compounds. KYP-2047 was able to better reach and inhibit the intracellular target enzyme, and the brain concentrations of KYP-2047 were similar in each brain area investigated. Thus, the PK properties of KYP-2047 can be considered as being more favorable than those of JTP-4819 and it may be better suited for use in the characterization of PREP as a CNS drug target.

6.2 PHARMACODYNAMIC STUDIES

6.2.1 Inhibition of brain PREP

The effect of JTP-4819 on brain PREP activity has been previously studied *ex vivo* in rats. JTP-4819 (3 mg/kg p.o. = 8.3 μmol/kg) produced 26-43% PREP inhibition in various brain areas at 1 h after administration and 35-50% inhibition at 3 h after administration (Toide et al., 1995a). Based on this data, practically all preclinical PD studies with JTP-4819 in rodents have been carried out using doses between 0.1-10 mg/kg (0.28-28 μmol/kg) p.o. (**I**).

At a dose of 9 μmol/kg i.p., KYP-2047 has been reported to inhibit rat brain PREP by 85-45 % for 6 h *ex vivo* (Venäläinen et al., 2006). However, it should be noted that both Toide and Venäläinen used unperfused brain tissue in their experiments (see discussion below).

The present results reveal that KYP-2047 produces a higher degree of brain PREP inhibition than JTP-4819 at an equimolar single dose. In KYP-2047 treated animals, the brain PREP activity was fully inhibited 10-60 min after administration, whereas JTP-4819 did not produce such complete inhibition of brain PREP activity at any time point.

The lower degree and greater variation of PREP inhibition achieved by JTP-4819 may be explained by the PK properties of this drug. The *in vitro* permeability data clearly demonstrated that the cell permeation properties of JTP-4819 are poor. In the *in vivo* studies, JTP-4819 was shown to have almost similar unbound extracellular brain/blood ratios than KYP-2047, but the comparison between the total brain tissue concentrations and the unbound extracellular concentrations suggested that JTP-4819 had been trapped unbound within the extracellular compartment, and could not easily pass the neuronal cell membranes to reach the intracellular PREP. In contrast, KYP-2047 was able to distribute from the ECF into the intracellular compartment, and it inhibited PREP more efficiently.

To the best of our knowledge, this is the first study where the degree of brain PREP inhibition has been measured from perfused brain tissue. With this approach, it is possible to avoid the problems associated with the residual blood remaining in the brain capillaries at the time of the decapitation (Figure 17). If one wishes to obtain reliable brain tissue drug levels, the residual blood should always be removed. The importance of the blood removal can be demonstrated by a simple calculation. The total arterial and venous blood volume in the brain of a 250 g rat has been estimated to be approximately 5% of its total 1.2 ml brain tissue volume (Davies and Morris, 1993; Duong and Kim, 2000; Liu et al., 2005). Thus, the physiological volume of intravascular space and blood in the rat brain can be calculated with the Equation 4

$$V_{(blood,\ brain)} = 0.05 \times 1.2\ ml = 0.06\ ml \qquad \text{Eq. 4}$$

The amount of drug (n) in the brain vasculature at the maximal brain concentration (C_{max}) can be calculated with the Equation 5

$$n = C_{max} \times V_{(blood,\ brain)} \qquad \text{Eq. 5}$$

For example, in the case of JTP-4819, n= 79 μmol/l × 0.00006 l = 4.74 nmol. At the same time, the total concentration of JTP-4819 in the bloodless brain tissue was measured as being 1.47 μM. If one assumes that JTP-4819 was distributed equally to the nonvascular brain volume (1.20 ml-0.06 ml= 1.14 ml), then the amount of drug that had passed through the BBB and distributed into the brain was 0.00114 l × 1.47 μmol/l = 1.68 nmol. This indicates that there would have been a 3-fold higher amount of JTP-4819 in the brain vasculature than in the brain tissue at the time of the sample collection.

Earlier *ex vivo* studies with PREP inhibitors have assayed the enzyme activity without removing the residual blood from the brain. On the basis of above calculation, it is clear that the drug in the brain vasculature could affect the enzyme activity measurement if it

were to be homogenized together with the brain. For example, JTP-4819 at 3 mg/kg produced not more than 50% brain PREP inhibition *ex vivo* (Toide et al., 1995a), although the cerebral blood contaminated the assay. This raises the question of to what degree the rat brain PREP has been inhibited in many PD studies with JTP-4819 at the most commonly used doses which have ranged from 0.1-3 mg/kg (Tables 1 and 3), when the degree of inhibition in bloodless brain at a dose of 50 μmol/kg (~18 mg/kg) was maximally 85% (Figure 10). Hence, it is difficult to estimate to what extent the previously reported effects of JTP-4819 in the PD studies were truly attributable to brain PREP inhibition. It is also possible that these effects are a result of an off-target effect, which may regulate other pathways than the originally targeted PREP.

6.2.2 Effect of KYP-2047 on spatial memory

Most PREP inhibitors were originally designed as antiamnesic drug candidates acting by elevating memory associated neuropeptides. Currently, there is a consensus that PREP inhibitors may have some positive effects on learning and memory (summarized in Table 3 and **I**).

Thus far, the only PREP inhibitor tested in the Morris water maze is JTP-4819. In this model, JTP-4819 has shown positive cognitive effects in aged rats and rats with middle cerebral artery occlusion or ibotenate lesion of nucleus basalis magnocellularis (**I**). These effects have been suggested to be mediated through reduced breakdown of proamnesic neuropeptides, such as SP and TRH by PREP, or through elevated levels of ACh. However, the cognitive effects were neither dose-dependent nor robust.

In the present study, the effect of KYP-2047 on scopolamine induced amnesia was tested in young and old rats in Morris water maze. In young rats, KYP-2047 showed some beneficial effects at a dose of 15 μmol/kg, but not at a dose of 3 μmol/kg. It is unlikely that the brain PREP was significantly inhibited at 3 μmol/kg. In old rats, the effect of KYP-2047 (15 μmol/kg) on scopolamine-amnesia was negligible. In contrast to the working hypothesis, the disruptive effects of scopolamine were weak in the old rats, thus complicating the interpretation of KYP-2047 -induced effects. Because of the poor scopolamine-response, the study should be repeated with a higher scopolamine dose. Also the dose of KYP-2047 could be higher to ensure a high degree of brain PREP inhibition during the trials.

The mechanism of action behind the positive cognitive effects in young rats remained unclear. The neuropeptide and IP_3 assays did not produce any evidence that the effects would have been mediated through elevated levels of SP, NT, or IP_3. Furthermore, our data on extracellular ACh levels showed that PREP inhibitors tend to decrease rather than increase striatal ACh levels (see chapter 6.2.5), indicating that the positive effects on cognition are not likely to be mediated through enhanced cholinergic system as hypothesized earlier (Toide et al., 1995a). However, we did not measure ACh levels in the hippocampus or cortex, which are generally considered to be the most important brain areas in terms of spatial learning and memory (see Deiana et al., 2011; Graef et al., 2011; Micheau and Marighetto, 2011). On the other hand, the striatal cholinergic system has an important interaction with the hippocampal cholinergic system *e.g.* in place and response learning (Chang and Gold, 2003; Pych et al., 2005a; Pych et al., 2005b). It is also unlikely that the DAergic system would be involved in the cognition-enhancing effects of PREP inhibitors (see chapter 6.2.6).

Taken together, this study confirmed the earlier findings that PREP inhibitors may have some positive effects on cognition. KYP-2047 may possess some potential as a cognitive enhancer, although it is disappointing that the positive behavioral effect was observed only in young rats. Further experimental investigations will be needed to clarify the mechanism of action of the cognitive effects.

6.2.3 Neuropeptide levels

The effect of PREP inhibition on the neuropeptide levels in rat brain was studied from tissue extracts (**III**) and microdialysates (**IV**). The difference between these experiments is that the microdialysate peptide levels reflect the situation in the extracellular space close to the target receptors of neuropeptides, whereas the total tissue extracts give an estimate of both the intracellular and extracellular peptide contents in the brain. The total tissue peptide samples are also subjected to *post-mortem* unspecific proteolysis, whereas the microdialysate is considered to be free of peptidase activity. For these reasons, the microdialysis peptide levels reflect the physiological situation more reliably than the total tissue levels.

The changes in the total tissue neuropeptide levels have been assessed with a number of PREP inhibitors, with perhaps JTP-4819 being the most widely studied (see **I**). Increased neuropeptide levels after JTP-4819 treatment have been described under various conditions, but the results are far from convincing. Also in the present study, JTP-4819 had no effect on either the total (**III**) or extracellular (**IV**) NT or SP levels in various brain areas. These findings are in agreement with many previous studies, where JTP-4819 has failed to increase peptide levels in the brain. In rats with middle cerebral artery occlusion, repeated administration of JTP-4819 (0.1-3 mg/kg, p.o. = 0.28-8.3 μmol/kg) did not affect the cortical or hippocampal SP levels (Shinoda et al., 1996). Furthermore, negative results were reported on the SP levels of the brains of aged rats after a single dose treatment with JTP-4819 (0.3-3 mg/kg) (Toide et al., 1995b). There are no earlier reports on the effects of JTP-4819 on brain NT levels.

In addition, the effects of JTP-4819 on the levels of other putative PREP substrates are conflicting, and the effects are seldom dose-dependent. A single dose of JTP-4819 (1-3 mg/kg p.o.) failed to increase the cortical levels of TRH (Toide et al., 1996). Furthermore, single or repeated administrations of JTP-4819 (1-3 mg/kg p.o.) had no effect on the AVP levels in the brains of aged rats (Toide et al., 1995b) or rats with middle cerebral artery occlusion (Shinoda et al., 1996).

One explanation for the above conflicting peptide results might be that the observed changes in the peptide levels are at least partly a result of unspecific proteolysis occurring during sample collection and preparation. Non-specific peptide degradation occurs rapidly after death leading to unphysiological changes in the peptide contents of the samples (Tenorio-Laranga et al., 2009). Furthermore, the interaction between the enzyme, inhibitor and the substrate may occur only in the brain homogenate *ex vivo*, but not in the true *in vivo* situation where the cellular compartments are intact, and the peptides released into the synaptic cleft are not accessible to the intracellularly located PREP.

Interestingly, when elevated peptide levels have been reported after JTP-4819 administration, the effects have generally been better in the cortex than in the hippocampus (Table 1). This might be partly explained by the higher JTP-4819 concentrations observed in the cortex (Table 5). For example, repeated administration of JTP-4819 (0.3-3 mg/kg p.o. once a day for 15 days) reversed the middle cerebral artery occlusion-induced decrease in TRH levels in the cortex but not in the hippocampus, and a single dose of 3 mg/kg p.o. could restore the age-related decrease in the SP content in the cortex but not in the hippocampus of old rats. In addition to neuropeptides, ACh release was found to be almost 2-fold higher in the cortex than in the hippocampus of young rats (Toide et al. 1995a).

The effects of JTP-4819 on extracellular peptide levels are unknown; there are no published results from microdialysis studies. However, in the review article of Toide et al. (1998) there is a statement that JTP-4819 (1000 μM at 0.5 μl/min for 2h) administered by retrodialysis *via* the microdialysis probe increased synaptic TRH levels in hippocampus and cortex, suggesting that JTP-4819 modulates TRH levels in the synaptic cleft. However, the TRH content was measured from the total tissue sample, not from the extracellular space. Furthermore, the dose used in the retrodialysis is extremely high and would be impossible

to reach after systemic administration; the periprobe extracellular concentrations have been probably 200-300 times higher than the peak concentrations (around 1.5 μM) measured after the i.p. administration of 50 μmol/kg (**II**).

In the present study, we have shown that KYP-2047 at a dose that produces extensive (100-60%) PREP inhibition in the rat brain (**II**) has no effect on the total tissue or extracellular levels of SP or NT in the rat brain (**III**, **IV**). Thus, this study did not reveal any evidence for the involvement of PREP in the digestion of SP or NT. Our extracellular data on unchanged peptide levels support the concept that the direct regulation of peptide processing by PREP is not likely due to the discrepancy in the localization of the peptides and the enzyme. The paradox in the neuropeptide theory suggesting that PREP regulates neuropeptide levels is that the putative peptide substrates of PREP are released and metabolized in the synaptic cleft (see Harrison and Geppetti, 2001; St-Gelais et al., 2006), whereas PREP is known to be located mainly intracellularly. A membrane-bound and less active form of PREP (mPREP) has also been characterized, but there is no evidence that mPREP is accessible from the synaptic cleft and its role in the extracellular metabolism of neuropeptides is unknown (Tenorio-Laranga et al., 2008). It is also known that a number of peptidases other than PREP are involved in the cleavage of SP and NT (see Harrison and Geppetti, 2001; St-Gelais et al., 2006). Therefore, the inhibition of PREP alone is not likely to elevate significantly the levels of these peptides.

Since the involvement of PREP in the extracellular cleavage of neuropeptides seems unlikely, it is tempting to speculate how PREP could regulate intracellular peptide signalling. For example, some putative PREP substrates, notably substance P, act as co-transmitters and are packed into storage vesicles and released together with other transmitters such as neurokinin A or gamma-aminobutyric acid (GABA) (see Hökfelt et al., 2001). These vesicles are not absolutely safe against peptidases, since for example monoamine oxidase (MAO) is an important intracellular metabolizing enzyme although its substrates are stored in vesicles. Hypothetically, if the substrate is moving, being released or taken up, it may encounter PREP. In addition, there is some evidence that putative PREP substrates are involved in the intracrine systems. Intracrines are molecules that have extracellular signalling functions but also have intracellular actions in the cell in which they were synthesized (Re and Cook, 2006). For example, a putative PREP substrate, NT, may have such an action (Toy-Miou-Leong et al., 2004). Interestingly, intracrines have been associated with long-term potentiation (LTP) and memory. It could be hypothesized that PREP is located sufficiently close to these intracellularly acting peptides and might be involved in the intracellular termination of their intracrine effects. However, there is no experimental data to support this speculation.

6.2.4 IP_3 levels

IP_3 is a second messenger that is known to be involved in the generation of LTP and long-term depression (LTD), phenomena that are important in the learning and memory processes *via* the release of Ca^{2+} within the cell (Khodakhah and Armstrong, 1997; Taufiq et al., 2005).

Several lines of investigation point to a role for PREP in the regulation of intracellular IP_3 levels (see chapter 2.1.5). The mutated social amoeba *Dictyostelium discoideum* missing the *Prep* gene (DpoA mutant) was resistant to the inositol and IP_3 depleting effect of lithium (Williams et al., 1999), and the same effect was seen in wild type *Dictyostelium* after enzymatic inhibition of PREP. Schulz et al. (2002) observed that there was an inverse correlation between the IP_3 concentration and PREP expression in mammalian neuronal cells. In primary neurons, lithium mediated growth cone spreading was reversed by PREP inhibition (Williams et al., 2002). Furthermore, PREP and IP_3 type 1 receptor were highly colocalized in distinct areas of rat brain (Myöhänen et al., 2008c).

This is the first study to assess the effect of PREP inhibition on the IP_3 levels *in vivo.* The IP_3 levels were determined in the rat cortex and hippocampus after a 2.5-day treatment with JTP-4819. No effect was seen, and thus, this study did not produce any evidence for the involvement of PREP in the regulation of IP_3 levels *in vivo*. One explanation for this result may be that the degree of brain PREP inhibition at the dose of 9 μmol/kg b.i.d was not sufficient to influence the IP_3 regulatory pathway. The lack of effect might be explained by a technical aspect as well; since only a fraction of cells in the brain contain PREP, the IP_3 elevating effect is lost once the brain tissue is homogenized and diluted with assay buffer. Therefore, even important changes in the IP_3 levels of the PREP containing cells may not be detected. Another and perhaps a more sensitive approach to study the effects of PREP inhibition on IP_3 levels *in vivo* would be to measure the changes in the intracellular Ca^{2+} release, which is the physiological cellular response that IP_3 triggers.

6.2.5 ACh levels

ACh is a key transmitter in cognitive processes (see Deiana et al., 2011; Graef et al., 2011; Micheau and Marighetto, 2011) and many biologically active PREP substrates have been associated with cholinergic transmission. For example, TRH (Toide et al., 1993) and SP (Vlasova and Dolgopol'skii, 2000; Perez et al., 2007) have been shown to increase ACh efflux in the brain. However, little is known about the effects of PREP inhibitors on brain ACh levels. The only study that has reported the effect of PREP inhibition on brain ACh levels was conducted by Toide et al. (1995a); JTP-4819 (1 or 3 mg/kg p.o.) significantly but not dose-dependently increased extracellular ACh levels in the frontal cortex (by ~160%) and hippocampus (by ~90%) of young rats, but in 24 month old rats, the effect was weaker (~40% increase). The authors hypothesized that JTP-4819 increased the brain levels of SP, TRH or AVP and in this way increased ACh release, but this mechanism has not been confirmed. Surprisingly, other PREP inhibitors have not been tested for their ability to modulate brain ACh levels.

Other reports have found only indirect evidence on the involvement of PREP in the cholinergic system. Another PREP inhibitor, Y-29794, was reported to potentiate TRH induced ACh release in the hippocampus of rat brain in a microdialysis study, suggesting that ACh release had been caused by increased TRH levels (Nakajima et al., 1992). In old rats, a 3-week treatment with JTP-4819 (1 or 3 mg/kg p.o.) reversed the age-related increase in choline acetyltransferase (ChAT) activity in cortex and also the decrease of choline uptake in the hippocampus (Toide et al., 1997), suggesting that JTP-4819 may have reversed age-related cholinergic dysfunction.

The present study was designed to assess the effect of PREP inhibitors on the striatal ACh and DA (see chapter 6.2.6) levels (**V**). Striatum was selected as the target tissue for the following reasons. Firstly, the cholinergic system in the striatum is involved in a diverse set of cognitive functions through interactions with other neurotransmitter systems such as the dopaminergic system (see Havekes et al., 2011). Secondly, there is emerging evidence from different behavioral tasks suggesting that the striatal cholinergic system undergoes important interactions with the hippocampal cholinergic system *e.g.* in place and response learning (Chang and Gold, 2003; Pych et al., 2005a; Pych et al., 2005b), and PREP has been associated with these functions. Thirdly, endogenously produced ACh in the striatum is crucial for the induction of corticostriatal LTP (Calabresi et al., 1999). Fourthly, ACh and DA are known to regulate each other's release in striatum (Abercrombie and DeBoer, 1997, Brady et al., 2008, Jeon et al., 2010, Lehmann and Langer, 1983, Millan et al., 2004, Smolders et al., 1997, Threlfell et al., 2010). Finally, PREP protein is widely expressed in the striatum and PREP has been reported to colocalize with striatal ACh (Myöhänen et al. 2008a) suggesting an interaction between ACh and PREP.

In the present study, both JTP-4819 and KYP-2047 decreased extracellular ACh levels. This does not support the earlier hypothesis suggesting that the positive effects of PREP

inhibitors on cognition would be mediated through elevated ACh levels. JTP-4819 decreased ACh both after systemic and intrastriatal administration, whereas KYP-2047 had an effect only after the intrastriatal administration. This difference may be attributable to the different PK characteristics of these molecules (**II**).

The ACh results in this study contradict the findings of the only earlier microdialysis study on the effects of PREP inhibition on brain ACh levels (Toide et al., 1995a). The difference may be explained by a number of factors. Firstly, different brain areas were investigated. Secondly, the doses used in the present study have been proven to inhibit PREP by at least 60-80% for 5 h. Most importantly, Toide and co-workers used an extremely high concentration (10 μM) of the AChE inhibitor physostigmine in the perfusate in order to obtain detectable quantities of ACh, and this could well have influenced the basal ACh release (Messamore et al., 1993). The ACh levels here were determined in the presence of 100 nM neostigmine, which is not believed to affect basal ACh release (Himmelheber et al., 1998; Kehr et al., 2010). It has even been postulated that the addition of low-concentration neostigmine is necessary for assessing the functional changes in the release of ACh (Chang et al., 2006).

On the basis of the current knowledge, it is difficult to attribute the decrease in ACh levels after JTP-4819 and KYP-2047 administration to the inhibition of PREP peptidase activity. Firstly, recent studies have found no evidence that PREP would regulate the brain levels of peptides (*e.g.* SP, TRH or AVP) that are associated with the regulation of ACh release (**III, IV**, Brandt et al., 2005; Nolte et al., 2009; Tenorio-Laranga et al., 2009). Furthermore, one would expect rather an increase in ACh release if PREP had elevated the levels of these neuropeptides. Secondly, the degree of brain PREP inhibition by JTP-4819 and KYP-2047 and the observed effect on ACh levels are not parallel; both compounds are known to inhibit brain PREP by 80-100% within 10-30 min after a single dose of 50 μmol/kg i.p. (**II**). In the present study, however, only JTP-4819 decreased ACh levels at this dose. When the compounds were administered directly into the brain by retrodialysis, both PREP inhibitors rapidly decreased ACh levels. This may be explained by the fact that the maximal extracellular concentrations in the periprobe tissue have been considerably higher after the intrastriatal administration than after the systemic administration. In summary, the decrease in ACh levels did not correlate with the degree of PREP inhibition, and higher brain drug levels would be needed to modulate ACh release than to inhibit PREP. This may point to an off-target mechanism for JTP-4819 and KYP-2047, or to a mechanism that is not related to the catalytic activity of PREP. Indeed, it has been suggested that PREP can regulate certain intracellular functions *via* cytosolic protein-protein interactions rather than *via* its peptidase activity (Schulz et al., 2005; Di Daniel et al., 2009). PREP inhibitors might modulate these functions by causing conformational changes in the tertiary structure of the enzyme and thus perturb the protein-protein interactions (Fuxreiter et al., 2005).

The spatial associations between PREP and ACh do not support a role for PREP in the regulation of ACh release (Myöhänen et al., 2008a; Peltonen et al., 2011). Immunohistochemical studies have shown that the colocalization of PREP protein and ACh in the brain is rather low. Moreover, the ACh synthesizing enzyme ChAT is only partially colocalized with PREP in the cortex, but not in the hippocampus or medial septum. In striatum, the colocalization of PREP with cholinergic neurons is poor (Myöhänen et al., 2008a). This further supports the present findings indicating that the effects of JTP-4819 and KYP-2047 on the striatal ACh levels are not mediated by inhibition of PREP catalytic activity.

In an attempt to find some clues to the possible mechanisms that could explain the decreased ACh levels, an *in vitro* pharmacological profiling was performed for JTP-4819 and KYP-2047 (**V**). This analysis of 70 pharmacological targets did not reveal any obvious off-targets that could account for the effect on ACh levels. Furthermore, JTP-4819 and KYP-2047 did not exert any inhibitory effect on several serine proteases and proline-specific

proteases *in vitro* at 10 μM concentration (**V**). Slight inhibition of lysosomal prolyl carboxypeptidase was observed at 10 μM, which is 1000-fold higher concentration than that needed to fully inhibit PREP *in vitro*. As far as we are aware, prolyl carboxypeptidase has not been associated with the regulation of ACh levels in the brain. Overall, these results validate the proposal that JTP-4819 and KYP-2047 have a high selectivity towards PREP.

In humans plasma AChE activity has been observed to gradually increase after multiple doses of JTP-4819 (Umemura et al., 1997). Theoretically, if this increase holds true in the brain after a single dose as well, it could explain the decrease in the extracellular ACh levels. The *in vitro* screen showed that there was no significant interaction between JTP-4819 or KYP-2047 and AChE, *i.e.* the increase in the AChE activity would appear to be mediated through an indirect mechanism. A further study could assess the effect of PREP inhibitors on the AChE activity in the rat brain *in vivo*.

One interesting off-target that was not included in the screening is ChAT, an enzyme which catalyzes the synthesis of ACh from choline and acetyl coenzyme A. Theoretically, inhibition of ChAT by PREP inhibitors would decrease the synthesis and release of ACh and explain the observed effect in ACh levels. Therefore, the effects of PREP inhibitors on ChAT activity should also be determined.

6.2.6 DA levels

As far as is known, this is the first report on the effects of PREP inhibitors on brain DA levels. PREP inhibitors had no significant effect on the striatal DA levels; JTP-4819 at doses of 15 and 50 μmol/kg i.p. and and KYP-2047 at a dose of 50 μmol/kg i.p. tended to decrease (ns) the levels, but the interindividual variation was high. Moreover, no effect was seen in DOPAC and HVA levels; only normal the fluctuation related to the circadian rhythm was observed (Castaneda et al., 2004), which are parallel with the minor changes in the DA levels.

Although DA and ACh systems exist in close proximity in the striatum, the minor change seen in DAergic transmission is most unlikely to explain the observed changes in the ACh levels. This result is consistent with immunohistochemical studies indicating that PREP is not present in the DAergic neurons in the striatum (Myöhänen et al., 2008a). Furthermore, destruction of DAergic neurons in the medial forebrain bundle with 6-hydroxydopamine had no effect on the PREP activity or immunoreactivity in the striatum, which is the terminal projection area of these DAergic neurons (Peltonen et al., 2011). NT, a PREP substrate *in vitro*, is known to regulate DA release in the brain (see St-Gelais et al., 2006), but our studies have shown that PREP inhibitors have no effect on either the total brain tissue or on the extracellular NT levels (**III, IV**).

In summary, current evidence points to no role for PREP inhibitors in the modulation of the DAergic system. Thus, the positive cognitive effects of PREP inhibitors are not likely to be mediated through this system. On the other hand, the lack of DAergic effect in the striatum suggests that PREP inhibitors do not have the potential to induce extrapyramidal side effects.

7 Summary and conclusions

Despite intensive research, the main physiological function and the general potential of PREP as a drug target remains obscure. No specific indication for the therapeutic use of PREP inhibitors has yet been found. Therefore, further studies are required to elucidate the physiological role of PREP both in the CNS and in the periphery, and to validate the pharmacological effects of PREP inhibitors.

The major findings of this study and their impact on the current understanding about PREP as a target for drug development have been summarized in Table 6. The present study aimed to characterize the PK and PD properties of two model PREP inhibitors, JTP-4819 and KYP-2047, in the rat. This is the first study to assess the brain pharmacokinetics of PREP inhibitors. It was shown that the model inhibitors were able to penetrate the brain in pharmacologically active concentrations.

The behavioral study confirmed the earlier findings that PREP inhibitors have some potential as cognitive enhancers, but the PD studies could not elucidate any specific mechanism of action for this effect. As long as the proof of mechanism is unknown, the potential of PREP inhibitors as memory enhancing drugs remains unconvincing.

This study provides novel information on the lack of effect of PREP inhibition on extracellular neuropeptide levels. Together with other recent findings, this result suggests that PREP is not involved in the extracellular cleavage of proline-containing peptides. This seems rational since the enzyme is located intracellularly and peptide cleavage occurs mainly extracellularly. A novel finding was also that PREP inhibitors were found to decrease the extracellular ACh levels in the rat striatum by some unknown mechanism.

Based on the results of the experimental section (**II**-**V**), the following specific conclusions can be drawn:

1. The model PREP inhibitors have different PK properties in the rat. KYP-2047 penetrates the brain better than JTP-4819, distributes homogenously in the brain, and inhibits brain PREP more efficiently after systemic administration of a single dose. This may be due to the poor ability of JTP-4819 to permeate through the cell membranes and to reach the intracellular PREP. KYP-2047 is a highly potent, brain penetrating, PREP specific inhibitor and it represents a suitable pharmacological tool to assess the potential of PREP as a drug target.
2. KYP-2047 has a positive effect on spatial learning in young rats in the Morris water maze. This confirms previous findings on the positive effects of PREP inhibitors on cognition. However, the mechanism of action remains unknown despite the fact that several potential mechanisms were investigated *in vivo* using the model inhibitors, JTP-4819 and KYP-2047. The high degree of PREP inhibition had no effect on the brain levels of memory enhancing neuropeptides NT or SP, failed to increase brain IP_3 levels, and had no significant effect on striatal DA levels.
3. JTP-4819 and KYP-2047 decrease the extracellular levels of striatal ACh by some unknown mechanism. However, the decrease does not seem to be attributable to the degree of PREP inhibition.
4. The lack of effect of PREP inhibitors on extracellular peptide levels and the known intracellular localization of PREP indicate that rather than being involved in the extracellular cleavage of proline-containing peptides, PREP may be a part of some

intracellular protein machinery *e.g.* as a component of the proteasome degradation pathway.

5. On the basis of recent findings, it seems that the most promising sources for future research involve clarifying the roles of PREP in neutrophil mediated inflammation, in α-synuclein aggregation and in cell proliferation and differentiation. The present findings provide the following targets for future research: the effect of PREP inhibition on the brain levels of other neurotransmitters known to be involved in cognitive processes, such as glutamate, serotonin, GABA and noradrenaline, should be determined. Furthermore, the effects of PREP inhibitors on ACh levels as well as on AChE and ChAT activities in other brain areas (*e.g.* the hippocampus and cortex) should be assessed. Finally, if one wishes to establish a link between IP_3 and PREP *in vivo*, a future study could assess the effect of PREP inhibition on the intracellular Ca^{2+} release in the brain.

Table 6: A summary of the major findings of the present study and their impact on the current understanding about PREP as a drug target.

Measure	**JTP-4819**	**KYP-2047**	**Impact of the finding(s)**
Brain penetration	+	++	The general potential of current PREP inhibitors to penetrate the BBB and reach the CNS target site is relatively poor.
Brain PREP inhibition	+	++	KYP-2047 is a suitable pharmacological tool to assess the potential of PREP as a CNS drug target.
Spatial memory	NA	+ Young ± Old	Confirms earlier findings on the positive effects of PREP inhibitors on learning and memory.
Whole tissue neuropeptide levels	±	±	Questions the role of PREP in cleavage of neuropeptides *in vivo*; regulation of the brain levels of classical PREP substrates by PREP inhibitors is unlikely.
IP_3	±	NA	PREP inhibition has no major role in controlling the brain levels of IP_3 *in vivo.*
Extracellular neuropeptide levels	NA	±	Questions the role of PREP in cleavage of neuropeptides *in vivo*. Indicates that PREP as an intracellular enzyme cannot affect the extracellular cleavage of neuropeptides.
Extracellular ACh levels	−−	−	Contradicts the earlier findings on the elevating effects of PREP inhibition on brain ACh levels. The positive cognitive effects of PREP inhibitors are not likely to be mediated by increased ACh levels.
Extracellular DA levels	±	±	PREP inhibition has no major role in modulating the striatal DA levels.
In vitro selectivity	±	±	Both model inhibitors have a high selectivity towards PREP.

+, positive finding; ±, no change in measure; −, negative finding; NA, not assessed.

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The Potential of Prolyl Oligopeptidase as a Drug Target

Prolyl oligopeptidase (PREP) cleaves proline containing small peptides such as substance P and neurotensin. PREP is an abundant peptidase in the mammalian brain and periphery, but the physiological role of PREP remains unknown. This dissertation aims at characterizing the pharmacokinetic and pharmacodynamic properties of specific PREP inhibitors in the rat. It provides new perspectives into the physiological role of PREP and evaluates the importance of PREP as a drug target.

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