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TIMO MYÖHÄNEN

Distribution of Prolyl Oligopeptidase and its Colocalizations with Neurotransmitters and Substrates in Mammalian Tissues

Doctoral dissertation

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ABSTRACT

Prolyl oligopeptidase (POP) is an ancient serine endopeptidase that hydrolyzes proline-containing peptides at the carboxyl end of the proline residue. POP is able to hydrolyze small peptides that are shorter than 30-mer. Substrates of POP include peripherally acting hormones, like angiotensins and vasopressin and also many neuroactive peptides, like substance P and thyrotropin-releasing hormone. Since many of these peptides are affecting to the memory and learning functions, these findings have served as a rationale to develop POP inhibitors as antiamnesic drugs. Furthermore, POP has also been associated with inositol 1,4,5-triphosphate (IP_3) signalling. Despite intensive research into POP inhibitors, the distribution and physiological role of the POP protein are poorly known.

In this work, we studied the distribution of the POP protein in mammalian tissues using immunohistochemistry based on a specific POP antibody, and the POP activity assay. In the mouse whole-body sections, POP is present in high and equal amounts in the brain, testis, thymus and kidney. Furthermore, the distribution of the POP protein differs extensively from the distribution of the POP activity both in the brain and peripheral tissues, and also from the distribution of POP coding mRNA in the brain. These findings point to a strict endogenous regulation of POP.

At the cellular level, POP is not cell type specific in the peripheral tissues though in the rat brain it is present only in the neurons, not in the glial cells. However, in the rat brain POP can be found both in the inhibitory (GABAergic), excitatory (glutamatergic) and cholinergic neurons, meaning that there is no neurotransmitter specificity. Most importantly, POP is preferentially localized in the nuclei of the peripheral cells but exclusively in the cytoplasm of the brain neurons. Moreover, the spatial association of POP and its well-characterized substrate, substance P, is poor throughout the brain. These findings point to role for POP in the cell proliferation/differentiation in the peripheral tissues and protein modification and trafficking in the brain. However, the strong colocalization of POP with IP_3 receptors in the adult hippocampus supports its role in the memory and learning functions.

National Library of Medicine Classification: QU 136, QV 38, QV 126, WL 300
Medical Subject Headings: Serine Endopeptidases; Tissue Distribution; Neurotransmitter Agents; Brain; Neurons; Testis; Thymus Gland; Kidney; Cell Nucleus; Cytoplasm; Substance P; Cell Proliferation; Cell Differentiation; Protein Transport; Inositol 1,4,5-Trisphosphate Receptors; Hippocampus; Memory; Learning; Immunohistochemistry; Mice; Rat

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Kuopio, May 2005

Timo Myöhänen

ABBREVIATIONS

6-OHDA	6-hydroxydopamine
α-MSH	α-melanocyte stimulating hormone
ACE	angiotensin-converting enzyme
ACh	acetylcholine
AVP	arginine-vasopressin
BSA	bovine serum albumin
ChAT	choline acetyltransferase
CNS	central nervous system
CREB	cAMP response element-binding
DPPIV	dipeptidyl peptidase IV
DAPI	4',6-diamidino-2-phenylindole
GABA	gamma-aminobutyric acid
GFAP	glial fibrillary acid protein
GI	gastrointestinal
GNF	Genomics Institute of the Novartis Research Foundation
FAP	fibroblast activation protein α
HPT-axis	hypothalamic-pituitary-thyroid-axis
IP_3	inositol (1,4,5)-triphosphate
IP_3R1	inositol (1,4,5)-triphosphate type 1 receptor
LTP/LTD	long-term potentiation / depression
MInsPP	multiple inositol polyphosphate phosphatase
NEP	neutral endopeptidase
NK	neurokinin
NK1R	neurokinin-1 receptor (substance P receptor)
NMDA	N-methyl-D-aspartic acid
PAP I/II	pyroglutamyl aminopeptidase I and II
PBS	phosphate buffered saline
PPT-A	pre-protachykinin-A
POP	prolyl oligopeptidase
PVN	paraventricular nuclei of hypothalamus
RER	rough endoplasmic reticulum
SEM	standard error of mean
SON	supraoptic nuclei of hypothalamus
SP	substance P
TH	tyrosine hydroxylase

TRH	thyrotropin-releasing-hormone (thyroliberin)
TRH-R1/R2	thyrotropin-releasing-hormone receptors type 1/2
V1R/ V2R	arginine-vasopressin receptors, type 1 and 2
$V1_aR$/ $V1_bR$	arginine-vasopressin type 1 receptor, subtype a and b
VPA	valproic acid
ZIP	Z-L-prolyl-L-prolinal insensitive Z-Gly-Pro-7-amino-4-methylcoumarin hydrolyzing peptidase

LIST OF THE ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text by Roman numerials **I-IV**.

I Timo T. Myöhänen, Jarkko I. Venäläinen, J.Arturo Garcia-Horsman, Marjo Piltonen, Pekka T. Männistö: Distribution of prolyl oligopeptidase in the mouse whole-body sections and peripheral tissues. Submitted

II Timo T. Myöhänen, Jarkko I. Venäläinen, Erkki Tupala, J.Arturo Garcia-Horsman, Riitta Miettinen, Pekka T. Männistö: Distribution of immunoreactive prolyl oligopeptidase in the human and rat brain. Neurochemical Research 32:1365-1374, 2007

III Timo T. Myöhänen, Jarkko I. Venäläinen, J.Arturo Garcia-Horsman, Marjo Piltonen, Pekka T. Männistö: Cellular and subcellular distribution of rat brain prolyl oligopeptidase and its association with specific neuronal neurotransmitters. Journal of Comparative Neurology 507: 1694-1708, 2008

IV Timo T. Myöhänen, Jarkko I. Venäläinen, J.Arturo Garcia-Horsman, Pekka T. Männistö: Spatial association of prolyl oligopeptidase, inositol 1,4,5-triphosphate type 1 receptor, substance P and its NK-1 receptor in the rat brain: An immunohistochemical study. Neuroscience, doi:10.1016/j.neuroscience.2008.02.047

CONTENTS

1 INTRODUCTION

Prolyl oligopeptidase (POP, EC 3.4.21.26) is an 80 kDa serine protease enzyme (Rawlings et al. 1994) of ancient origin (Venäläinen et al. 2004). POP preferentially hydrolyses peptides at the carboxyl side of proline residues and mammalian POP appears to cleave only peptides shorter than 30 amino acids. POP has been implicated in the hydrolysis of many bioactive peptides such as angiotensins, neurotensin, arginine-vasopressin, substance P and thyrotropin releasing hormone (for reviews, see Garcia-Horsman et al. 2007a, Männistö et al. 2007). Several of these peptides are thought to be involved in the regulation of memory and learning (Huston et al. 1995).

Alterations in POP enzyme activity in serum and brain tissue have been observed in aging (Agirregoitia et al. 2003a) and in several pathological conditions, including psychiatric diseases and Alzheimer's and Parkinson's diseases (Maes et al. 1995, Mantle et al. 1996). Moreover, several studies have recently reported that POP is involved in inositol 1,4,5-phosphate (IP_3) signalling in the brain (Williams et al. 1999, Schulz et al. 2002, Williams et al. 2002, Harwood et al. 2003, Cheng et al. 2005). An *in vitro* study by Cheng et al. (2005) even suggested that the enzyme may be a possible target of certain mood stabilizing drugs. These findings have served as the rationale for the development of POP inhibitors. POP inhibitors have been shown to prevent the amnesic effects of scopolamine in rats (Toide et al. 1995a, Morain et al. 2002), improve cognition in untreated old rats (Toide et al. 1997) and they have restored declining neuropeptide levels (Toide et al. 1995b, Toide et al. 1996, Bellemere et al. 2005) However, the effects of POP inhibitors on neuropeptide levels in the brain are still controversial (Jalkanen et al. 2007, Männistö et al. 2007).

POP is widely distributed among different organisms, including bacterial and archeal species (Venäläinen et al. 2004, Garcia-Horsman et al. 2007a). In human and rat, POP enzyme activities have been found in most tissues (Kato et al. 1980b, Daly et al. 1985, Fuse et al. 1990, Irazusta et al. 2002) and even body fluids contain some POP-like activity (Goossens et al. 1996). At the subcellular level, POP is known to be mainly cytosolic (Dresdner et al. 1982) and Schulz *et al*. (2005) reported a close association between POP and cytoskeletal component microtubules in glial and neural cell lines though POP-like activity has also been detected in membranes (O'Leary et al. 1995). However, previous studies have been made using enzyme activity measurements and by determining the mRNA coding POP, but the distribution of POP protein has not been characterized.

Despite intensive research, the true physiological role of POP is still virtually unknown. The following review of the literature provides a short summary of properties

and the possible physiological role of POP in CNS, peripheral tissues and plasma. The experimental part of this dissertation focuses on the characterization of the distribution of the POP protein in the body, particularly in the brain, and the spatial association of the POP protein with neuronal neurotransmitters and potential targets of POP.

2 REVIEW OF THE LITERATURE

2.1 Proline

The imino acid, proline, is unique with its structure among amino acids. Its side chain R-group (-CH_2-CH_2-CH_2) is bonded both amino group and α-carbon (-CH) resulting in a cyclic structure (Fig. 1).

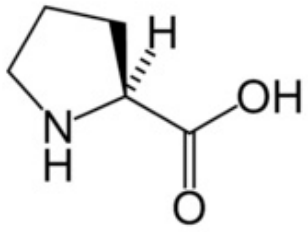

Figure 1. Structure of proline

The five-membered cyclic structure of proline prevents the rotation around the α-carbon-N bond and therefore, its structure allows only few conformations. Due to its cyclic nature proline is unlikely to be compatible with α-helix and furthermore, it lacks the amide H-atom that is necessary for hydrogen bonding. These characteristics of proline are crucial for its physiological functions. The presence of proline residues in the polypeptide precursors reduces the sensitivity of the polypeptide chain to proteolysis by aminopeptidases and carboxypeptidases limiting the enzymatic modification of the precursors (Yaron et al. 1993, Cunningham et al. 1997a, Berg et al. 2006). Moreover, the *cis-trans* isomerization of proline may alter the function of protein and changes in prolyl isomerization have been considered as a molecular timer with several biological processes, such as cell cycle, cell signalling and gene expression (Lu et al. 2007).

2.2 Overview of prolyl oligopeptidase (POP)

Prolyl oligopeptidase (POP, EC 3.4.21.26) was identified first by Walter et al. (1971) in the human uterus where it was found to cleave oxytocin. POP is an 80 kDa serine protease enzyme belonging to the family S9 of the SC clan (Rawlings et al. 1994). The closest relatives to POP are dipeptidyl peptidase IV (DPPIV, EC 3.4.14.5), acylaminoacyl peptidase (ACPH, EC 3.4.19.1) and oligopeptidase B (OB, EC 3.4.21.83) (Rawlings et al. 1994, Venäläinen et al. 2004). The POP family has ancient

origins and it is widely distributed from bacterial and archaeal species to human, and only fungi do not contain POP enzyme (Venäläinen et al. 2004).

The POP gene has been cloned from several species, including mouse (Ishino et al. 1998), cows (Yoshimoto et al. 1997), pigs (Rennex et al. 1991) and also from human brain (Tarrago et al. 2005), human lymphocytes (Vanhoof et al. 1994) and T-cells (Shirasawa et al. 1994), as well as bacterial sources such as *Flavobacterium meningosepticum* (Yoshimoto et al. 1991) and *Pyrococcus furiosus* (Robinson et al. 1995). POP is located in chromosome 10 B2/B3 in mouse (Kimura et al. 1999) and in 6q22 in human (Goossens et al. 1996). In animal species, POP is 710 amino acids long. POP has a cylindrical shape with a height of 60 Å and a diameter of 50 Å (Fig. 2). The peptidase domain is formed by N- and C-termini (residues 1-72 and 428-710) containing the catalytic triad (Ser554, Asp641, His680). The seven bladed β-propeller domain is radially arranged around the central tunnel and is embedded within the cylinder (Fig. 2). Concerted movements of peptidase and β-propeller domains are required for enzyme function as substrate induces an opening at the interface of the two domains while entering into the active site of POP (Szeltner et al. 2004, Juhasz et al. 2005). The relatively small size of the interface opening presumably prevents large peptide substrates from entering into the active site.

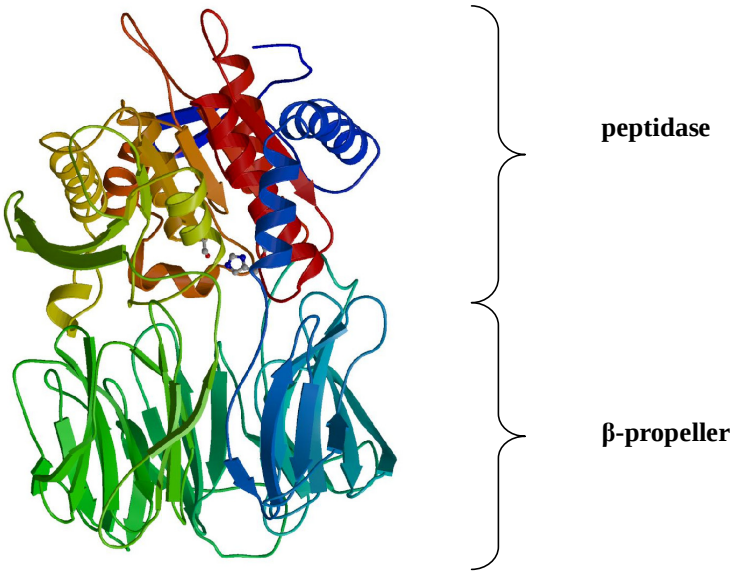

Figure 2. The 3D-structure of POP. The ribbon diagram is color-ramped blue to red from the N to the C terminus (modified from Fülop et al. 1998).

The POP ability of cleaving peptides at the carboxyl side of proline is a rare quality among peptidases. Proline is unique among amino acids due to its cyclic structure and most peptidases are unable to cleave peptides at a proline residue. POP hydrolyses the -Pro-Xaa- bond, where Xaa is any amino acid other than proline, since POP it is not able to break the -Pro-Pro- bond (Polgar 1994, Cunningham et al. 1998). Several bioactive neuropeptides, such as substance P (SP), thyrotropin-releasing hormone (TRH), arginine-vasopressin (AVP), angiotensins I-IV, bradykinin and neurotensin (Table 1), have been found to be POP substrates (for reviews, see Polgar 1994, Garcia-Horsman et al. 2007a, Männistö et al. 2007). Many of these neuropeptides are associated with memory and learning (Huston et al. 1995, Cunningham et al. 1997a) and changes in the levels of these peptides during aging and degenerative diseases in the brain have been reported (Hasenohrl et al. 2000, Harrison et al. 2001, Hökfelt et al. 2003). Furthermore, changes in POP enzyme activity or expression in tissues have been detected during aging (Agirregoitia et al. 2003a, Rossner et al. 2005) and in various diseases such as Parkinson's and Alzheimer's disease, suggesting a role for POP in these disorders via the neuropeptide cleavage (Mantle et al. 1996, Kato et al. 1997, Shinoda et al. 1997).

Table 1. Potential substrates of POP (modified from Garcia-Horsman et al. 2007a).

Substrate	Sequence	Type of evidence*
SP	R-P-K-**P-Q**-Q-F-F-G-L-M	A, B, C, D(t), E, F
TRH	pQ-H-**P–NH**$_2$	A, D(t)
AVP	C-Y-F-Q-N–C-**P-R**-G	A, D, E, F(t)
Angiotensin I	D-R-V-Y-I-H-**P–F**-H-L	A
Angiotensin II	D-R-V-Y-I-H-**P–F**	A, B(t), C, D
Angiotensin III	R-V-Y-I-H-**P–F**	A
Angiotensin IV	V-Y-I-H-**P–F**	A
Bradykinin	R-P–P-G-F-S-**P–F**-R	A, B(t), G
Oxytocin	C-Y-I-Q-N–C-**P-L**-G-NH$_2$	A
β-Endorphin	Y-G-G-F-M-T-S-E-K-S-Q-T-**P-L**-V-T-L-F-K-N-A-I-I-K-N-A-Y-K-K-G-E	A
Neurotensin	pQ-L-Y-E-N-K-**P-R**-R-**P-Y**-I-L	A
α-MSH	S-Y-S-M-E-H–F-R-W-G-K-**P-V**-NH$_2$	D(t)
β-Casomorphin	Y-**P–F-P-G-P-I**	A
LVV-hemorphin-7	L-V–V-Y-**P-W**-T-Q-R-F	C
Morphiceptin	Y-**P–F-P–NH**$_2$	A
Urotensin II	D-T-**P-D**-C–F-W-K-Y-C-V	A
Humanin	M-A-**P-R**-G-F-S-C-L-L-L-L-T-S-E-I-D-L-P-V-K-R-R-A	A

The cleavage bonds are shown in bold.
* A, Digestion by purified POP; B, digestion by tissue crude preparations; C, digestion sensitive to POP inhibitors by crude tissue preparations; D, modulation of peptide levels by POP inhibitors *in vivo*; E, peptide effect potentiated by POP inhibitors *in vitro*; F, peptide effect potentiated by POP inhibitors *in vivo*; G, genetic modulation of POP by the peptide or analogues; (t) tissue or cell-type dependent.

These findings have served as the rationale for the development of POP inhibitors in an attempt to discover a novel drug to combat memory and learning disorders, which would act by modifying the neuropeptide levels in the brain. Several inhibitors have been developed and characterized in the literature with Z-Pro-prolinal, JTP-4819, ONO-

1603, SUAM-1221, S17092 and ZTTA being the best characterized inhibitors (Wilk et al. 1983, Tanaka et al. 1994, Toide et al. 1995a, Katsube et al. 1996, Shishido et al. 1996, Barelli et al. 1999, Männistö et al. 2007, Garcia-Horsman et al. 2007, see also Table 2). Most of the POP inhibitors are substrate-like compounds that interact with the three subsites of enzyme's substrate binding site: S1, S2 and S3, in which S1-site is most likely specific for proline. Therefore most of these substances contain a proline or proline analogue residues at their P1 and P2 sites. An electrophilic P1 site substituent, such as aldehyde (in Z-Pro-Prolinal) or hydroxyacetyl (in JTP-4819), forms a covalent adduct to the serine554-residue in catalytic site being crucial for the inhibitor binding (Venäläinen 2005).

Z-Pro-Prolinal, JTP-4819, S17092 and ZTTA are the most tested POP inhibitors in different animal models of memory and learning. Of these, Z-Pro-Prolinal, JTP-4819 and S17092 have been shown to prevent the amnesic effects of scopolamine in rats in a passive avoidance test, especially with higher doses (> 1 mg/kg) (Yoshimoto et al. 1987, Toide et al. 1995a, Morain et al. 2002). Furthermore, repeated administration of JTP-4819 improved performance in passive avoidance test and in Morris water maze in rats after middle cerebral artery occlusion (Shinoda et al. 1996) and enhanced cognition (Morris water maze) in untreated old rats (Toide et al. 1997). Interestingly, after scopolamine treatment, single dose (5 mg/kg) of KYP-2047 inhibitor improved performance in Morris water maze with young rats but not with old (Jalkanen et al. 2006). Repeated administration of ZTTA has also shown memory impairement effects in passive avoidance test after basal forebrain lesion (Shishido et al. 1998) and some improvements was observed after a single dose in 3-panel runaway task after cerebral ischemia (Shishido et al. 1996). Moreover, repeated administration of S17092 have prevented the cognitive symptoms of Parkinson's model induced by chronic low dose of MPTP (1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine) in monkeys (Schneider et al. 2002).

In some studies, POP inhibitors have restored declining neuropeptide levels in rat cerebral cortex and hippocampus, e.g. SP (Toide et al. 1996, Bellemere et al. 2003), TRH, AVP (Toide et al. 1995b, Toide et al. 1996, Bellemere et al. 2005) and α-melanocyte stimulating hormone (α-MSH, Bellemere et al. 2003). Especially these effects were seen after single dose of inhibitor in young rats. Only in the study of Toide et al. (1995b), repeated administration of JTP-4819 caused significant increase to the neuropeptide levels. Moreover, some studies have reported that POP inhibitors have been able to prevent the generation of β-amyloid protein in Alzheimer's disease (Kato et al. 1997, Shinoda et al. 1997). However, the changes in POP activity in the generation of β-amyloid are more likely to occur from neuronal damage than from a role for the

enzyme in β-amyloid production (Petit et al. 2000, Laitinen et al. 2001, Rossner et al. 2005). Moreover, when critically evaluating the results of POP inhibitor studies, the effects of POP inhibitors on neuropeptide levels in the brain are less convincing (Table 2) and studies with opposite results have been published (Jalkanen et al. 2007).

Table 2. Effects of POP inhibitors to neuropeptide levels in the brain (modified from Männistö et al. 2007).

Peptide	Compound	Rat	Dosing	Brain area and result	Reference
SP	JTP-4819	Young	Single	CTX +, HC +	Toide et al. 1996
		Aged	Single	CTX +, HC 0	Toide et al. 1995b
		Aged	Repeated	CTX +, HC +	Toide et al. 1995b
		Young	Repeated	CTX 0, HC 0	Shinoda et al. 1996
		Young	Single	CTX 0, HC 0, HT 0	Jalkanen et al. 2007
		Young	Repeated	CTX 0, HC 0, HT 0	Jalkanen et al. 2007
	S-17092	Young	Single	STR +	Lestage et al. 1998
		Young	Repeated	STR +	Lestage et al. 1998
		Young	Single	CTX +, HT +	Bellemere et al. 2003
		Young	Repeated	CTX 0, HT 0	Bellemere et al. 2003
	KYP-2047	Young	Single	CTX 0, HC 0, HT 0	Jalkanen et al. 2007
		Young	Repeated	CTX 0, HC 0, HT 0	Jalkanen et al. 2007
TRH	JTP-4819	Young	Single	CTX 0, HC +	Toide et al. 1996
		Young	Repeated	CTX +, HC 0	Shinoda et al. 1996
	S-17092	Young	Single	CTX +, AMG 0	Bellemere et al. 2005
		Young	Repeated	CTX +, AMG 0	Bellemere et al. 2005
AVP	JTP-4819	Young	Single	CTX +, HC +	Toide et al. 1996
		Aged	Single	CTX 0, HC 0	Toide et al. 1995b
		Aged	Repeated	CTX 0, HC 0	Toide et al. 1995b
		Young	Repeated	CTX 0, HC 0	Shinoda et al. 1996
	S-17092	Young	Single	CTX 0, HC +	Bellemere et al. 2005
		Young	Repeated	CTX 0, HC 0	Bellemere et al. 2005
α-MSH	S-17092	Young	Single	CTX +, HT +	Bellemere et al. 2003
		Young	Repeated	CTX 0, HT 0	Bellemere et al. 2003

AMG, amygdala; CTX, cortex; HC, hippocampus; HT, hypothalamus; STR, striatum; +, elevation; 0, no change.

Nevertheless, some additional roles other than direct neuropeptide cleavage have been proposed for POP. Williams et al. (1999) found that POP may be involved in the regulation of IP_3 signalling. They observed that a mutant *Dictyostelium,* lacking of POP gene, was resistant to the effects of lithium. Furthermore, administration of a POP inhibitor restored lithium induced depletion of IP_3 levels in the wild-type *Dictyostelium* cells (Williams et al. 1999). Similar results were obtained in mammalian cells by Schulz et al. (2002) (see section 2.3.2.4 for more detailed description). The mechanism of this action is still unclear, but POP may be able to regulate the synthesis of IP_3 via the multiple inositol polyphosphate phosphatases (MInsPP) (Williams et al. 2000, Harwood et al. 2003) or intracellular calcium levels via the short sequence of PEP-19, a calmodulin binding polypeptide (Brandt et al. 2005). Furthermore, several studies have suggested that POP and its effects to IP_3 levels in the cell may be a common mechanism for several types of mood stabilizing drugs, such as Li^+, valproic acid (VPA) and carbamazepine (Williams et al. 2002, Harwood et al. 2003, Williams 2005). Recently, Cheng et al. (2005) even suggested that POP could be the direct target for VPA. These findings may point to a role for POP in psychiatric disorders. However, subchronic administration of a POP-inhibitor (JTP-4819) had no significant effects on the IP_3 levels in rat cerebral cortex and hippocampus (Jalkanen et al. 2007).

POP has also been suggested as a potential treatment for celiac disease. Gluten proteins of wheat, barley and rye evoke inflammation of the small intestine villi in celiac disease, and these grains contain α-gliadin which has several immunogenic peptides that are resistant to gastrointestinal tract proteases (Hausch et al. 2002, Shan et al. 2002). These peptides are proline-rich and therefore, POP has been suggested as representing a potential treatment for celiac disease since it could be able to degrade these immunogenic peptides. In some studies, bacterial and to lesser extent also mammalian POP have been able to accelerate the cleavage time of these peptides (Piper et al. 2004, Stepniak et al. 2006, Garcia-Horsman et al. 2007b).

POP has also been associated with several other diseases such as cancer, inflammation, hypertension and eating disorders based on its enzymatic activity and/or altered levels of its potential substrates. Furthermore, roles in cell death, cell proliferation and differentiation have also been proposed (for review, see Brandt et al. 2007). However, despite of these findings, the true physiological importance of POP is still largely undefined.

2.3 Possible physiological role of POP and the functions of its proposed substrates in peripheral tissues and CNS

2.3.1 POP in the peripheral tissues

POP activities have been localized in various organs (Kato et al. 1980a, Kato et al. 1980b, Daly et al. 1985, Fuse et al. 1990, Goossens et al. 1996, Agirregoitia et al. 2005) establishing that POP is widely present outside the CNS. However, the physiological role of POP in the peripheral tissues is even more unclear than that of POP in the CNS.

2.3.1.1 The distribution and regulation of POP activity in peripheral tissues

The knowledge of distribution of POP in peripheral tissues is based on enzyme activity measurements. Enzyme activity measurements have been made using substrates with a suitable Pro-X-bond where X is a fluorescent compound such as β-naphthylamine or 4-methylcoumarin, which is activated after POP cleavage. POP activities have been measured from peripheral tissues such as the rat skeletal muscle (Daly et al. 1985, Fuse et al. 1990), testis, liver, kidney, lung, renal cortex, heart and gut (Kato et al. 1980a, Kato et al. 1980b, Fuse et al. 1990, Goossens et al. 1996, Agirregoitia et al. 2005). The results of these studies are somewhat controversial (Fig. 3), possibly due to the use of different POP substrates and their concentrations. Generally, in rat, the highest POP activities have been found in the brain (Kato et al. 1980b, Irazusta et al. 2002, Agirregoitia et al. 2005), but Fuse et al. (1990) found the highest activities in the kidney. In humans, the highest enzyme activities were found from cancerous tissues and in healthy samples, from the epithelial cells, renal cortex and testis (Goossens et al. 1996). However, brain POP activity was not measured in that study.

In the high-throughput gene expression profiling, the highest levels of human POP mRNA have been found in the testis and different types of lymphocytes. In contrast to the enzymatic activity studies, the amount of POP mRNA was approximately equal with internal organs, such as the liver, lungs and pancreas, and the brain. In the rat, the uppermost POP mRNA expression was observed from the kidney and endothelial cells while in the mouse the expression was the highest in the thymus. These results are suggesting that, in contrast to POP enzyme activity, the highest amount of POP mRNA are not located in the brain tissue (Genomics Institute of the Novartis Research Foundation (GNF) SymAtlas, Su et al. 2002).

Interestingly, in nonneural cell lines, POP activity has also been detected from the nucleus (Ishino et al. 1998) although POP is mainly a cytosolic enzyme (Dresdner et al. 1982). Furthermore, in *Sarcophaga peregrina* (flesh fly), POP has been detected widely in the tissues, both in cytoplasm and nucleus (Ohtsuki et al. 1997a, Ohtsuki et al. 1997b). A more detailed discussion of this nuclear POP and its functions is provided in section 2.3.1.3.

An endogenous POP inhibitor has been described in several studies (Yoshimoto et al. 1982, Salers 1994, Yamakawa et al. 1994). It was originally found and purified from the rat pancreas (Yoshimoto et al. 1982) and a pancreatic cell line (Salers 1994) and thereafter identified also from the regenerating rat liver (Yamakawa et al. 1994). This substance is a 6.5 kDa POP specific inhibitor (Ki value 2.6 μM) and it is localized within the cytosolic compartment (Soeda et al. 1985, Salers 1994). However, this compound has been biologically rather poorly characterized and its regulation and functions are still obscure. From other interfering endogenous substances, estradiol-17β, progesterone (Ohta et al. 1992) and cortisol (Yasuda et al. 1992) are able to increase POP activity in the peripheral tissues.

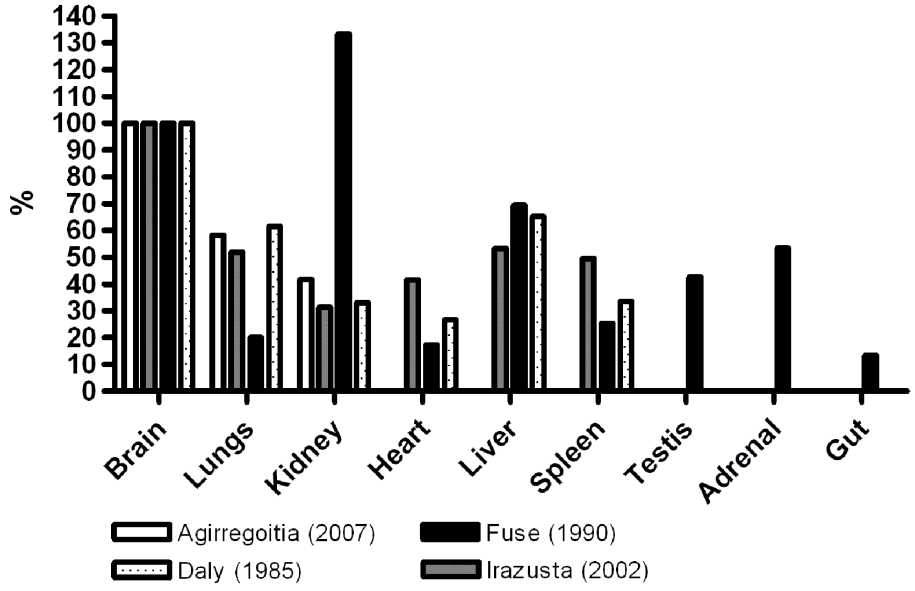

Figure 3. Distribution of enzyme activity of POP in the rat in studies by Agirregoitia et al. (2005), Irazusta et al. (2002), Fuse et al. (1990) and Daly et al. (1985). In order to

compare the results, enzymatic activity of the brain has been set as 100 % and other organs are compared to that value.

2.3.1.2 POP and its proposed substrates in peripheral tissues

POP has been implicated in the hydrolysis of various neuropeptides in the CNS (Table 1) that are also present as transmitters in the peripheral tissues. This review of the literature below is focused only to SP, TRH and AVP since they have been the best characterized, also in the POP inhibitor studies.

SP

SP has been widely studied after its discovery in the 1930s (for reviews, see Leeman et al. 1974, Leeman 1980, Raffa 1998, Harrison et al. 2001, Severini et al. 2002, Hökfelt et al. 2003, Almeida et al. 2004). The amino acid structure of SP (H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2) was first identified by Chang et al. (1971) in the bovine hypothalamus. There are two potential cleavage sites for POP; Pro-Lys and Pro-Gln, of which the latter bond has been determined as being the main target for POP (Kato et al. 1980a, Yoshimoto et al. 1981). Mammalian SP derives from the pre-protachykinin-A (PPT-A) gene whose gene transcript produces three mRNAs (α-PPT-A, β-PPT-A and γPPT-A) that all encode for SP as well as other tachykinins (Carter et al. 1990). SP, and these other neuropeptides, are synthesised in the ribosomes and confined in the perikaryon. Thereafter, SP is packed into vesicles and axonally transported to the terminal endings for final enzymatic processing (Harrison et al. 2001, Hökfelt et al. 2003). SP is preferentially released from the synapse, not only in nerve endings but also from dendrites and the soma, under burst or high frequency firing, and it is metabolized by extracellular peptidases (Hökfelt et al. 2003). It is known that several peptidases are involved in the metabolism of SP. In addition to POP, also neutral endopeptidase (NEP, EC 3.4.24.11), SP-degrading enzyme (SP-DE, EC 3.4.24), angiotensin-converting enzyme (ACE, EC 3.4.15.1), dipeptidyl aminopeptide IV (DPIV, EC 3.4.14.5) and cathepsin D (EC 3.4.3.24) and E (EC 3.4.23.34) can degrade SP. NEP and ACE are thought to be the most important metabolizing enzymes of SP (Harrison et al. 2001).

SP is able to activate all tachykinin (neurokinin, NK) receptors – NK1, NK2 and NK2 – but its affinity is highest to the NK1 –receptor (NK1R, Harrison et al. 2001, Almeida et al. 2004). NK1R is a G-protein coupled receptor and its activation leads to intracellular IP_3 turnover with a resulting elevation of the intracellular calcium level (Almeida et al. 2004). It should be noted that POP is also implicated in the regulation of

IP_3 and calcium-signalling (see section 2.3.2.4), possibly through a regulation of IP_3 synthesis (Harwood et al. 2003).

SP and its receptors, NK1, NK2 and NK3 are distributed widely in peripheral tissues. SP-immunoreactive neurons projecting from sensory spinal ganglia have their terminals in the skin (epidermis, blood vessels and hair follicles or glands in the dermis), joints, submucosa and myenteric plexuses of gastrointestinal (GI) tract, respiratory tract, endothelial cells of blood vessels, genouritary tract and immune system (lymph nodes, spleen and thymus). SP has also been detected in smooth muscle, carcinoid tumors, cromaffin and acidophil cells and bones (for reviews, see Ribeiro-da-Silva et al. 2000, Harrison et al. 2001, Severini et al. 2002, Liu et al. 2007). Furthermore, NK1Rs of SP are widely present in the same organs (Quartara et al. 1998). Interestingly, the distribution of SP and NK1R follows only partially the distribution of POP enzyme activity in the peripheral tissues. Moderate POP activities have been measured from the GI tract (Fuse et al. 1990), lungs (Daly et al. 1985, Irazusta et al. 2002, Agirregoitia et al. 2005) and from spleen and lymphoid cells (Goossens et al. 1996). However, accurate and quantative determinations of POP locations in peripheral tissues have not been reported and therefore, spatial comparison of these substances is difficult.

The physiological responses of SP in the peripheral tissues are mediated via SP release from the peripheral endings of capsaicin-sensitive primary sensory neurons. Several substances and transmitters can affect the release of SP in both inhibitory (i.e. opiates, 5-HT agonists) or excitatory (i.e. bradykinin, prostaglandins, eicosanoids) directions. The binding of SP to the NK1R activates the phospholipase C (PLC) mediated IP_3-turnover leading to an increase in the intracellular level of Ca^{2+} (Harrison et al. 2001, Severini et al. 2002).

In the cardiovasculature, SP induces cGMP accumulation and activates Ca^{2+}-dependent nitric oxide (NO) synthesis in the endothelial cells of blood vessels, and therefore evokes vasodilatation and plasma extravasation (Quartara et al. 1998, Walsh et al. 2006), this latter effect being important in neurogenic inflammation (Harrison et al. 2001). The respiratory effects of SP are mediated through the dense NK1 (and partially NK2) receptor populations in the smooth muscle of bronchus. SP may induce bronchoconstriction or in some cases, bronchodilatation via NK1R mediated NO release in the endothelial cells. Moreover, the SP stimulated inflammatory response may be crucial in the hyperresponsiveness of asthma, and NK1R mRNA is increased in the respiratory smooth muscle cells of asthmatic subjects (Quartara et al. 1998, Harrison et al. 2001). In the gut, SP can affect both motility and secretion. The motility functions are excitatory, though some differences between species do exist (Harrison et al. 2001,

Severini et al. 2002). Moreover, intra-arterial infusion of SP increases the intestinal secretion of water and electrolytes and pancreatic juice (Severini et al. 2002). Even though these effects are mediated mainly via the NK2 receptors, and only to a minor extent via the NK1R, it has been found that NK1Rs are upregulated during inflammatory gut diseases, such as Crohn's disease, pseudomembranous colitis (Ribeiro-da-Silva et al. 2000, Harrison et al. 2001, Almeida et al. 2004) and pancreatis (Harrison et al. 2001), which have been interpreted as evidence for the inflammatory functions of SP. Furthermore, SP rich nerve fibres have been found in suburothelial layer and smooth muscle layer of the renal pelvis and ureter (Harrison et al. 2001). An i.v. infusion of SP increases the motility of the genitourinary tract and also causes plasma extravasation (Harrison et al. 2001, Severini et al. 2002).

SP may also be associated with the immune system, since it is abudantly present in various immune system organs and cell types. Its proposed participation in inflammation is supporting this association. It is known that SP induces B- and T-cell proliferation, immunoglobulin secretion, cellular chemotaxis, and lymphocyte migration both *in vitro* and *in vivo*. However, the mechanism of these actions has remained unclear (Quartara et al. 1998, Harrison et al. 2001, Severini et al. 2002). Furthermore, several studies (for review, see Liu et al. 2007) have linked SP to bone metabolism since SP can increase the proliferation and resorption of bones by an action on bone marrow.

Although there is a lack of studies concerning the involvement of POP in the SP hydrolysis in the peripheral tissues, some studies have reported higher POP activity in rheumatoid arthritis (Kamori et al. 1991) as well as in *Mycobacterium tuberculosis*-induced inflammation (Kakegawa et al. 2004). Furthermore, the activity and expression of POP in the lymphocytes and T-cells (Shirasawa et al. 1994, Vanhoof et al. 1994, Goossens et al. 1996) may link it to SP.

TRH

TRH was first found and characterized by Boler et al. (1969). Its tripeptidic structure (pyro-Glu-His-Pro-NH_2) contains the Pro-NH_2 bond that can be degraded by POP (Table 1). TRH is derived from TRH-precursor (pro-TRH) which is also a source of several other neuropeptides closely related to TRH but with different functions (for review, see Nillni et al. 1999). Pro-TRH is synthesized in the ribosomes and thereafter processed in the trans-Golgi-network before transportation to the immature secretory granules. Cleavage of the precursor to active TRH occurs by the action of prohormone convertases (PC1 and 2) and carboxypeptidase E (Cruz et al. 1996, Nillni et al. 1999).

TRH is metabolized extracellularly by pyroglutamyl aminopeptidase I and II (PAP I and II), thyroliberinase and POP (Nillni et al. 1999).

TRH has two known receptors, TRH-R1 and TRH-R2. These receptors belong to the G-protein-coupled receptor superfamily and their activation leads to the activation of calcium-dependent protein kinase via IP_3 turnover and/or to the activation PKC and MAPK, which can induce gene transcription via three transcription factors i.e. cAMP response element-binding (CREB), AP-1 and Elk-1. Actions of TRH-R1 and TRH-R2 are rather similar, but the activation of the transcription factors seems to be dominate in the TRH-R2 expressing cells (for review, see Sun et al. 2003). TRH-immunoreactivity and TRH-receptors (both type 1 and 2, TRH-R1/R2) are present in various organs. Cao et al. (1998) determined TRH-R1 from rat heart, spleen, liver, lung, skeletal muscle, kidney and testis using northern blot. Moreover, immunoreactive TRH-R1 and TRH-R2 was found extensively in rat GI tract (Auerbach's nervous branch, Meissner's nervous branch and mucosa of the stomach), testis and retina, while in adrenal medulla only TRH-R1 immunoreactivity was seen (Mitsuma et al. 1995, Mitsuma et al. 1999). Intense expression of TRH is also present in the pancreas, where TRH is synthesized in the insulin-producing β-cells (Leduque et al. 1989).

Even though TRH has receptors in various internal non-neuronal tissues, its peripheral functions are not well understood and even many of its peripheral actions are mediated via the CNS and the autonomic nervous system. The most important effect of TRH to peripheral tissues is its indirect involvement to the functions of thyroid gland. However, since this action is mediated via thyroid-stimulating hormone (TSH), it will be not discussed in this text. Similar to SP, TRH can also affect the GI-tract. Intracerebroventricular injections of TRH induce gastric acid secretion, gastric emptying and intestine motility in rats and rabbits, pointing to a role for central TRH in the vagal stimulation of GI motility. The abundant presence of TRH receptors in the GI-tract is also and indication that there may be peripheral release of TRH, but central TRH is far more potent than i.v. TRH in inducing GI effects (Nillni et al. 1999, Fujimiya et al. 2000, Beglinger et al. 2002). Moreover, TRH inhibits food and water intake but this function is regulated via the hypothalamus (for review, see Nillni et al. 1999). The cardiovascular effects of TRH have also been studied. TRH can modulate cardiac contractility as an autocrine regulator in a concentration-dependent manner (Socci et al. 1996) and i.v. administration of TRH to rats with ischemic cardiomyopathy evokes an increase in heart rate, arterial pressure and cardiac output via the regulation of autonomic nervous system (Jin et al. 2004). In the respiratory system, TRH is able to raise the respiratory level at high doses. However, even though TRH receptors are distributed in the lungs, the respiratory control of TRH is mostly mediated via the

respiratory motoneurons in the medulla (Nillni et al. 1999). The most important paracrinal effects of TRH occur in the pancreas, where TRH and insulin secretions are inversely related (Ebiou et al. 1992), i.e. TRH inhibits pancreas secretion and enhances arginine-stimulated glucagon release (Fragner et al. 1997). Furthermore, high levels of TRH are present in the genitourinary tract, and TRH may act as a paracrinal regulator in the reproductive system (Nillni et al. 1999).

The association of POP with the peripheral functions of TRH has been seldom studied. High POP activity is present in the porcine pancreas (Yoshimoto et al. 1982) and in the rat pancreatic cell lines (Salers 1994), though Fuse et al. (1990) failed to detect any POP in rat pancreatic homogenates. The possible involvement of POP in controlling the level of pancreatic TRH in rats during the development has been studied by Salers et al. (1992). Even though POP degraded TRH *in vitro*, it did not hydrolyze TRH *in vivo,* evidence that the pancreatic TRH content appears to be principally regulated during the biosynthetic steps.

AVP

AVP, also known as antidiuretic hormone, was one of the first neuropeptides recognized, being first described in 1895. It is primarily known as the key regulator of water and electrolyte balance (for reviews, see Bisset et al. 1988, Rose et al. 2002, LeJemtel et al. 2007, Rinaman 2007, Caldwell et al. 2008). AVP's amino acid structure, Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH2, can be cleaved by POP at the Pro-Arg bond (Table 2), and it was also one of the first peptides to be confirmed as being degraded by POP (Walter 1976). Although most of the AVP neurons are located in the brain supraoptic and paraventricular nuclei (SON and PVN) of hypothalamus, AVP receptors are also extensively distributed in peripheral tissues, pointing to various physiological roles for this peptide. AVP has two main receptor types, V1R and V2R, of which V1R is divided into two subtypes, $V1_aR$ and $V1_bR$ (or V3R). $V1_aR$ are mostly found in the liver, kidney, adrenal cortex, blood vessels, platelets, lymphocytes and monocytes and blood vessels (for reviews, see Lee et al. 2003, Caldwell et al. 2008), while the $V1_bR$ mRNA is found in the kidney, thymus, heart, lung, spleen, uterus, and breast (Lolait et al. 1995). The most well known peripheral AVP receptor is V2, which is present in the surface of the principle cells of the collecting tubules of kidney, regulating water reabsorption (Phillips et al. 1990, Lolait et al. 1995, Birnbaumer 2000, Lee et al. 2003, Caldwell et al. 2008). Even though AVP is found in most peripheral tissues, its functions are relatively unknown, with the exception of its well known role in the kidneys and vascular smooth muscles.

The most important peripheral action of AVP is the regulation of water reabsorption. Changes in plasma osmolality are detected by hypothalamic osmoreceptors and activation of these neurons releases AVP into the circulation from the axon terminals of posterior pituitary. The binding of AVP to the V2 receptors in the collecting tubules of kidney activates G_s protein mediated cAMP synthesis, and the synthesis of aquaporin-2 (AQ2) water channel proteins, which shuttle to the apical surface of collecting duct, allowing free water to pass across the apical membrane (for reviews, see Birnbaumer 2000, Lee et al. 2003).

The vascular effects of AVP are mediated via the $V1_bR$ subtype. AVP release is stimulated via the cardiopulmonary and sinoaortic baroreceptors, which detect reductions in blood pressure, especially during dehydration, intense hypotension or shock. The release of AVP is accompanied by the activation of the renin-angiotensin system. The binding of AVP to $V1_bR$ of the vascular smooth muscle activates the G_q protein mediated IP_3 second messenger system, resulting in the release of intracellular Ca^{2+} and arterial vasoconstriction (for reviews, see Schrier et al. 1993, Lee et al. 2003). Furthermore, the hypothalamic AVP release is associated with liver growth and increased bile flow after partial hepatectomy, through the $V1_aR$ (Nicou et al. 2003). Furthermore, in the liver, AVP has been linked to the glycogen homeostasis, since it is able to modify glycogen phosphorylase activity (Kirk et al. 1979). Also $V1_aR$ knock-out mice exhibit major deficiencies in glucose homeostasis (Aoyagi et al. 2007). Within the blood cells, AVP can cause platelet aggregation and the coagulation of monocytes and lymphocytes (Inaba et al. 1988).

The effects of POP on peripheral AVP has been poorly studied, but interestingly, the activity of POP is decreased in atria and increased in ventricles as a consequence of hypertension such as that seen in left renal artery obstructed rats with nephrectomy (Cicilini et al. 1994). However, it is not known how the resulting hypertension affects AVP and whether there is a correlation between POP activity and the effects of AVP on kidney/blood vessels. Furthermore, renal hypertensive patients displayed a significant correlation between the POP and ACE activities in serum (Goossens et al. 1996). Nevertheless, this action of POP may be partially mediated by the hydrolysis of angiotensins I and II to angiotensin (Garcia-Horsman et al. 2007a) and the true nature of serum POP activity is unclear (see chapter 2.3.3).

2.3.1.3 The role of POP in cell proliferation and differentiation

POP has been implicated in several studies to cell proliferation and differentiation *in vitro* and even *in vivo* after POP was found localized in the nuclei of nonneural cell

lines (Ishino et al. 1998) and in the *Sarcophaga peregrina* (fresh fly). Ohtsuki et al. (Ohtsuki et al. 1994, Ohtsuki et al. 1997b) postulated that POP may have a crucial role in cell differentiation since ZTTA, a specific POP inhibitor, prevented differentiation of the imaginal discs of *Sarcophaga peregrina* (fresh fly). They also found that ZTTA inhibited DNA synthesis, and therefore cell proliferation, in a *Sarcophaga* cell line (Ohtsuki et al. 1997a). Similar results have been obtained with the mouse Swiss 3T3 cell line (Ishino et al. 1998).

POP activity in several rat organs is also high during embryonic and early development stages and this activity becomes reduced in adulthood (Fuse et al. 1990, Matsubara et al. 1998, Agirregoitia et al. 2003a, Agirregoitia et al. 2007). Kimura et al. (2002) observed changes in the localization of POP mRNA during sexual maturation in mouse testis, suggesting that POP may be involved in meiosis and maturation of spermatozoa. Furthermore, high POP activity in the cancerous tissues (Goossens et al. 1996) may reflect to a role for this enzyme in cell division and/or differentiation.

The mechanism of how POP can modulate DNA synthesis and/or cell differentiation has remained unknown. Possible substrate for POP participating to these functions has not been identified. Interestingly, Ikura et al. (2008) recently found that POP may act similarly to the peptidyl-prolyl isomerases (PPIase) and accelerate the change of prolyl *cis-trans* isomerization. These workers studied the isomerization of the Ala-Pro-bond in N-succinyl-Ala-Ala-Pro-Phe-4-methylcoumaryl-7-amide (AAPF-MCA) and observed that POP was able to catalyse this reaction with moderate activity although at a slow catalytic rate when compared to the other PPIases in the same study. However, changes in the prolyl *cis-trans* isomerization may lead to different actions of the same protein, the conformation changes are associated with several functions in molecular timing, involving the cell cycle, cell signalling, gene expression, immune response and neuronal functions (Lu et al. 2007). This may offer novel physiological roles for POP and explain the involvement of POP in the cell cycle and proliferation. However, more studies clarifying the role of POP in the DNA synthesis/cell proliferation are needed.

2.3.2 POP in the CNS

POP has been studied most intensively in the brain. In enzyme activity measurements, the highest activities have been generally found in the brain, especially in the cerebral cortex where bioactive neuropeptides, such as SP, TRH and AVP are partially located (Kato et al. 1980b, Daly et al. 1985, Irazusta et al. 2002, Agirregoitia et al. 2005). Moreover, the basis of POP inhibitor development was to generate a novel

drug which would prevent the metabolism of bioactive neuropeptides in the brain (Kato et al. 1980a, Cunningham et al. 1997a). These findings have raised the preconception that POP must have an important physiological function in the brain.

2.3.2.1 POP enzyme activity and mRNA distribution in the brain

POP enzyme activities have been found in various brain areas. Kato et al. (1980) studied the distribution of POP activity in the human brain and found the highest activity in the cerebral cortex, while other areas exhibited much lower activities. Similar results were obtained from the rat brain by Daly et al. (1985) who also found a rather high POP activity in the cerebellum and slightly lesser activity from the brain stem. Cortex has been the most POP-active brain area also in the other distribution studies conducted in rat brain (Fuse et al. 1990, Irazusta et al. 2002, Agirregoitia et al. 2005). In the rat brain, lower POP activities were measured in the hypothalamus (Fuse et al. 1990, Irazusta et al. 2002), hippocampus, cerebellum and amygdala (Irazusta et al. 2002). Moreover, Agirregoitia et al. (2005) measured the same levels of enzyme activities in the rat cortex, striatum and cerebellum. However, a valid comparison of enzyme activities between studies is rather difficult, since most of these studies were made using different substrates and under different conditions. Furthermore, the presence of an endogenous POP inhibitor (Yoshimoto et al. 1982, Salers 1994, Yamakawa et al. 1994) and other regulators of POP activity may have influenced the results (see section 2.3.2.3).

Bellemere et al. (2004) studied the distribution of the POP mRNA in rat brain and pituitary by quantitative RT-PCR analysis and in situ hybridization. The highest amounts of POP mRNA were found in the cerebellum and hypothalamus. Interestingly, the mRNA amounts in the cerebral cortex were only approximately one half of the amounts in the cerebellum (Bellemere et al. 2004), even though in terms of enzyme activity measurements, the situation was reversed. Minor amounts of POP mRNA were observed in the substantia nigra, medulla oblongata and spinal cord. In the high-throughput gene expression profiling, the POP mRNA levels have been generally rather equal among different brain areas. In human brain, the highest POP mRNA levels have been found from the hypothalamus and prefrontal cortex, while the lowest levels were observed from the cerebellum. In the mouse brain, substantial amounts of POP mRNA were found from the cerebellum and preoptic area, while in the rat the highest levels were observed from the hippocampus and dorsal striatum. In these species, the expression of POP mRNA in the rest of the brain areas was rather equal (GNF SymAtlas, Su et al. 2002).

The distribution of POP between different neuronal cell types has been analyzed in a few studies. Mentlein et al. (1990) measured the highest POP enzyme activity from rat neurons, followed closely by astrocytes, and concluded that there was expression of POP in both the neurons and glial cells. The enzyme activity was significantly lower in the oligodendrocytes. Somewhat similar results were obtained by Schulz et al. (2005), when they measured POP activities in rat neuron-, astrocyte-, oligodendrocyte- and microglial-rich primary cultures. However, in this study, the difference between neurons and glial cells was significant, and moderate POP activity was seen only in the astrocytes. Furthermore, *in vivo* in the mouse brain, no expression of POP was seen in the glial cells (Rossner et al. 2005).

The conclusions from these studies is that in the CNS POP is mostly present in the neurons, but minor expression can also be present in the glial cells. However, discrepancies between POP enzyme activity and mRNA point to some post-translational or inhibitor-mediated regulation of POP activity in the CNS.

2.3.2.2 Subcellular localization of POP in the CNS

POP is mainly considered as a soluble cytosolic enzyme (Dresdner et al. 1982, Schulz et al. 2005) although the membrane-bound form exists (O'Leary et al. 1995). Membrane-bound POP has been found in the membranes of various cell lines (Chappell et al. 1990) and in the synaptosomal fractions of bovine brain (O'Leary et al. 1995). This form of POP is able to hydrolyze the same substrates as the soluble cytosolic form (O'Leary et al. 1996) but its activity or amounts are somewhat lower than that of cytosolic POP (Irazusta et al. 2002, Agirregoitia et al. 2005).

POP activity has been found in the nuclear, mitochondrial, synaptosomal and microsomal fractions of rat and human brain while the highest activities were located in the cytosolic fractions (Irazusta et al. 2002). However, the enzymatic activity of POP in brain nuclear fraction may be questionable since no POP activity or protein had been seen in the nucleus of neuronal cells in the other studies (Dresdner et al. 1982, Rossner et al. 2005, Schulz et al. 2005). Moreover, Schulz et al. (2005) observed using immunofluorescence techniques that POP was attached to the main component of microtubulin cytoskeleton, tubulin, in human glioma cell lines. Furthermore, POP appeared to be mostly localized in the perinuclear space of the cell in human glioma and neuroblastoma cell lines, with no POP being detected in the nucleus.

2.3.2.3 Regulation of POP enzyme activity and expression in the brain

The regulation of POP enzyme activity and gene expression has been studied to some extent but it is still far from clear. An endogenous POP inhibitor may be influencing the enzymatic activity of POP (see section 2.3.1.1). However, this compound has been poorly biologically characterized.

Several substances are able to affect to the POP activity in the brain. Polyamines (e.g. spermine, spermidine) have increased POP activity in the brain and they are able to reverse the effect of POP endogenous inhibitor *in vitro* (Soeda et al. 1986). Moreover, treatment with a non-competitive NMDA-receptor antagonist, MK-801, was able to increase POP activity in the hippocampus and cerebral cortex of rat, while a $GABA_A$-receptor blocker, pentylenetetrazol, and atypical antipsychotic drug, clozapine, decreased POP activity in the same brain areas (Ahmed et al. 2005, Arif et al. 2007). Moreover, oxidizing agents may inhibit POP activity, at least in cell lines (Tsukahara et al. 1990). Even changes in plasma volume and/or osmotic pressure have been reported to affect to brain POP activity (Irazusta et al. 2001).

Furthermore, in a microarray analysis of the effects of aging on gene expression in the mouse hypothalamus and cortex (Jiang et al. 2001), the POP gene expression in 22 months old mice was 11-fold in hypothalamus and in cerebral cortex it was 2.7 fold higher than the values in 2 months old mice. In further support, Rossner et al. (2005) used immunohistochemistry to demonstrate increased POP expression in the hippocampus of aged mouse. Furthermore, POP gene expression was down-regulated by 2.6-2.7-fold after exposures of 3 and 6 hours to an enriched environment (Rampon et al. 2000), pointing to the age- and learning-dependent regulation of POP expression

2.3.2.4 POP and its proposed substrates in the CNS

POP has been implicated in the hydrolysis of several neuropeptides in the brain (Table 1) of which, SP, TRH and AVP have been the best characterized in the POP inhibitor studies and therefore, the following text is limited to these neuropeptides. Furthermore, the involvement of POP in IP_3 signalling system will be discussed in the text below.

SP

SP and NK1Rs are distributed rather widely in the brain. The highest expression of SP in the rat brain has been found in the cortical amygdaloid nucleus, ventral pallidum, substantia nigra, globus pallidus and spinal cord laminae I-II followed by striatum,

nucleus accumbens and periaqueductal gray (for reviews, see Tohyama et al. 1998, Ribeiro-da-Silva et al. 2000). Interestingly, in the cerebral cortex, only sparse expression of SP has been observed (Tohyama et al. 1998, Ribeiro-da-Silva et al. 2000) although the highest POP activities have been detected in the cortex (see section 2.3.2.1). NK1Rs are also widely distributed in the CNS, but similarly to SP, their expression is only moderate in the cerebral cortex (Yip et al. 2000, Yip et al. 2001).

SP has been a target of pharmaceutical interest, since it has been associated with various physiological functions and diseases in the CNS. In general, SP is recognized as a neurotransmitter involved in pain signalling from peripheral tissues to CNS, since there are high concentrations of SP and NK1R in the dorsal root of spinal cord (for review, see Harrison et al. 2001). However, involvement of SP in memory and learning served originally as a rationale to develop POP inhibitors to prevent the hydrolysis of this peptide (Kato et al. 1980a). Injections of SP to the brain areas have shown positive reinforcement effect on learning and systemic injection has been effective in alleviating age-related learning deficits (Huston et al. 1995, Hasenohrl et al. 2000). The mechanism of action for SP in memory and learning is unclear, but it presumably operates as a reinforcer of cortical cholinergic neurons, affecting their activity and acetylcholine (ACh) release (Hasenohrl et al. 2000), and possibly acting as an enforcer of long-term potentiation (LTP) in hippocampus (Langosch et al. 2005). In some studies, POP inhibitors have shown memory enhancing effects (Toide et al. 1995a, Toide et al. 1997) but the effects of these inhibitors on the SP levels in the brain are debatable (Table 2). SP is also claimed to be associated with fear-related behaviour since it is released by adverse stimulation, and anxiolytic and antidepressant drugs are able to reduce SP levels in the brain (Raffa 1998, Hasenohrl et al. 2000). Moreover, in animal models, SP agonists have showed anxiogenic effects while SP antagonists have reduced anxiety (Hasenohrl et al. 2000, Hökfelt et al. 2003). Nevertheless, the association of POP with SP in pain transmission or in anxiety has not been adequately studied although some changes in serum POP activity have been observed in patients suffering from fibromyalgia and psychiatric disorders (Table 3).

SP has been linked to the neurodegenerative diseases such as Alzheimer's and Parkinson's disease. In both diseases, depletion of SP in the brain has been observed but not invariably (Beal et al. 1987, Raffa 1998). The association of SP to Parkinson's disease appears to be more convincing, since GABAergic efferent nigrostriatal neurons are known to use SP as a neurotransmitter (Emson et al. 1979, McGinty 2007). Moreover, parallel alterations of brain POP enzyme activity have been reported in both diseases (Mantle et al. 1996). POP has also been associated with the generation of β-amyloid protein in Alzheimer's disease (Kato et al. 1997, Shinoda et al. 1997).

However, the changes in POP enzyme activity in brain are not unambiguous and POP activity changes in the generation of β-amyloid are likely to result from neuronal degeneration (Petit et al. 2000, Laitinen et al. 2001, Rossner et al. 2005).

Several studies have connected SP to the early development of the brain due to the changes in its expression during the embryonic period, perhaps reflecting a role for SP as a neuroprotective agent or even as a growth factor in the brainstem (for review, see Raffa 1998). Interestingly, also POP enzyme activity levels have been shown to increase during embryonic development and to decrease during the postnatal period (Agirregoitia et al. 2003b, Agirregoitia et al. 2007) and some studies have postulated that POP may function as a neuroprotective agent (Rossner et al. 2005). Nevertheless, it is not known whether POP is involved in these events through its hydrolytic or non-catabolic functions.

TRH

TRH and TRH-receptors are distributed throughout the human and rat brain while the highest amounts are located in the hypothalamus (Lechan et al. 1982, Parker et al. 1983, Mantyh et al. 1985), especially in the paraventricular nucleus (Jackson et al. 1985). Rather high amounts of TRH/TRH-receptors have also been detected in the olfactory bulb, amygdala, dentate gyrus of hippocampus and entorhinal cortex (Parker et al. 1983, Mantyh et al. 1985). The distribution pattern of the mRNA's of TRH-R1 and TRH-R2 are somewhat different. TRH-R2 is present preferentially in the cortex and thalamus of rat brain, while R1 is mostly present in the hypothalamus (Sun et al. 2003). Interestingly, similarly to the situation with the TRH/TRH-receptors, high amounts of POP mRNA (Bellemere et al. 2004) and moderate POP enzyme activities have been measured in the hypothalamus (Fuse et al. 1990, Irazusta et al. 2002).

The most important CNS function of TRH is the control TSH release via the stimulatory input from the hypothalamus in the hypothalamic-pituitary-thyroid (HPT) axis (for reviews, see Fliers et al. 1998, Nillni et al. 1999, Lechan et al. 2004). However, in the context of the POP research, the ergotropic effects (arousal, sleep, cognition, locomotor activation and antidepressant) of TRH, especially in cognition, have been a focus of interest. The cognitive aspects of TRH are likely to be mediated by enhanced cortical ACh turnover in the frontal cortex and hippocampus (Toide et al. 1993, Itoh et al. 1994, Ogasawara et al. 1996). As mentioned before, POP is able to deaminate TRH or His-Pro-NH_2 product after PAP I/PAP II/thyroliberinase cleavage *in vitro* (Yanagisawa et al. 1980) but POP inhibitors have failed to modify TRH levels in cell line experiments (Mendez et al. 1990, Salers et al. 1991). However, in animal studies,

treatment with a POP inhibitor JTP-4819 has restored decreased TRH-like immunoreactivity (TRH-LI) in the cortex but not in the hippocampus of young rats (Shinoda et al. 1995, Toide et al. 1996) and another POP inhibitor, S17092, was able to increase TRH-LI in the rat cortex in a dose dependent manner (Bellemere et al. 2005). However, the lack of effect in cell line experiments and the controversial results *in vivo* cast serious doubt on the ability of POP inhibitors to influence the TRH/TRH-LI levels in the brain. Interestingly, despite the above findings, no effects of POP inhibitors to the regulation of physiologically important HPT-axis have been reported.

AVP

AVP synthesis takes place together with another proposed POP substrate, oxytocin (Walter et al. 1971), within the neurons of SON and PVN. AVP is synthesized with its carrier protein or neurophysins (NPI and NPII) as a part of a precursor or pre-prohormone in the perikarya of the mangocellular neurons. This precursor is packaged into granules and transported to the axon terminals in the neurohypophysis and the cleavage and maturation of the peptide occurs during this transport. Although most of the AVP neurons are located in the SON and PVN, projecting to the hypophysis, a small population of these AVP synthesizing parvocellular neurons are located in the PVN, bed of nucleus of the stria terminalis, medial amygdala and suprachiasmatic nucleus and their projections remain within the brain (for reviews, see Bisset et al. 1988, Rinaman 2007, Caldwell et al. 2008). The main regulator of AVP release under normal conditions is plasma osmolality or blood volume/pressure mediated via baro-, stretch- and chemoreceptors (Bisset et al. 1988, LeJemtel et al. 2007). Changes in plasma osmolality can also affect the gene expression of AVP (Hayashi et al. 2006). Furthermore, cholinergic and GABAergic neurons may act as inhibitory or excitatory transmitters for AVP release (Bisset et al. 1988). Sex hormones are also able to regulate the release of AVP (Fink et al. 1996).

In the peripheral tissues, $V1_aR$ is mostly present in the liver, smooth muscle (Birnbaumer 2000) and its mRNA is abundantly present in the hypothalamus and moderately in the hilus of hippocampus, granule cells of cerebellum, lateral septum, ventral tegmental area and brain stem (Ostrowski et al. 1994). However, its physiological function remains rather unclear except that it may be involved in the behavioral effects of AVP (see below). $V1_bR$ stimulates the release of adrenocorticotropin from the anterior pituitary but these receptors are widely distributed throughout the CNS. High amounts of immunoreactive $V1_bR$ are present in the hypothalamus, olfactory tubercule, hippocampal CA fields, while it is moderately

present in the frontal cortex, caudate putamen, substantia nigra, dentate gyrus of hippocampus and granule cell layer of cerebellum (Hernando et al. 2001). Autoradiographie studies have confirmed the V1R distribution in the CNS (Brinton et al. 1984). V2R is present mostly in the peripheral tissues, in the distal nephron, regulating water reabsorption (see section 2.3.1.2). These three AVP receptors are G-protein coupled receptors. V1Rs can induce IP_3-mediated Ca^{2+}-release from endoplasmic reticulum while V2R activation triggers the activation of cAMP mediated opening of the water channel thus increasing water reabsorption (Birnbaumer 2000, Caldwell et al. 2008). The distribution of CNS receptors of AVP is rather similar with the mRNA (Bellemere et al. 2004) and enzyme activity distribution of POP (Fuse et al. 1990, Irazusta et al. 2002).

Similar to the situation with TRH, the interest of POP and AVP has focused on the role of AVP as a behavioral regulator. AVP has been implicated as being involved in social behaviors such as aggression and affiliation, social and non-social memory and to anxiety and depression. The behavioral effects of AVP are evidently mediated through $V1_aR$ and $V1_bR$, since the aggression, social and spatial memory are impaired in $V1_aR$ and $V1_bR$ knock-out mice. Furthermore, injection of AVP to lateral septum can increase social recognition and male aggression in mice (for review, see Rose et al. 2002, Caldwell et al. 2008). The exact mechanism of the AVP regulated behavioral effects are not known, but interactions with serotonin (5-HT) (Ferris et al. 1999) and LTP in the hippocampus have been reported (Sarvey et al. 1989, Dubrovsky et al. 2003). POP inhibitors have shown possibly AVP-mediated memory and cognitive improvements in some animal models (Toide et al. 1995a, Toide et al. 1997) and acute administration of S17092 and JTP-4819 has increased AVP-like immunoreactivity (AVP-LI), especially in the hippocampus of young rats (Table 2, Toide et al. 1996, Bellemere et al. 2005). However, no changes of the AVP levels in the hippocampus or cortex were seen after repeated administration of POP inhibitors (Table 2, Toide et al. 1995a, Toide et al. 1995b, Shinoda et al. 1996, Bellemere et al. 2005) pointing to some discrepancies in the effects of POP inhibitors. Similarly to TRH, no studies have reported the involvement of POP inhibitors in the antidiuretic properties of AVP nor has the effect of POP inhibitors been examined on the AVP levels in the hypothalamus.

IP_3

IP_3 is a second messenger that especially affects the release of Ca^{2+} from the rough endoplasmic reticulum (RER) (Ferris et al. 1992, Berridge 1993, Hokin et al. 1993). IP_3 mediated Ca^{2+} release has been linked to several important cell functions such as

activation of transcription factors, e.g. CREB (Furuichi et al. 1995) and NO (Iino 2006). Furthermore, IP_3 signalling is involved in LTP and long-term depression (LTD), two well established parameters in learning and memory (Lynch et al. 1991, Khodakhah et al. 1997, Jun et al. 1998, Fujii et al. 2004). IP_3 regulated LTP and LTD has been observed in hippocampal CA1 pyramidal cells (Jun et al. 1998, Fujii et al. 2004), one of the most important areas of memory and learning, and in cerebellar Purkinje cells (Khodakhah et al. 1997). In hippocampus, LTP is induced by the activation of N-methyl-D-aspartate (NMDA) glutamate receptors (Bliss et al. 1993, Fujii et al. 2004).

Recently, several studies have connected POP and IP_3 signalling. Firstly, Williams et al. (1999) observed that a lithium resistant mutant *Dictyostelium discoideum* was lack of POP encoding gene. Thereafter, they observed that mutant *Dictyostelium* had 3-fold IP_3 levels after 10 mM of LiCl administration when compared to the wild type cells (Williams et al. 1999). As a further support, Schulz et al. (2002) generated POP antisense glial cell line and observed an inverse correlation between IP_3 and POP expression. Similar relationships between POP enzyme activity and IP_3 levels were obtained when POP inhibitors were applied to the wild-type *Dictyostelium* (Williams et al. 1999) and human glioma and neural cell lines (Schulz et al. 2002). These findings have proposed that POP may be involved in regulation of IP_3 signalling pathway. Interestingly, administration of POP inhibitor did not affect to the functions cAMP pathway, but cAMP was increased in the POP antisense glial cell line (Schulz et al. 2002).

The mechanism of how POP control over IP_3 synthesis is not clear. POP is not a direct target for Li^+ since it had no effect to the POP activity (Williams et al. 1999, Cheng et al. 2005). Furthermore, POP does not degrade IP_3 metabolizing enzymes, since Williams et al. (1999) showed increased IP_3 degradation in the mutant *Dictyostelium*. Therefore, POP may be able to inhibit MInsPP that generates IP_3 from IP_6 (Harwood et al. 2003). POP inhibition activates MInsPP and therefore increases the synthesis of IP_3. Another connection for POP to the IP_3 and Ca^{2+} signalling is the PEP-19 that is able to influence Ca^{2+} signalling via binding to calmodulin. Brandt et al. (2005) revealed that during incubation of porcine brain homogenates with POP, a peptide sequence that is minimum domain of PEP-19 to bind calmodulin, disappeared. Moreover, Williams et al. (2000) studied the effects of Li^+, VPA and carbamazepine in the POP mutant *Dictyostelium* cell line. They observed that lack of POP gene made mutant *Dictyostelium* resistant also to VPA induced IP_3 depletion. Furthermore, the administration of two different POP inhibitors, Z-Pro-Prolinal and BOC-Glu (NHO-Bz)-Pyr, to the rat neural explant cultures block the expansive effects of Li^+, VPA and carbamazepine to the growth cone area. Similar effect was obtained using pure inositol

(Williams et al. 2000). Recently, Cheng et al. (2005) revealed that *in vitro* VPA inhibited recombinant human POP activity at therapeutic blood levels of the drug (1 mM) and they even suggested that POP may be a direct target for VPA. These findings are suggesting that IP_3 signalling pathway is involved in mood control and may be a common target of a very diverse group of mood stabilizing drugs, such as Li^+, VPA and carbamazepine (Hokin et al. 1993, Williams et al. 2000, Agam et al. 2002, Cheng et al. 2005, Williams 2005) and that POP may be involved in the regulation of these actions.

Interestingly, all previously discussed neuropeptides (SP, TRH and AVP) influence the IP_3 second messenger system via their G-protein receptors. However, the possible association between POP, neuropeptides and IP_3 signalling has not been clarified. One serious complication against the hypothesis that POP significantly influences the metabolism of neuropeptides in the brain is their different cellular localization. Neuropeptides are released outside the cell directly from the storage vesicles whereas POP is mainly an intracellular enzyme. How is it possible for an intracellular enzyme to metabolize neuropeptides released outside the cell? Membrane-bound POP may be able to cleave neuropeptides in the vicinity of the cell membrane assuming that it is orientated extracellularly. However, relatively low activity and/or amounts of membrane-bound POP exist in the brain (Irazusta et al. 2002, Agirregoitia et al. 2003b) and this is probably insufficient to significantly contribute to the metabolism of neuropeptides. Furthermore, even though intracellular receptors for SP (Baude et al. 1998, Levesque et al. 2007) and TRH (Sun et al. 2003, Cook et al. 2004) have been found, these are more likely internalized receptors being recycled in a desensitization pathway (Hökfelt et al. 2003, Sun et al. 2003, Cook et al. 2004) and their agonists are not able to activate the receptors when they are in the intracellular space. This contradiction may be the reason for inconsistent effects of POP inhibitors on neuropeptide levels in the brain. Therefore, the participation of POP on IP_3 signalling in the brain could explain some of the neuropeptide-like results obtained with POP inhibitors in animal studies, such as the beneficial effects of POP inhibitors to memory and learning. Nevertheless, no significant changes in rat brain cortical or hippocampal IP_3 levels were seen *in vivo* after five consecutive doses (9 µmol/kg) of JTP-4819 administered every 12 h (Jalkanen et al. 2007). However, the distribution of POP is apparently restricted to only a moderate amount of neurons in the cerebral cortex and therefore the effects of POP inhibitors to the IP_3 levels may be overlooked in these measurements.

2.3.3 POP in the serum

Various studies have reported some changes of POP activity in human serum in several diseases (Table 3). These analyses have been made with a fluorometric method, using Z-glycyl-prolyl-4-methylcoumarinyl-7-amide as a substrate, by the method of Goossens et al. (Goossens et al. 1992, Goossens et al. 1996).

Table 3. Effects of various diseases to POP enzyme activity in human serum

Disease	**Effect to POP activity**	**Reference**
Alcoholism	↓	Maes et al. 1999a
Anorexia nervosa	↓	Maes et al. 2001
Depression	↓	Maes et al. 1994, Maes et al. 1995
Fibromyalgia	↓	Maes et al. 1998b
HIV infection	↓	Goossens et al. 1996
Malaria infection	↓	Goossens et al. 1996
Mania	↑	Maes et al. 1995
Prostate carcinoma	↑ (hormonally treated) ↓ (untreated)	Goossens et al. 1996
Post-traumatic stress disorder	↑	Maes et al. 1999b
Renal hypertension	↑	Goossens et al. 1996
Schizophrenia	↑	Maes et al. 1995
Stress induced anxiety	↑	Maes et al. 1998a

↑, increased POP serum activity; ↓, decreased POP serum activity

However, the true nature of POP activity in the serum is questionable. The examined POP was partially purified from bovine serum, and interestingly, this serum POP failed to hydrolyze several fluorometric substrates and had significantly lower specificity towards several well-established POP substrates - such as bradykinin and SP - than tissue POP, suggesting that perhaps it represents a previously unknown protease with POP-like functions (Cunningham et al. 1998). Cunningham and O'Connor (1997) also found that another POP-like enzyme, named as Z-Pro-Prolinal-insensitive POP (ZIP) in bovine serum. This enzyme was resistant to the classical POP inhibitor Z-Pro-Prolinal, but still capable of hydrolyzing Z-glycyl-prolyl-4-methylcoumarinyl-7-amide

substrate (Cunningham et al. 1997b). After this finding, ZIP was purified from bovine serum and it was identified as an 87 kDa monomer enzyme exhibiting several similarities to POP (Birney et al. 2001). ZIP was found to have the ability to hydrolyze some proline-containing neuropeptides (angiotensin and TRH) but failed to degrade others (SP, bradykinin and neurotensin) (Birney et al. 2001). In a further study, the ZIP protein sequence was analyzed and it was identified as seprase/fibroblast activation protein α (FAP, EC 3.4.21.B28). FAP is resistant to another well documented POP inhibitor, JTP-4819, but still capable of hydrolyzing the Z-glycyl-prolyl-4-methylcoumarinyl-7-amide substrate (Collins et al. 2004). Nevertheless, ZIP or FAP/Seprase (Aoyama et al. 1990) can be inhibited with cysteine protease inhibitors (Birney et al. 2001). Breen et al. (2004) studied the POP and ZIP enzyme activities in the serum of bipolar disorder and schizophrenic patients using Suc-Gly-Pro-MCA as the substrate. However, no significant differences were found in POP activity between two groups (Breen et al. 2004). Therefore, the alterations of serum POP-like activity in diseases may reflect the enzymatic activities of ZIP/FAP rather than POP, especially when measured with Z-glycyl-prolyl-4-methylcoumarinyl-7-amide.

FAP was first identified as a 170 kDa homodimer, containing two 97 kDa subunits, in the malignant melanoma cell line LOX (Aoyama et al. 1990). Similar to the DPPIV, the closest relative to POP (Venäläinen et al. 2004), FAP is a type II membrane protein, capable of degrading Pro-Xaa –bond, and its sequence is rather similar to that of DPPIV (Havre et al. 2008). However, FAP has generally not been found in normal adult tissue but it is believed to be involved in wound healing and various cancers (Garin-Chesa et al. 1990, Havre et al. 2008). This may connect FAP to the increased POP-like activity in cancer (Goossens et al. 1996), but the membrane-bound nature of FAP is a true dilemma considering its POP-like activity in serum.

3 AIMS OF THE STUDY

The objective of this study was to localize POP protein in various tissues, also at the cellular and subcellular levels, and to determine associations of POP with specific neuronal neurotransmitters and substrates in the brain. The specific aims of this neuroanatomical study were:

1. To examine the distribution and amounts of POP protein in the mouse whole-body and peripheral tissues, and compare them to the enzymatic activity (**I**).

2. To determine and quantitate the amounts of POP protein in the main functional areas of human and rat brain and compare it to the previous results of POP distribution as assessed by enzyme activity measurements and mRNA in situ hybridization **(II).**

3. To clarify the expression of POP at the cellular and subcellular levels in rat brain and to study its associations with specific neuronal neurotransmitters (**III**).

4. To study the spatial associations of POP and its suggested substrates, SP and IP_3 receptor type 1, in the brain (**IV**).

4 MATERIALS AND METHODS

4.1 Chemicals

All chemicals used were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless an alternative source is specified in the text. NaCl was from FF-Chemicals AB (Yli-Ii, Finland) and ethanol from Altia (Helsinki, Finland).

4.2 Study subjects and human brain sampling (II)

The study group consisted of 5 Caucasian individuals (4 males, 1 female; age-range 36–77 years; mean age 53.5 ± 10.6 years; postmortem delay 14.8 ± 9.2 h; mean ± SD) free of psychiatric illnessis. The subjects died suddenly because of myocardial infarction (4) and rupture of the aorta (1).

Human brains used were obtained during clinical necropsy at the Department of Forensic Medicine, University of Oulu, Finland, and the Department of Forensic Medicine, University of Kuopio, Finland. The Ethics Committee of the University of Oulu and the University of Kuopio and the National Institute of Medicolegal Affairs, Helsinki, Finland, approved the study.

Frozen left hemisphere was cryosectioned using a heavy-duty cryomicrotome (LKB 2250, LKB, Stockholm, Sweden) into 100 µm horizontal (canto-meatal) sections, which were transferred to the gelatinized glass plates (100 × 220 × 1 mm) as previously described (Hall et al. 1996). The sections were allowed to air dry before they were stored with dehydrating agents at − 25 °C until use.

4.3 Animals and tissue preparation (I-IV)

Wistar rats **(II-IV)** were supplied from National Laboratory Animal Center, University of Kuopio **(II-IV)**. NMRI mice **(I)** were supplied from National Laboratory Animal Center, University of Helsinki. Animals were housed under controlled conditions and had free access to food and water.

For whole-body immunohistochemistry **(I)**, mice were deeply anesthetized using chloral hydrate, perfused, and quickly placed into isopentane cooled with dry ice for 30 sec and then stored at -70 °C until sectioning. Frozen animal was cryosectioned to sagittal sections using a heavy-duty cryomicrotome (LKB 2250, LKB, Stockholm,

Sweden). For paraffin embedded tissue samples **(I)**, animals were deeply anesthetized using chloral hydrate and then perfused with 4% paraformaldehyde solution. After perfusion, the internal organs were removed and placed to the 10% paraformaldehyde solution until paraffin embedding and sectioning.

For frozen sections immunohistochemistry, rats were killed by decapitation and brains were immediately removed, frozen and then stored at -70 °C until sectioning to the coronal brain sections by cryostat **(II)**. For floating sections **(III-IV),** rats were deeply anesthetized using chloral hydrate and then perfused using saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB), decapitated, and the brains were removed and placed into cryo-protectant solution and stored at -20 ºC until sectioning. For electron microscopy **(III)**, rats were perfused using saline followed by fixative containing 4% paraformaldehyde and 0.05% glutaraldehyde in 0.1 M PB, pH 7.4. The brains were removed and washed with PB before placing into the cryo-protectant solution and stored at -20 ºC. After vibratome sectioning, selected brain areas were cut to 1 mm^2 pieces and placed in 10% and 20% glycerol in PBS for 1 hour and 30% glycerol in PB overnight before freeze-substitution.

For enzyme activity measurements **(I)**, tissues were removed, quickly frozen in liquid nitrogen and thereafter stored at -70 °C until homogenized in 5 vol. of assay buffer (0.1 M Na-K-phosphate buffer, pH 7.0). The homogenate was centrifuged at 16,000 g, 4 °C, for 20 min. Aliquots of supernatant were frozen and stored at -70 °C.

All animal procedures were conducted according to the Council of Europe (directive 86/609) and Finnish guidelines, and approved by the local animal ethics committee and State Provincial Office of Eastern Finland.

4.4 6-OHDA (6-hydroxydopamine) lesion (III)

Stereotaxic surgery was performed under isoflurane inhalation anaesthesia. The rat was placed in a stereotaxic apparatus (Stoelting, Wood Dale, Illinois, USA) and 4 µl of the 6-OHDA-infusion (2.0 µg/µl) were injected for 8 min to the final infusion site near substantia nigra using motor-driven slow-motion syringe pump (CMA/102, CMA Microdialysis, Solna, Sweden). Six weeks after the lesion, the animal was perfused, decapitated and brains were removed and stored as described above.

4.5 Preparation and specificity of polyclonal POP antibody (I-IV)

The preparation of polyclonal POP antibody has been described in Venäläinen et al. (2006). Briefly, purified E. coli expressed recombinant human POP (Venäläinen et al. 2002) was used to generate antibodies against POP in a hen. Two 50 μg protein dosages (in 50% Freund's complete adjuvant), with two weeks interval, were injected to hens. Egg yolks were collected and IgY´s were isolated by the water dilution method (Kokko et al. 1994) and POP-specific IgY was then purified by affinity chromatography using a HiTrap NHS column coupled with purified POP (Venäläinen et al. 2006). Second patch of polyclonal POP antibody was generated using the same methods. In the specificity tests it proved to have same characteristics as the first patch.

Antibody specificity in rat brain was assayed by western blotting using rat brain homogenates from the whole brain, cerebral cortex and hypothalamus while purified recombinant pig POP served as a control **(II-IV)**. In all experiments, only one band at around 80 kDa, corresponding to the full length POP, was detected (Fig. 4A). We also studied whether the antibody was able to react with human dipeptidyl peptidase IV (DPPIV, EC 3.4.14.5, a gift from GlaxoSmithKline), the closest relative of POP (Venäläinen et al. 2004). Amounts as high as 2.5 μg of pure DPPIV did not react with the anti-POP IgY in western blot experiments (data not shown). Moreover, preadsorption control was used. POP-antibody solution (dilution 1:1000) was preadsorbed with purified recombinant pig POP (24 μg) for 60 min at room temperature and thereafter the staining was carried out normally. Control section remained free of POP-immunoreactivity (**III**).

Antibody specificity in mouse tissues was assayed by western blotting **(I)** using mouse tissue homogenates from the whole brain, liver, kidney and testis while purified recombinant pig POP served as a control. In all experiments, only one band at around 80 kDa, corresponding to the full length POP, was detected (Fig. 4C).

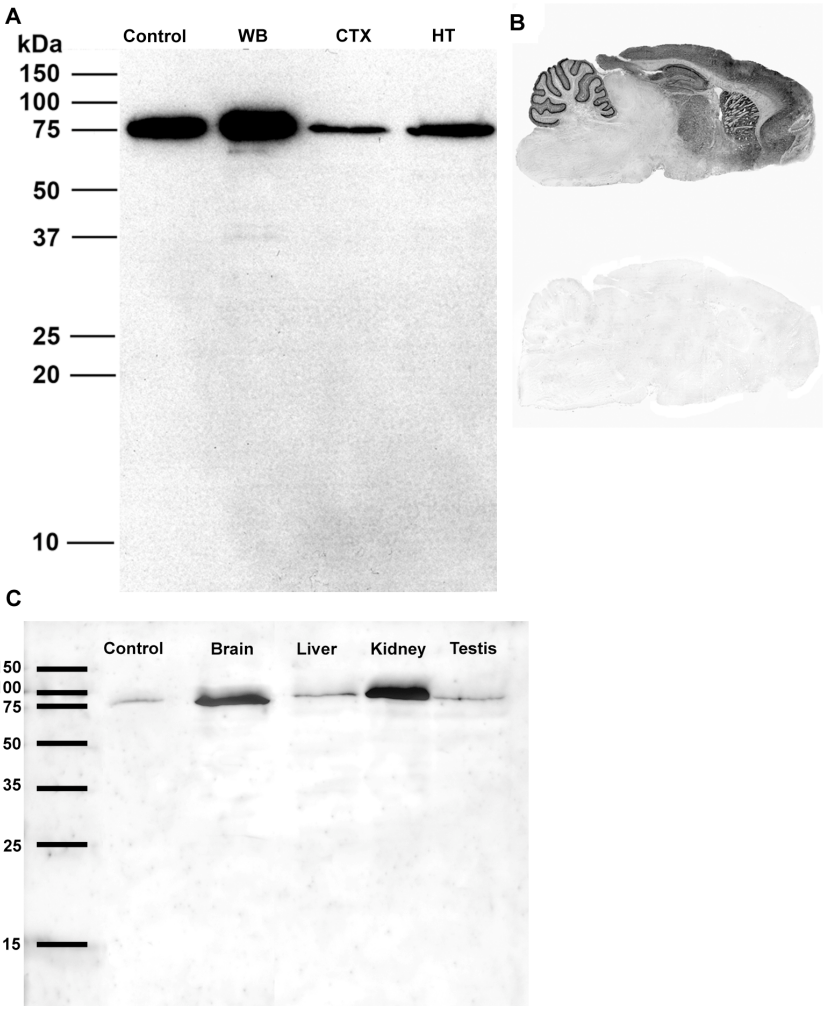

Figure 4A-C. The specificity of POP antibody confirmed by western blot from rat brain homogenates (A) and mouse tissue homogenates (C), and by preadsorption control from rat brain section (B). In all experiments, only one band at around 80 kDa was seen. CTX, rat cerebral cortex; HT, rat hypothalamus; WB, rat whole brain.

4.6 Immunohistochemical and microscopic methods

4.6.1 Light microscopic immunohistochemistry (I-III)

For frozen and whole-body sections **(I, II),** a basic immunohistochemical staining procedure was used. Endogenous peroxidase was blocked with 0.1 % NaN_3 and 0.3 % H_2O_2 in phosphate buffered saline (PBS) and sections were incubated in blocking solution (3 % BSA in PBS) for 30 min to reduce non-specific staining. Primary antibody against POP was added and the sections were incubated for 48 hr at +4 °C, followed by 2 hour incubation in room temperature with HRP conjugated secondary antibody. Details of the antibody dilutions are shown in Table 4. Immunocomplexes were detected by using DAB with a nickel enhancer in whole-body and human brain sections, and with a cobalt enhancer in rat brain sections.

Immunohistochemistry for the free-floating sections **(III)** was performed with a protocol modified for frozen sections. Sections were incubated in blocking solution (10 % normal goat serum (Chemicon International Inc., Temencula, CA, USA) in PBS containing 0.5% Triton X-100) for 40 min to minimize non-specific staining. Thereafter, the primary antibody against POP was added and the sections were incubated for 48 hr at +4 °C. After the primary antibody incubation and the subsequent PBS washes, there was an overnight incubation at +4 ºC with HRP conjugated secondary antibody (Table 4). Immunocomplexes were detected by using DAB with a nickel enhancer.

For paraffin-embedded mouse tissues (**I**), immunohistochemistry was performed using modified frozen-sections protocol. Sections were dewaxed in xylene, rehydrated with graded alcohols and washed with 0.1 M PBS. The antigen retrieval was processed in a microwave oven in citrate buffer (pH 6.0) and thereafter the staining was carried out normally. Finally, the slides were counterstained with Mayer's haematoxylin, washed, dehydrated and mounted with Depex (BDH, Poole, UK).

Light-microscopy immunohistochemistry photomicrographs **(I, III)** were captured by a digital camera connected to the Olympus BX40 microscope and DP50 Digital Camera (Olympus Corporation, Tokyo, Japan) and corrections to brightness and contrast, were made with Adobe Photoshop CS2 software (version 9.0, Adobe Systems Incorporated, Mountain View, CA, USA). The identification and nomenclature of the brain structures **(II, III)** was based on the rat brain atlas by Paxinos et al. (1997), human

brain atlas by Mai et al. (2004) and cresyl violet staining of the corresponding specimen level of human brain sections. The identification and nomenclature of mouse internal organs **(I)** were based on the atlas by Iwaki et al. (2001).

Whole-body section photomicrographs **(I)** were captured using ImageQuant ECL RT imaging device (GE Healthcare, Buckinghamshire, UK) and immunohistochemically processed frozen-sections **(II)** were scanned by a Bio-Rad (Hercules, CA, USA) scanner (GS-710 Calibrated Imaging Densitometer).

Immunohistochemically processed mouse whole-body section photomicrographs **(I)** and rat and human brain sections **(II)** were diverted from grayscale images to color coded images with Scion Image software (version alpha 4.0.3.2, Scion Corporation. Frederick, MD, USA). Only minor corrections to brightness and contrast and pixel errors were made with Adobe Photoshop CS2 (version 9.0, Adobe Systems Incorporated).

4.6.2 Immunofluorescent microscopy (I, III, IV)

In colocalization studies, the double and triple-labelling immunofluorescence technique was used by modifying free-floating sections protocol (see above). All the primary antibodies and their dilutions used are presented in Table 4. Double- **(I, III, IV)** or triple-labelling immunofluorescence **(I, IV)** with POP-antibody followed after the first or second secondary antibody incubation. The procedure in the second and/or third staining was similar to the first and 40 min incubation with 10 % NS blocking solution was used to minimize cross-reactions between antibodies. Furthermore, in order to minimize the cross-reactions, the primary antibodies used in double and triple-label immunofluorescence were chosen from different species as shown in Table 4. Vectashield with DAPI (Vector Laboratories, Burlingame, CA, USA) was used as a mounting medium to demonstrate the nuclei of the cells.

In double- and triple-labelled immunofluorescence, control stainings were carried out by omission of primary antibodies. No evidence of any staining was observed in these negative controls.

Immunofluorescence photomicrographs were captured by a digital camera connected to the Olympus BX40 microscope and DP50 Digital Camera (Olympus Corporation, Tokyo, Japan) and corrections to brightness and contrast, and converting of red images to magenta **(III)** were made with Adobe Photoshop CS2 software (version 9.0, Adobe Systems Incorporated).

Table 4. Details of antibodies

Antigen	**POP (I-IV)**	**Ki-67 (I)**	**GFAP (III)**	**Glutamic acid decarboxylase 65/67 (GAD 65/67) (III, IV)**	**Choline acetyltransferase (ChAT) (III)**	**Tyrosine-hydroxylase (TH) (III)**	**Substance P (SP) (IV)**	**Neurokinin-1 Receptor (NK1R) (IV)**	**IP_3R1 (IV)**
Marker for	POP protein	Ki-67, cell proliferation marker	Astrocytes (glial fibrillary acidic protein)	GABAergic cells	Cholinergic cells	Dopaminergic cells	Substance P	Neurokinin-1 receptor (SP receptor)	Inositol 1,4,5-triphosphate type 1 receptor
Species	Chicken IgY	Mouse IgG	Rabbit IgG	Rabbit IgG	Rabbit IgG	Rabbit IgG	Guinea-pig IgG	Rabbit IgG	Rabbit IgG
Immunogen	see details in materials and methods	Monoclonal rat anti-mouse IgG, clone TEC-3	Purified bovine GFAP	Synthetic peptide. Sequence: K-DIDFLIEEIERLGQDL		Denaturated tyrosine hydroxylase from rat	Synthetic peptide. Sequence: CRPKPQQFFGLM	Synthetic peptide corresponding to 23 amino acid sequence (385-407) of the rat NK1R	Synthetic peptide corresponding to amino acids 1829-1848 of human IP3R1
Manufacturer	see details in materials and methods	Dako, Glostrub, Denmark	Chemicon International Inc., Temencula, CA, USA	Sigma-Aldrich, St. Louis, MO, USA	Chemicon International Inc.	Chemicon International Inc.	Abcam, Cambridge, UK	Chemicon International Inc.	Calbiochem; San Diego, CA, USA
Product # and lot #	-	Product M7249	Product AB5804; 0604027868	Product G5163; 016K4851	Product AB5042; 0606031669	Product AB152; 0607034655	Product AB10353	Product AB5060; 0512016863	Product NR09; D32568
Dilution used	1:500 (rat) 1:1000 (human	1:50	1:1000	1:1000	1:1000	1:500	1:800	1:400	1:500
Literature reference	-	Scholzen et al. 2000	Hammerle et al. 2003, Jungling et al. 2003	Hart et al. 2001, Colonnese et al. 2006	Benecke et al. 1993	Haycock 1987, Witkovsky et al. 2005	-	Piggins et al. 2001	Hayashi et al. 2001
Secondary antibody	Rabbit anti-chicken IgY HRP/FITC, Pierce Biotechnology, Rockford, IL, USA	Goat anti-mouse IgG Alexa Fluor 568, Invitrogen, Frederick, MD, USA	Goat anti-rabbit IgG Texas Red, Pierce Biotechnology	Goat anti-rabbit IgG Texas Red, Pierce Biotechnology **(II)**, Goat anti-rabbit Alexa Fluor 405, Invitrogen, Frederick, MD, USA **(III)**	Goat anti-rabbit IgG Texas Red, Pierce Biotechnology	Goat anti-rabbit IgG Texas Red, Pierce Biotechnology	Rabbit anti-guinea-pig IgG Texas Red, Abcam, Cambridge, UK	Goat anti-rabbit IgG Texas Red, Pierce Biotechnology	Goat anti-rabbit IgG Texas Red, Pierce Biotechnology

4.6.3 Laser scanning microscopy (I, III, IV)

Fluorescent double-labelled **(I, III, IV)** sections were analyzed and photographed using Ultra VIEW Confocal Imaging System (PerkinElmer, Cambridge, UK) equipped with an argon-krypton laser mounted on an inverted Nikon Eclipse TE300 microscope (Nikon Corporation, Tokyo, Japan). Triple-labelled **(IV)** sections were photographed using an upright microscope (Leica DM5000B, Leica Microsystems Inc.) with broadband laser confocal device (Leica TCS SP5, Leica Microsystems Inc.).

After capturing images with an imaging device, confocal double- and triple-labelled immunofluorescence micrographs were converted to RGB images and colorized with the corresponding colors (blue, green and red (**I, IV**), and red to magenta **(III)**) using Adobe Photoshop CS2 software (version 9.0, Adobe Systems Incorporated). Only minor corrections to brightness and contrast in the pictures were made.

4.6.4 Post-embedding immunoelectron microscopy (III)

Tissue samples were processed with the freeze-substitution technique (Reichert AFS, Leica/Reichert, Vienna, Austria) and low-temperature embedding using Lowicryl HM20 (Electron Microscopy Sciences, Hatfield, PA, USA). Ultra thin cryosections were sectioned using an ultramicrotome (Ultracut E; Reichert-Jung, Austria) and POP-immunohistochemistry was performed using the following protocol: Sections were first incubated in 50 mM NH_4Cl blocking solution for 10 min and in 5% BSA (bovine serum albumin, containing 0.1% fish skin gelatin) solution for 20 min followed by washes in 0.1% acetylated BSA (BSA-c) and 0.1% Tween 20 in PBS. Primary antibody incubation for 1 hr with POP-antibody was performed and followed by washes using 0.1% BSA-c and 0.1% Tween 20 in PBS. POP-immunocomplexes were detected using rabbit anti-chicken IgY with 15 nm gold label (Product #825.222, Aurion, Wageningen, The Netherlands) as a secondary antibody. After 30 min secondary antibody incubation, 6x5 min PBS washes followed. Thereafter the sections were post-fixed using 5% glutaraldehyde in PBS for 5 min before further fixation with 5% uranyl acetate and lead citrate. Omission of primary antibodies served as negative staining and remained free of gold particles (see Fig. 10A in **III**).

Sections were viewed with JEM-1200EX (JEOL Ltd., Tokyo, Japan) transmission-electron microscope (TEM).

4.7 Tissue fractioning and western blot analysis (I)

NE-PER Nuclear and Cytoplasmic Extraction Reagents (Product #78833, Pierce Biotechnology) was used to extract nuclear and cytoplasmic fractions from the brain, liver, kidney and testis. The tissues were homogenized in cytoplasmic reagent with EDTA-free Halt protease inhibitor cocktail (Product #78410, Pierce Biotechnology). After homogenization, secondary cytoplasmic reagent was added and the fraction was centrifuged for 5 min at 16,000g, supernatant (cytoplasmic fraction) was removed and stored at -80 ºC. Remaining pellet was washed twice using PBS and thereafter resuspended to the nuclear reagent, incubated for 40 min in ice and then centrifuged for 10 min at 16,000g. Supernatant (nuclear fraction) was removed and stored at -80 ºC until use.

The purities of nuclear and cytoplasmic fractions were confirmed by western blot (Fig. 9B in **IV**) using anti-histone H3 goat anti-rabbit polyclonal antibody (nuclear marker, dilution 1:5000, Product #H0164, Sigma-Aldrich) and anti-tubulin monoclonal antibody (cytoplasmic marker, dilution 1:5000, Product #05-829, clone DM1A, Upstate, Temecula, CA, USA). In all western blot analyses, standard SDS-PAGE, transfer and blocking techniques were used and chemiluminescence images were captured using ImageQuant ECL RT imaging device (GE Healthcare).

The distribution of POP between nuclear and cytoplasmic fractions of the brain and peripheral tissues was assayed by western blot analysis using anti-POP (described above) as the first antibody, and goat anti-chicken IgY-HRP conjugate (dilution 1:10000) as a second reporter.

4.8 Enzyme activity assay (I)

The POP activity assay was performed as described earlier (Venäläinen et al. 2002). Briefly, tissues were frozen in liquid nitrogen after removal and stored at −70° until homogenized in 5 vol. of assay buffer (0.1 M Na–K-phosphate buffer, pH 7.0). The homogenate was centrifuged at 10,000 *g*, 4 °C, for 20 min. Aliquots of supernatant were frozen and stored at −70 °C. An enzyme solution (10 µL of tissue homogenates) was preincubated in a 48-microtiterplate with 465 µl of assay buffer for 30 min at 30°. The reaction was initiated by adding 25 µl of substrate (4 mM Suc-Gly-Pro-AMC) and the plates were incubated for 60 min at 30 °C. The reaction was terminated by the addition of 500 µl of 1 M sodium acetate buffer (pH 4.2). The formation of AMC (7-amino-4-methylcoumarin) was measured fluorometrically using a Wallac 1420 VICTOR2

fluorescence plate reader (PerkinElmer, Waltham, MA, USA). The excitation and emission wavelengths were 360 and 460 nm, respectively. AMC dilutions served as a control in the measurements. The variation of results between 10 different measurements was ± 6.80 % (± SEM).

4.9 Semiquantitative analysis (I-IV)

Immunohistochemically processed sections **(I, II)** were analyzed using Bio-Rad QuantityOne 4.5.1 software. In optical density (OD) analysis brain regions were delineated with the freehand-tool of the software. The OD values of different brain areas were compared to the mean values of corpus callosum **(I)** or brain **(II).** The background values of each brain area (background obtained by control staining without primary antibody) were subtracted from raw data values of the same brain area. The averages and SEM of optical densities from each area were calculated using an Excel spreadsheet.

The percentages of colocalizations in double-labeling immunofluorescence were calculated comparing colocalized neurons with POP containing neurons and using Abercrombie's correction (Abercrombie et al. 1946) **(III, IV)**. The colocalization percentage for each area was calculated from at least 3 different animals and from 2-4 different immunostainings. The average of counted cells per region was approx. 20 **(III, IV)** and the number of counted slides per brain area varied from 3 to 7.

4.10 Statistical analyses (I-IV)

Statistical analyses were performed using GraphPad Prism (version 4.03, GraphPad Software, Inc., San Diego, CA, USA). One-way ANOVA followed by Newman-Keuls multiple comparison test was performed to analyze differences between the groups. To detect a significant difference between groups **(II, III)**, two-tailed student t-test was performed. Statistically significant differences were considered at $P < 0.05$.

5 RESULTS

5.1 Body distribution of POP protein and POP activity in the mouse (I)

5.1.1 Whole-body immunohistochemistry

In mouse whole-body sections, POP protein was widely but unevenly distributed (Fig. 5A). The highest amount of POP protein was observed in the brains (Fig. 5A, Fig. 5B), but almost equally high levels of POP immunoreactivity was found in the kidney, testis and thymus (Fig. 5A, Fig. 5B). POP was also clearly present in the urinary bladder, lungs (Fig. 5A, Fig. 5B) and heart. However, only a low amount of POP protein was detected in the liver (Fig. 5A, Fig. 5B).

5.1.2 Enzyme activity assay

In the enzyme activity measurements (Fig. 5A), the highest POP activity (pmol/min * mg tissue) was located in the liver followed by testis. The activity was moderate in the heart, lungs, thymus and brain. Lower levels of activities were measured from the urinary bladder, adrenal gland and kidney. Surprisingly, the lowest activities were assayed in the kidney although this organ contained almost the highest amount of POP protein, and a high activity was measured from liver regardless of its small protein amount. Moreover, the enzyme activity in the brain was only moderate despite the fact that it contained highest protein amount. Generally, the POP enzyme activities in various organs differed clearly from the POP protein distribution measured from the whole-body immunohistochemistry (Fig. 5A).

5.2 Cellular and subcellular distribution of POP protein (I-II)

5.2.1 Cellular distribution of POP in mouse tissues

POP was generally widely present in various cell types, both in their nuclei and cytoplasm. With respect to the liver, POP was moderately present in the cytoplasm and the nucleus of the liver hepatocytes and nuclei of the Kupffer's cells (Fig. 4A-B in **I**). POP was also present in the hepatic endothelial cells (Fig. 4B in **I**). In the lungs, a substantial amount of POP protein was observed both in the cytoplasm and nuclei of type I and type II cells of the alveolar walls. A substantial amount of POP protein was

also found in the nuclei of smooth muscle cells in the wall of the bronchioles (Fig. 4C-F in **I**).

In kidney, high POP-immunoreactivity was observed in the medulla and it was less abundant in the cortex (Fig. 5C). From moderate to high degree POP-immunoreactivity was present in the glomerular podocytes (Fig. 5C) and the walls of tubules, but only minor expression was found in the rest of the cortical labyrinth (Fig. 5C). In medulla, POP was clearly present in the cells of walls of the tubules (Fig. 5C in **I**)

In the adrenal glands, POP immunoreactivity was high both in the cortex and medulla (Fig. 5A in **I**). In particular, cells in the zona glomerulosa and zona reticularis were intensively immunostained (Fig. 5E in **I**). In the cortex, POP was preferentially present in the nuclei, whereas in the medullary chromaffin cells, POP was present both in the nuclei and cytoplasm (Fig. 5F in **I**). In the heart, POP was moderately present in the nuclei and sarcomeres of the cardiac muscle.

In the testis, POP was amply present in the nuclei of Sertoli cells of lamina propria, spermatogonia and spermatocytes (Fig. 5D). Moderate POP immunoreactivity was seen in developing spermatids, but not in the mature spermatids (Fig. 5D). Moderate amounts of POP protein were observed in the thymus, both in cortex and medulla. POP was also present in the nuclei of various types of epithelioreticular cells. POP was intensively present in the nuclei and cytosol of the epithelium cells of the urinary bladder (Fig. 5E-H). Moderate POP-immunoreactivity was seen in the connective tissue (Fig. 7A-B in **I**) but only sparse staining was observed in the smooth muscle cells (Fig. 7A in **I**).

5.2.1.1 Nuclear POP in peripheral tissues and cell proliferation marker

We used triple-label immunofluorescence of POP, DAPI (a nuclear marker) and Ki-67 (a proliferation marker) to determine whether the presence of POP in the nuclei is associated with cell proliferation in peripheral cells. A rather poor colocalization between POP, DAPI and Ki-67 was seen (Fig. 5E-H) despite the abundant colocalization of POP and DAPI (Fig. 5E-H). The colocalization between cytosolic POP and Ki-67 was moderate, (Fig. 8 in **I**) pointing to a possible role of POP in the cell proliferation.

In order to substantiate the significant nuclear localization of the POP protein in the peripheral tissues, in contrast to the brains, where no nuclear localization was seen, this finding was confirmed by a western blot assay of POP protein from nuclear and cytosolic fractions of the brain, liver, kidney and testis (Fig. 9 in **I**). In peripheral tissues, POP was present also in the nuclear fraction while in the brain nuclear POP was not seen. However, excluding the liver, the expression of POP protein was significantly

smaller in the nuclear than in the cytoplasmic fraction, indicating that POP is preferably present in the cytoplasm even in the peripheral tissues. The identity of nuclear and cytoplasmic fractions was confirmed by nuclear (Histone H3) and cytoplasmic markers (tubulin) (Fig. 9 in **I**).

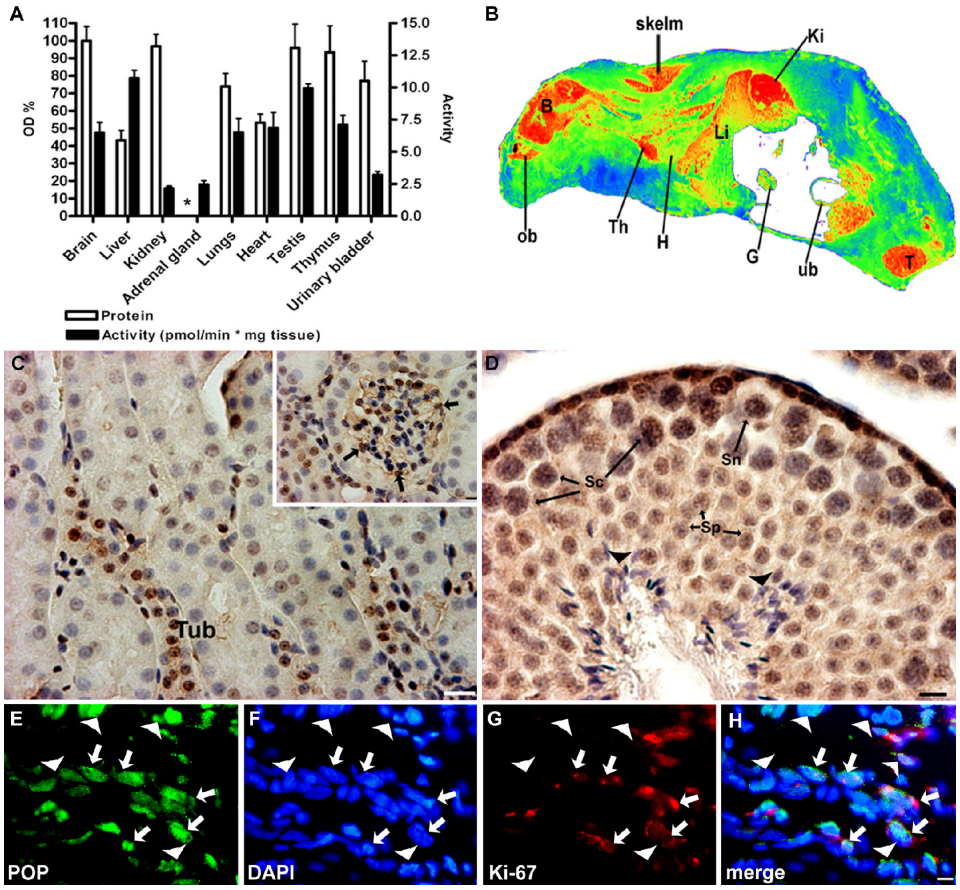

Figure 5A-H. The distribution of POP in the peripheral tissues, determined by immunohistochemistry from mouse whole-body sections (**A, B**) and enzyme activity measurements (**A**). OD value of brain is set as 100% and other values are compared to that value. Enzymatic activity is presented as pmol/min * mg tissue. Color codes in **B** are similar to Fig. 4. In the photomicrographs, nuclei are blue, POP is visualized brown and the colocalization gives dark blue/brown color. In the cortex of kidneys (**C**), POP was present in the walls of tubules (Tub) and in the glomerulus podocyte cells (small view, black arrows). Rather intensive POP immunostaining was seen in the testis

spermatocytes (Sc) and in the Sertoli nuclei (Sn) (**D**). POP was moderately present in the immature spermatids (Sp), but not in the mature spermatids (black arrowheads, **D**). In Fig. **E-F**, triple-labeled immunofluorescence photomicrographs illustrating the colocalization between POP (green), DAPI (blue) and Ki-67 (red) in the urinary bladder epithelial cells. Photomicrographs are merged and the colocalizations of POP, DAPI and Ki-67, in the same cells are pointed with white arrows. The abundant colocalization of POP and DAPI is presented with white arrowheads. Scale bars: C, D, 20 µm; E-H, 10 µm.

5.2.2 POP distribution in the human brain (II)

In humans, POP was expressed quite evenly and widely in all analyzed brain areas (optical density analysis, Fig. 6A). Generally, POP was present in the gray matter while axons devoid the POP immunoreactivity (Fig. 6B). The expression of POP was the highest in caudate nucleus followed closely by putamen and overall, POP was extensively present in nigrostriatal pathway. However, POP was only moderately present in red nucleus (Fig. 6B).

There was intense expression of POP in cerebellum. Immunostaining was clearly evident in the cortex, especially in molecular cell layer and Purkinje cells, but only to a moderate degree in the granular cell layer (Fig. 6B).

POP was very evenly and extensively present in all cortical areas, especially in insular cortex (Fig. 6B). A large amount of POP protein was present in claustrum (Fig. 6B). Furthermore, POP was intensively immunostained in all parts of hippocampus (Fig. 6B).

Lateral and medial geniculate bodies along with superior colliculus were immunostained very intensively. Somewhat lesser degree of expression of POP was observed in tegmentum (Fig. 6A).

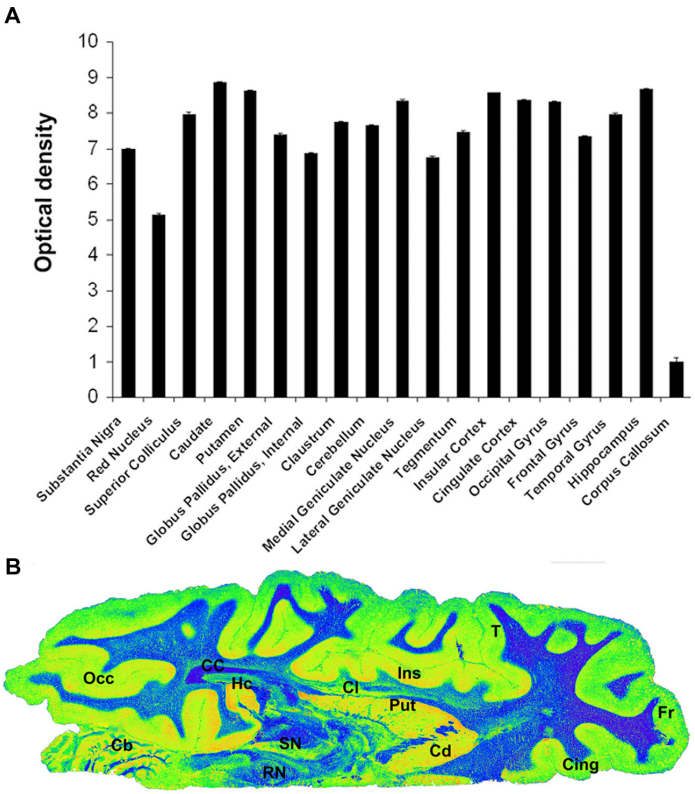

Figure 6A-B. The distribution pattern of POP in human brain **(A)**. The optical density (OD) values of analyzed brain areas are compared to corpus callosum OD value (set as 1). The color coded (from low levels = blue color or cooler to high levels = red tones or warmer) image depicts the distribution of POP in horizontal human left whole-hemisphere **(B)**. Cb, cerebellum; CC, corpus callosum; Cd, caudate; Cing, cingulate cortex; Cl, claustrum; Fr, frontal cortex; Hc, hippocampus; Ins, insular cortex; Occ, occipital gyrus; Put, putamen; RN, red nucleus; SN, substantia nigra; T, temporal gyrus.

5.2.3 POP distribution in the rat brain (II, III)

The overall distribution of immunoreactive POP in the rat brain areas is presented in Fig. 7A and Table 5.

Cerebral cortex and forebrain

The intensity of POP staining was high in most of the regions of the cerebral cortex, especially in motor, primary auditory, primary visual, granular insular and somatosensory cortices **(II)**. Generally, the staining was particularly intense in the large and medium sized pyramidal cells and apical dendrites of layers II-VI (Fig. 7B). POP immunoreactivity was also very visible in the piriform cortex. POP immunoreactive neurons were moderately present in retrosplenial, lateral entorhinal and agranular insular cortices in the large and medium sized pyramidal cells in layers III and V, especially in the retrosplenial cortex (Fig. 1F in **III**). Prelimbic, infralimbic and cingulated cortices were less immunostained and the expression of POP was apparent mostly in the pyramidal cells in layers II-III and V (Fig. 1G in **III**).

In striatum, POP was clearly visible in small to medium sized spiny neurons while leaving myelin sheathed axons unstained (Fig. 7C). The spiny neurons of ventral pallidum were also extensively immunostained with POP. A medium degree of POP-immunostaining was present in the small to medium sized spiny neurons of nucleus accumbens core and shell region of this brain site, while the POP level was slightly lower in the nucleus accumbens core section (Fig. 7A).

In hippocampus, a high level of POP was seen in the pyramidal neurons and apical dendrites descending to the stratum lacunosum moleculare in CA1 field (Fig. 7D). Other parts of hippocampus (CA2, CA3, dentate gyrus and subiculum) were immunostained with POP only from a moderate to a low degree (Fig. 7D). Furthermore, in the areas with close connections to the hippocampus such as indusium griseum, POP-immunoreactivity was particularly rich in the pyramidal neurons. POP was abundant in somatospiny LS IIb neurons of the medial part of lateral septal area (Fig. 5 in **III**), whereas in medial septum, no POP immunoreactivity was observed.

Midbrain

The highest POP densities in rat brain were measured from substantia nigra (Fig. 7A,E). The reticular and compact parts of the substantia nigra were intensively immunostained, especially in the dendrites (Fig. 7E). Other midbrain nuclei such as ventral tegmental area, superior and inferior colliculus were devoid of POP immunoreactivity.

Thalamus and hypothalamus

Moderate POP staining was found in different thalamic nuclei, such as the ventroposterior nucleus group, posterior nucleus group, laterodorsal nuclei (Fig. 7 in **III**), mediodorsal thalamic nucleus and ventromedial thalamic nucleus. Similar to human, in lateral and medial geniculate nucleus (Fig. 7A), the expression of POP was high.

In the hypothalamus, practically no POP was detected with the exception of the medial mamillary nucleus where a moderate level of POP was observed (Fig. 7A).

Cerebellum, pons and medulla

In the cerebellum, the expression of POP was high in the cortex and in the Purkinje's cells which were intensively immunostained both in somas and in dendrites of the molecular cell layer (Fig. 7F). The granular cell layer virtually lacked POP immunoreactive cells (Fig. 7F). POP was not present in pons and medulla oblongata and in the myelin sheathed axon bundles such as corpus callosum (Fig. 7A) and anterior commissure.

POP and astrocytes

Localization of POP in astrocytes was studied using double-staining immunofluorescence and using glial fibrillary acid protein (GFAP) as a glial marker. However, POP was not detected in astrocytes in any of the sections made throughout the brain (Fig. 9H).

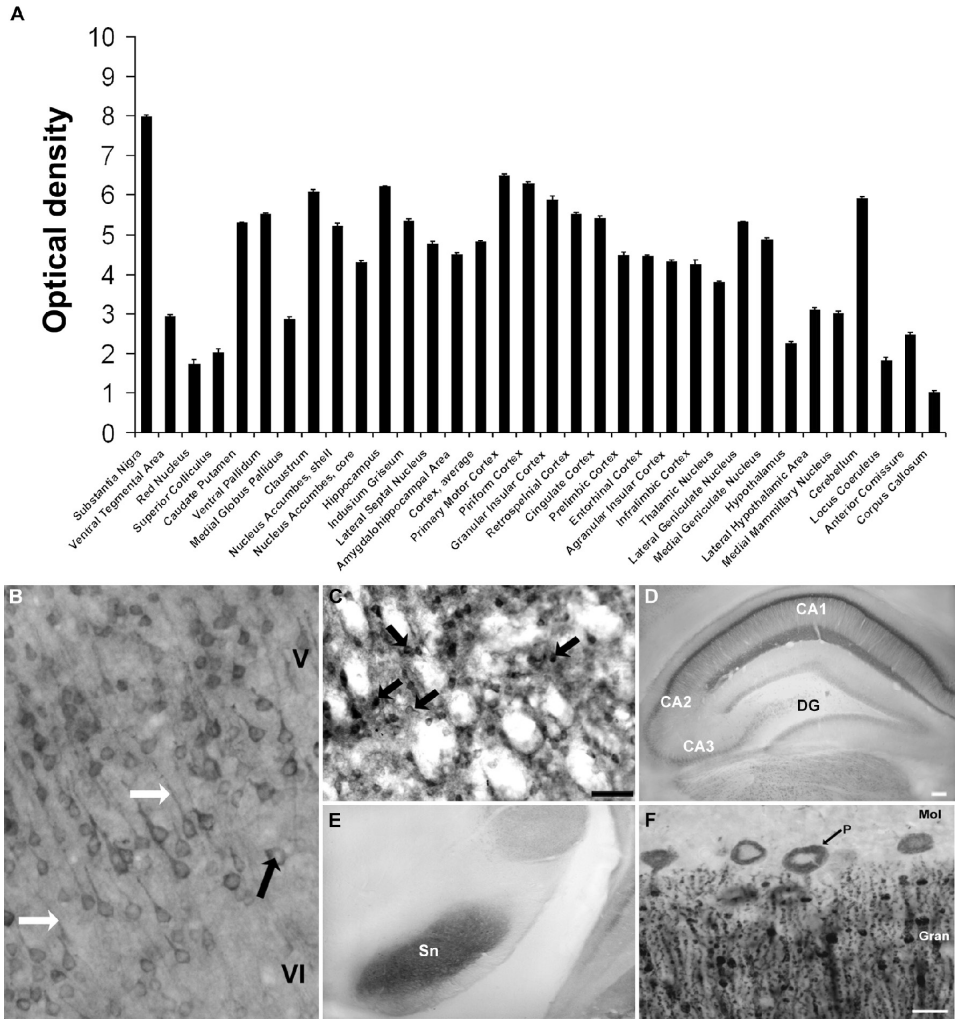

Figure 7A-F. The distribution of immunoreactive POP in the rat brain. The optical density **(A)** values of analyzed brain areas are compared to *corpus callosum* OD value (set as 1). In the somatosensory cortex layers V and VI **(B)**, POP was intensely present in the pyramidal cells somas (black arrow) and dendrites (white arrows). High POP immunoreactivity was seen in the spiny neurons of striatum (**C**, black arrows) and CA1 field of hippocampus **(D)**. Substantia nigra **(E)** was abundantly immunoreactive for POP similar to Purkinje cells **(F)** of the cerebellum. CA1, CA2, CA3, hippocampal CA fields; DG, dentate gyrus; Gran, granular cell layer of cerebellum; Mol molecular cell layer; P, Purkinje cell; Sn, substantia nigra. Scale bars: B,C, 30 µm; D, 50 µm; E, 150 µm; F, 10 µm.

5.2.4 Subcellular localization of POP in the rat brain

In post-embedding immunoelectron microscopy, POP was identified using 15 nm fully round gold particles coupled to the secondary antibody. We studied rat brain tissue samples from those areas where POP immunoreactivity was clearly visible i.e. striatum, primary somatosensory cortex, Purkinje's cells, substantia nigra and the CA1 area of hippocampus.

The enzyme was generally and most abundantly found freely-moving in the cytoplasm (Fig. 8A). However, POP seemed also to be attached to cell membranes (Fig. 8B), especially to the intracellular membranes of rough endoplasmic reticulum (RER, Fig. 8C) and Golgi apparatus (Fig. 8D). Some POP was also found inside the axons (Fig. 10F in **III**) and within their myelin sheaths (Fig. 10F in **III**).

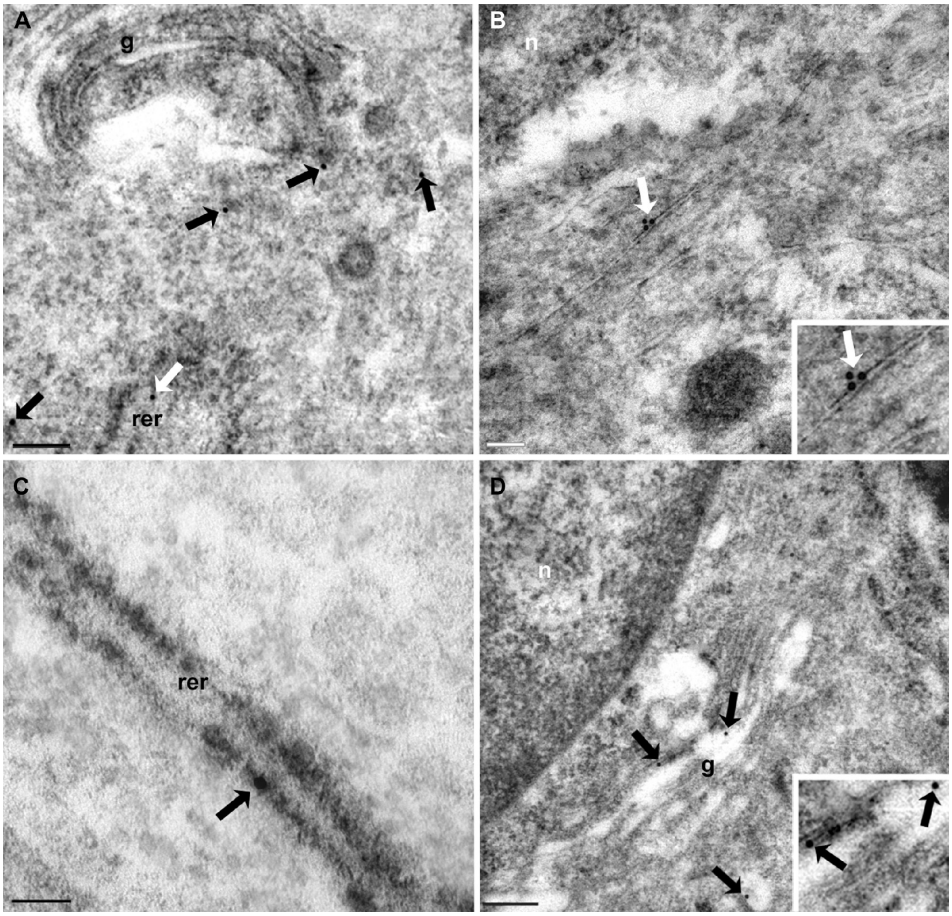

Figure 8A-D. High magnification electron photomicrographs depicting subcellular distribution of POP in rat brain. POP was present freely in cytoplasm (**A**, black arrows point to the 15 nm gold particles, white arrow points to the immunoreactive POP in the rough endoplasmic reticulum, rer) and in the cell membranes (**B**, small view is a magnification from the larger picture, white arrows indicate POP in the cell membrane and n marks the nucleus). POP was present in the membrane of RER (**C**, black arrow) and Golgi apparatus (g) (**D**, small view is a magnification from the larger picture, black arrows indicate POP in the Golgi, n points to the nucleus). Scale bars: A, 0.1 µm; B, 50 nm; C, 0.2 µm; D, 0.1 µm.

5.3 Spatial associations of POP protein with specific neurotransmitters and its presumed substrates in the rat brain (III, IV)

Double-label immunofluorescence of POP with GABA **(III)**, ACh **(III)**, SP **(IV)** and its NK1-receptor (NK1R) and IP_3 type 1 receptor (IP_3R1) **(IV)** was evaluated throughout the whole rat brain and the percentages of colocalizations are shown in Table 5. Furthermore, the colocalization of POP with a dopamine marker in the nigrostriatal path was studied by double-label immunofluorescence and specific lesioning of dopaminergic neurons (**III**).

5.3.1 GABA, ACh and dopamine (III)

In addition to the pyramidal cells (see section 5.2.3), which are known to be highly glutaminergic, POP was also present in the cerebral cortex in large and medium sized GABAergic (Fig. 2A-C in **III**) and cholinergic (Fig. 2G-I in **III**) interneurons (Table 5).

In striatum and nigrostriatal path nuclei, POP-immunoreactive neurons were mostly GABAergic (Fig. 9A, Table 5) and colocalization with cholinergic neurons was poor (Table 5). Interestingly, both double-label immunofluorescence and neuronal morphology indicated that, POP was not present in TH-containing dopaminergic cells in striatum (Fig. 3D in **III**). In confirmation, lesioning of nigrostriatal dopaminergic neurons using 6-OHDA, removed tyrosine hydroxylase (TH) reactive dopaminergic neurons from striatum while leaving the POP-containing cells intact (Fig. 3E-J in **III**).

The pyramidal neurons of hippocampus CA1 containing POP were seldom GABAergic or cholinergic (Fig. 4C-D in **III**, Table 5), whereas in indusium griseum, POP immunoreactive neurons were usually GABAergic (Fig 4E in **III**, Table 5). POP-immunoreactive neurons of lateral septal area were GABAergic and no colocalization between POP and ChAT was observed (Fig 5B-D in **III**, Table 5).

Thalamic POP was quite evenly present in GABAergic (Fig. 8B-D in **III**) and cholinergic medium sized projection neurons (Fig. 8E-G in **III**, Table 5)

In the cerebellar Purkinje's cells, there was clear colocalization with the GABAergic marker (Fig.9B, Table 5).

5.3.2 POP and IP_3R1 (IV)

In the cerebral cortex, we found moderate to substantial POP colocalization with IP_3R1 (Fig. 1A-F in **IV**), the percentages in the cerebral cortex varying from 31% (primary visual cortex) to 63% (piriform cortex, Table 5).

In the high POP-immunoreactive nuclei in nigrostriatal path, such as striatum (Fig. 2A-C in **IV**), lateral globus pallidus and substantia nigra, generally a low degree of colocalization of POP and IP_3R1 was observed (Table 5).

In POP-immunoreactive hippocampal areas, such as CA1, CA2 and CA3, POP was highly colocalized with IP_3R1 containing pyramidal cells (Fig. 9C, Table 5). In the lateral septum (Fig. 3D-F in **IV**), POP colocalization was only 34% (Table 5).

The colocalization of POP and IP_3R1 in the thalamus was extremely high. In the middle section of thalamus, where POP is abundantly present in neurons, the percentage of colocalization varied from 66 to 86% (Fig. 9D, Table 5).

In cerebellum, the colocalization of POP and IP_3R1 was 77% in the Purkinje cells and molecular layer (Fig. 5A-C in **IV**).

5.3.3 POP and SP (IV)

The colocalization between POP and SP was generally rather low throughout the brain. POP was weakly colocalized with SP-immunoreactive neurons (Table 5). In cerebral cortex (Fig. 9E, Table 5), the degree of colocalization varied from 20% (agranular insular cortex) to 39% (auditory cortex). Similar minor coexpression was true also in forebrain nuclei, such as striatum (Fig. 7A-C in **IV**), lateral globus pallidus and nucleus accumbens, areas which contain high amounts of POP protein (Table 5).

In hippocampal CA-fields (Fig. 8F) and in the lateral septum (Fig. 8D-F in **IV**), the coexpression was rather weak (Table 5). The colocalization was also poor throughout the thalamus, as well as in the midbrain nucleus and substantia nigra, both regions rich in POP protein (Table 5).

In cerebellum, POP and SP were abundantly colocalized in the Purkinje cells but less extensively in the molecular layer (Fig. 9A-C in **IV**).

5.3.4 POP, GABAergic cells and SP (IV)

We also studied the coexpression of POP, GABAergic cells and SP in the rat striatum and motor cortex using triple-label immunofluorescence. In the striatum, 26 ± 2% (average ± SEM) of those GABAergic cells having POP-immunoreactivity contained also SP (Fig. 10A-D in **IV**). About 65 % of GABAergic cells in the striatum contain POP (Table 5) and therefore we detected a significant population of GABAergic and POP-immunoreactive cells without SP, as well as POP-containing cells with SP, but no GABA (Fig. 10D in **IV**). In cortex, the mutual cellular localization of POP, GABA and SP was less impressive (19 ± 5%, when compared to POP-containing cells) than in the striatum (Fig. 10E-H in **IV**).

5.3.5 POP and NK-1R (IV)

Double-label immunofluorescence between POP and SP receptor, NK-1R, was made throughout the rat brain. However, virtually no colocalization of these proteins was observed (Fig. 9G). The size and shape of NK-1R positive cells clearly differed from POP immunoreactive cells and only in a few areas (Fig. 11I in **IV**) were the projections of NK-1R positive cells in the vicinity of POP containing cells.

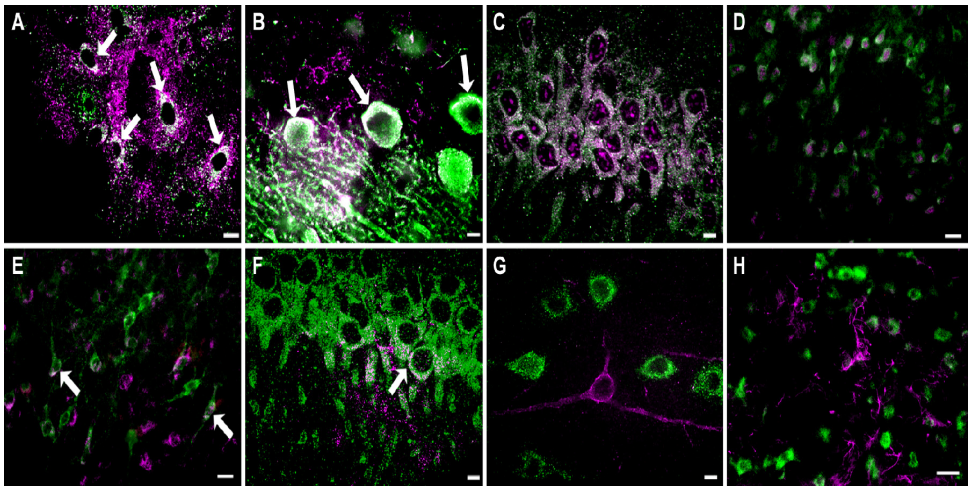

Figure 9A-H. Confocal (A-C, F-G) and fluorescent (D-E, H) photomicrographs presenting the high colocalization of POP with GAD 65/67 (GABA-marker) in striatum (A) and cerebellar Purkinje cells (B), and with IP_3R1 in hippocampal CA1 field (C) and thalamus (D). Poor colocalization is seen with POP and SP in cortex (E) and hippocampal CA1 (F), and with NK1R (G) and astroglial markers (GFAP, H) in the cortex. POP is presented in green color, other substance in magenta and the colocalization is visualized as white color and white arrows. Scale bars A-C,F-G, 6 µm; D-E,H, 20 µm.

Table 5. The distribution of POP immunoreactivity in the rat brain and its colocalizations with GABAergic (GAD 65/67), cholinergic (ChAT), IP_3R1 and SP markers. The colocalization percentage is calculated comparing the GABA, Ach, IP_3R1 and SP immunoreactive neurons with POP-immunoreactive neurons.

Structure	Expression of POP	Colocalization of POP with GAD 65/67 (%)*	Colocalization of POP with ChAT (%)*	Co-localization of POP with IP_3R1 (%)*	Co-localization of POP with SP (%)*
Forebrain and cerebral cortex					
Agranular insular cortex	++	58 ± 2.6	42 ± 3.1	58 ± 4.7	20 ± 3.5
Auditory cortex, primary	+++	37 ± 4.4	41 ± 2.9	46 ± 5.7	39 ± 2.9
Cingulated cortex	+	23 ± 1.7	31 ± 3.9	42 ± 3.8	31 ± 5.6
Granular insular cortex	+++	34 ± 2.2	36 ± 2.0	47 ± 2.8	33 ± 3.5
Infralimbic cortex	+	50 ± 4.7	38 ± 3.3	53 ± 2.4	27 ± 2.5
Lateral entorhinal cortex	++	45 ± 4.0	36 ± 3.3	57 ± 1.0	35 ± 2.7
Motor cortex, primary	0	-	-	-	-
layer 1	++	54 ± 4.9	21 ± 2.8	56 ± 1.5	29 ± 4.8
layer 2	+++	50 ± 2.4	32 ± 2.5	31 ± 2.9	35 ± 3.8
layer 3	++	43 ± 1.4	30 ± 7.0	35 ± 1.5	34 ± 0.5
layer 4	+++	35 ± 4.2	37 ± 3.3	40 ± 2.5	35 ± 2.3
layer 5	+++	45 ± 5.3	40 ± 2.7	37 ± 2.5	22 ± 2.9
layer 6					
Piriform cortex	+++	43 ± 3.6	53 ± 3.4	63 ± 9.8	25 ± 1.2
Prelimbic cortex	+	34 ± 4.7	38 ± 3.4	52 ± 2.1	35 ± 4.8
Retrosplenial cortex	++	46 ± 2.7	57 ± 4.6	38 ± 4.2	25 ± 5.6
Somatosensory cortex, primary	0	-	-	-	-
layer 1	+++	37 ± 1.5	33 ± 5.6	40 ± 7.1	24 ± 1.9
layer 2	+++	43 ± 6.7	39 ± 4.2	32 ± 4.3	28 ± 1.1
layer 3	++	37 ± 4.4	38 ± 1.7	53 ± 6.4	29 ± 3.6
layer 4	+++	42 ± 2.3	36 ± 3.8	54 ± 7.0	38 ± 3.0
layer 5	+++	44 ± 5.6	39 ± 2.8	57 ± 2.8	39 ± 6.4
layer 6					
Visual cortex, primary	++	35 ± 3.4	28 ± 3.6	31 ± 2.3	33 ± 1.9
Claustrum	+++	43 ± 3.3	44 ± 6.4	51 ± 4.8	26 ± 3.4
Lateral globus pallidus	++	44 ± 2.3	26 ± 1.8	38 ± 2.7	30 ± 4.5
Striatum	+++	61 ± 2.9	29 ± 3.2	36 ± 5.2	26 ± 2.2
Ventral pallidum	+++	45 ± 2.9	28 ± 3.6	59 ± 7.9	29 ± 6.1
Nucleus accumbens					
core	++	37 ± 3.2	42 ± 4.9	38 ± 3.0	31 ± 2.8
shell	+	32 ± 3.1	45 ± 3.3	42 ± 2.3	35 ± 1.5
Hippocampus					
CA1	+++	27 ± 2.1	31 ± 3.7	87 ± 7.6	27 ± 2.9
CA2	++	25 ± 2.2	29 ± 4.3	83 ± 3.3	28 ± 4.3
CA3	++	23 ± 3.4	26 ± 3.6	87 ± 4.6	26 ± 3.2
dentate gyrus	+				

stratum laconosum	+++				
Indusium griseum	++	55 ± 4.1	26 ± 2.5	-	-
Lateral septal area	+++	36 ± 7.0	-	34 ± 4.5	21 ± 4.7
Medial septum	0				
Nucleus basalis mangocellularis	0				
Diagonal band of Broca	0				
Midbrain					
Substantia nigra	++	58 ± 3.5	27 ± 4.4	25 ± 4.1	23 ± 2.9
Ventral tegmental area	0				
Red nucleus	0				
Superior colliculus	0				
Inferior colliculus	0				
Thalamus and hypothalamus					
Ventroposterior nuclei	++	43 ± 3.9	34 ± 5.2	78 ± 3.9	32 ± 3.5
Posterior thalamic nuclear group	++	46 ± 2.4	43 ± 2.5	86 ± 4.3	40 ± 3.8
Laterodorsal thalamic nucleus	++	42 ± 2.4	34 ± 3.2	85 ± 3.5	32 ± 3.7
Mediodorsal thalamic nucleus	++	45 ± 6.4	35 ± 2.6	83 ± 1.6	35 ± 3.6
Ventromedial thalamic nucleus	++	42 ± 4.9	34 ± 1.5	66 ± 7.2	38 ± 6.6
Medial geniculate nucleus	++	38 ± 5.2	34 ± 1.9	71 ± 6.6	25 ± 4.6
Lateral geniculate nucleus	++	49 ± 5.5	35 ± 3.3	44 ± 7.4	30 ± 2.2
Medial mammillary nucleus	+				
Cerebellum and pons					
Cerebellum					
Purkinje cells and molecular layer	+++ 0	90 ± 3.7	-	77 ± 5.8	45 ± 6.1
Granular layer					
Medulla	0				

The densities of POP immunoreactive cells in brain structures: 0, no POP immunoreactivity; +, low density of POP; ++, moderate density of POP; +++, high density of POP; - (not detectable); * Mean ± SEM

6 DISCUSSION

6.1. Whole-body distribution of POP protein and POP activity (I)

We have studied comprehensively the distribution of the immunoreactive POP protein in mouse peripheral tissues and in rat and human brain. Our whole-body immunohistochemistry revealed that the POP protein is widely, but still specifically, distributed in peripheral tissues. Based on the enzyme activity studies conducted in rats (Daly et al. 1985, Fuse et al. 1990, Irazusta et al. 2002, Agirregoitia et al. 2005), there has been a belief that the highest amounts of POP are located in the brain, although the results have not been unanimous (see section 2.3.1.1). Here we have demonstrated that the POP protein is equally abundantly present in the mouse brain, kidney, testis and thymus, but much less in the liver. Moreover, the enzymatic activity of POP differed significantly from the relative amounts of POP protein, both in the brain and peripheral tissues.

In the mouse high-throughput gene expression measurements (GNF SymAtlas, Su et al. 2002), highest peripheral POP mRNA has been generally found from the thymus, followed by the lungs and adrenal gland. Interestingly, in the rest of the analyzed tissues, the amounts of POP mRNA were rather equal. The distribution of POP mRNA in mouse peripheral tissues was partially similar with POP protein, since rather high amounts of POP protein were determined from the lungs and thymus. However, some differences also occurred. We found the highest amounts of POP protein in the kidney, while POP mRNA was only moderately present in that tissue.

This raises an interesting question concerning the regulation of POP activity and protein. Endogenous POP inhibitor (Yoshimoto et al. 1982, Salers 1994, Yamakawa et al. 1994) may influence to the different localization between POP protein and activity, although its function and mechanism are still poorly characterized. One rationalization might be the regulation of POP enzymatic activity via its substrate expression. However, uneven distribution of POP substrates, e.g. SP, TRH and AVP, in the brain and peripheral tissues explains only partially the distribution of POP protein or POP activity (see chapters 2.3.1.2 and 2.3.2.4). As a further support, spatial locations of POP protein and SP are poor - at least in the rat brain - pointing to the fact that the locations of POP with its presumed substrates are not identical or even adjacent *in vivo* meaning that direct hydrolysis seems unlikely. Somewhat different distribution of POP mRNA and POP protein in certain peripheral tissues may occur from different methods, but post-translational modification or protein trafficking is a conceivable possibility.

This discrepancy between the density of POP protein and its enzymatic activity is interesting, and points to a strict endogenous regulation of POP. Furthermore, the presence of POP in specific internal organs supports some role for POP in these tissues, although actions of peripheral POP are still unclear and conclusions of the physiological role of POP in peripheral tissues cannot be drawn based on our findings.

6.2 Cellular and subcellular distribution of POP protein (I-III)

6.2.1 POP in peripheral organs of mouse (I)

In peripheral tissues, POP protein was present in various cell types of different organs, excluding cell-type specificity. However, most importantly, we observed in our immunohistochemical and western blot studies that POP was generally present also in the nuclei of the peripheral tissue cells. Generally, also in the brain tissue, POP has been considered as a cytosolic enzyme but a membrane-bound form has been reported (Dresdner et al. 1982, O'Leary et al. 1995, Schulz et al. 2005, Garcia-Horsman et al. 2007a). POP protein and enzymatic activity have been detected in the nucleus of the nonneuronal cell lines (Ishino et al. 1998), but probably also in neuronal cell cultures early in development (Moreno-Baylach, Felipo, Männistö and Garcia-Horsman, unpublished results 2007). This is in contrast to the brain where we were not able to detect any POP protein in the nuclei **(III, IV)** and it may be important when defining the physiological role of POP (see section 6.4).

6.2.2 POP in the rat and human brain (II-III)

In the rat and human brain tissue, POP has been previously localized by measuring enzyme activities (Kato et al. 1980b, Fuse et al. 1990, Goossens et al. 1996, Gallegos et al. 1999, Irazusta et al. 2002, Agirregoitia et al. 2003b, Agirregoitia et al. 2005) and POP coding mRNA levels by in situ hybridization (Bellemere et al. 2004). Interestingly, based on the results in the literature, POP activity significantly differed from the amount of the POP protein also in the brain tissues (for references, see sections 6.1 and 6.4).

Moreover, the distribution of POP protein in our studies and POP coding mRNA (Bellemere et al. 2004) in the brain were considerably different. For example, POP mRNA was present in high amounts in the hypothalamus (Bellemere et al. 2004), where POP substrates, such as TRH and AVP are also located (see chapter 2.3.2.4). However, we failed to determine immunoreactive POP protein in the hypothalamus, with the

exception of medial mammillary nucleus where minor amount of POP protein was seen. A similar discrepancy between the expression of POP mRNA and POP protein was observed also from substantia nigra and various other brain areas. In the rat GNF SymAtlas (Su et al. 2002), similar to the POP protein distribution, the highest POP mRNA amounts were found from the hippocampus followed by the frontal cortex and dorsal striatum. However, also in this databank equal amounts of POP mRNA were found from the cerebral cortex, hypothalamus and locus coeruleus even though we did not observe any POP protein in the hypothalamus or locus coeruleus. Although there are some differences between high-throughput gene expression measurements and in situ hybridization of POP mRNA (Bellemere et al. 2004), discrepancy between POP protein and POP mRNA occurs. These differences may be due to posttranslational modification of POP or differential targeting and/or mRNA turnover regulation. It still remains unclear how the POP enzyme is transported from these synthesis sites to the protein expression areas. Sequence analysis of POP protein and its gene indicates that this enzyme is cytosolic but it has not revealed any potential sites for post-translational modification or trafficking signal sequences nor is there any promoter signal which would point to the transport or modulation of the protein (Venäläinen et al. 2004).

The specific functions of the brain areas rich in POP may offer some explanations for the role of POP in the CNS. The high or moderate POP levels in the nigrostriatal systems of both rat and human, specifically in substantia nigra, globus pallidus, ventral pallidum and caudate putamen may point to a role for POP in motor functions (Paxinos 1990, Paxinos 2004) and indeed fluctuations of POP activity have been reported in the Parkinson's disease (Mantle et al. 1996). Furthermore, the high expression of POP in cerebellar Purkinje cells, pyramidal cells of motor cortex and projecting areas of the thalamus also postulates involvement of POP in the control of movements (Thach et al. 1992, Paxinos 2004).

Moreover, POP has been generally associated with the cognitive functions (Männistö et al. 2007). The occurrence of POP in human and rat hippocampus, particularly in the CA1 field pyramidal neurons, is evidence of some potential modulation of limbic activity (Paxinos 1990, Paxinos 2004). POP-immunoreactivity was also high in several other areas believed to participate in cognitive functions, such as the lateral septum and indusium griseum (Paxinos 2004). This indicates that POP may actively participate in CA1 neurotransmission to subiculum and to deep layers IV-VI of the entorhinal cortex (Amaral 1993, Paxinos 2004), and to the lateral septum as well (van Groen et al. 1990). However, POP was present only to a moderate extent in the prefrontal cortex, lateral entorhinal cortex and nucleus accumbens, other areas associated with the memory and learning functions (Paxinos 2004).

The abundant presence of POP in the projection neurons of sensory motoric areas of the cerebral cortex (i.e. motor and sensory cortices, gustatory cortex, visual and auditory cortices) and the corresponding projection areas of the thalamus may hint at its physiological role. POP expressing projecting pyramidal cells were detected especially in layers V and VI of the somatosensory and motor cortex and these cells are known to have strong afferent connections to the POP immunoreactive thalamic nuclei, such as ventral posterolateral and -medial nuclei (Paxinos 2004). These results suggest that POP may be involved in the regulation of thalamocortical neurotransmission in several thalamocortical loops, possibly as a regulator of neuropeptide levels or some other regulatory function. Further support for the role of POP in the regulation of neurotransmission may come from the abundant expression of protein in the cerebellar Purkinje cells that are known to be the major output cells of the cerebellum (Thach et al. 1992, Braitenberg et al. 1997).

Our study revealed, that POP was not located in the astrocytes in any part of the rat brain studied. This finding is in contrast to POP activity measurements performed with the cell lines *in vitro*, where enzyme activities have been found both in neuronal and glial cells (Koshiya et al. 1984, Mentlein et al. 1990, Schulz et al. 2005) but agrees with the study in which POP was found exclusively in neurons of both adult and aged mouse neurons and as well as human Alzeimer's diseased brains (Rossner et al. 2005). Furthermore, in the study of Bellemere et al. (2004), the POP coding mRNA was not present in the astrocytes. However, we were able to locate small amounts of POP protein in the myelin sheaths using electron microscopy and some enzyme activities have also been detected from oligodendrocytes (Koshiya et al. 1984). The reason for this discrepancy of the astroglial expression between *in vitro* and *in vivo* results may derive from the different localization – and probably different functions as well – of POP in the proliferating tissues, such as peripheral tissues and cell cultures (see section 6.4).

6.2.3 Subcellular localization of POP in the rat brain (III)

The subcellular localization of POP supports the proposed role of POP in peptide and protein trafficking, processing and secretion (Schulz et al. 2005, Garcia-Horsman et al. 2007a). We found that POP was associated with the Golgi apparatus and rough endoplasmic reticulum (RER) membranes, well known organelles where protein processing and secretion occurs. Using confocal microscopy, Schulz et al. (2005) found that POP was attached to the microtubules, i.e. a cytoskeletal component. We did not detect the POP protein in the microtubules. However, due to the fixatives required for

electron microscopy immunohistochemistry, we may have overlooked the presence of POP in microtubules *in vivo*. Nevertheless, the localization of POP in the Golgi apparatus and RER may point to a function for POP as participating in processing the immature form of proteins, possibly even its substrates, such as SP and TRH.

As anticipated, the majority of POP was present free in the cytosol (Dresdner et al. 1982, Polgar 1994). The cytosolic form may participate in the cleavage of proline-containing peptides being co-localized with POP, and evidently also acts as a regulator of IP_3-signalling while IP_3 itself is not a substrate of POP (Williams et al. 2000, Williams et al. 2002). The presence of POP in the intracellular RER also may point to a role for POP in IP_3-signalling, since IP_3 type 1-receptors reside in the RER membranes (Vermassen et al. 2004).

Some POP was found also in the cell and cytoplasmic membranes. Even though there is some evidence for a membrane-bound POP activity (O'Leary et al. 1995), the identity of this enzyme has not been conclusively confirmed. Furthermore, POP in the RER was also attached to the intracellular membranes, and it is not known whether this POP is the membrane-bound or cytosolic form of POP.

6.3 Spatial associations of POP with specific neuronal neurotransmitters and its substrates (III, IV)

Since the associations between POP and neuronal neurotransmitters are unknown, we considered it important to study this matter. Since POP is mainly an intracellular enzyme, it may participate in the neurotransmission via an intracrine mechanism (Re et al. 2006). Furthermore, there is an abundance of data for an intracellular colocalization of neuropeptides with classic neurotransmitters like ACh, GABA, histamine and catecholamines.

Cortical GABAergic interneurons contain several neuropeptides, such as SP and somatostatin, both potential POP substrates (Table 1, Emson et al. 1979, Kawaguchi et al. 2002, Garcia-Horsman et al. 2007a) and the colocalization of POP with GABA in the cerebral cortex was rather rich. However, only a poor degree of colocalization with POP and SP was seen in the cerebral cortex and the coexpression of POP, SP and GABA among the POP containing cells was only 19%. Therefore, due to the poor spatial colocalization of POP and SP, it seems unlikely that POP is involved to any significant extent in SP-mediated neurotransmission in the cerebral cortex or at least an effective transport system for SP and/or POP itself is needed.

Moreover, in striatum POP was present to a major extent in the GABAergic neurons but not in the TH-immunoreactive dopaminergic neurons and hardly at all in the cholinergic neurons, pointing to a role of POP in the inhibitory regulation of nigrostriatal path (Paxinos 2004). Interestingly, GABAergic neurons which are rich in POP protein, do use SP as a neurotransmitter (Besson et al. 1990, McGinty 2007). SP also participates in the "direct pathway neurotransmission" by slowly modifying the signalling activated after loss of dopaminergic input to the striatum, e.g. in Parkinson's disease. GABA is known to inhibit SP release and SP, on the other hand, releases AVP, histamine and ACh (Skidgel et al. 2006, Standaert et al. 2006). Therefore, we studied the coexpression of POP, GABA and SP using triple-label immunofluorescence and observed that in striatum, SP was present only in 26% of GABAergic POP-containing cells. Furthermore, since it is known that SP rich GABAergic neurons are an integral component of the neurotransmission in the direct nigrostriatal pathway (Paxinos 2004), it seems likely – based on our results - that POP is more involved in the inhibitory neurotransmission of the indirect nigrostriatal path than in the neurotransmission of projection neurons in direct path.

In cerebellar Purkinje cells, POP was highly colocalized with GABA and IP_3R1 and also substantial colocalization of POP and SP was found in those cells. The presence of IP_3R1 in the Purkinje cells has been linked to cerebellar LTD (Khodakhah et al. 1997) and the possible involvement of POP in the IP_3-signalling (see above) may suggest a role for POP also in the cerebellar LTD. SP is a regulator of cerebellar circuits since it controls the firing of the Golgi cells and in this way can modulate the cellular activity of Purkinje cells through parallel fibers (Inagaki et al. 1982, Del Fiacco et al. 1988, Nakaya et al. 1994). Moreover, high colocalization of POP with GABA points to a role as a regulator of the dominant GABAergic neurotransmission in the Purkinje cells. These results may indicate a role for POP as a signal modulator in the cerebellar Purkinje cells, possibly via the regulation of SP levels. However, it is not known how POP is able to hydrolyze SP located inside the vesicles.

POP was partially colocalized with the brain cholinergic system (Table 5). The associations of POP and ACh are poorly understood, but repeated administration of POP inhibitors has increased M_3-muscarinic receptor mRNA levels (Katsube et al. 1996) and ACh activity in the cortex of old rats (Toide et al. 1997). The mechanisms of these kinds of actions are not known and blockade of neuropeptide cleavage, especially TRH (Toide et al. 1993), is one possibility. However, the rather poor colocalization of POP with ACh (Table 5) - especially in the cortex areas important for memory and learning, such as lateral entorhinal cortex – is not support for the participation of POP in ACh mediated memory and learning functions. As a further support, no POP

immunoreactivity was observed in the medial septum, diagonal band of Broca and nucleus basalis magnocellularis, the most important cholinergic memory and learning areas projecting to the hippocampus and cortex (Paxinos, 2004).

The cortical spiny pyramidal cells, also neurones very rich in POP, are known to be mostly glutamatergic (DeFelipe et al. 2002, Paxinos 2004). Furthermore, changes in POP activity were recently observed in the rat cortex and hippocampus after administration of an N-methyl-D-aspartate (NMDA)-antagonist to induce schizophrenia-like symptoms (Ahmed et al. 2005, Arif et al. 2007). These findings support the participation of POP in the glutamatergic neurotransmission in the brain - possibly by modifying the levels of the POP substrates, such as TRH (Kasparov et al. 1994). Moreover, it is known that glutamatergic neurotransmission and changes in the morphology of cortical pyramidal cells are associated with schizophrenia (Lewis et al. 2000, Lewis et al. 2003) and this may explain why there are changes in the activity of serum POP which show a correlation with the severity of schizophrenic symptoms (Maes et al. 1995). However, the variations of enzymatic activity of POP in plasma should be looked with a doubt (see section 2.3.3).

In the hippocampus CA1 area, a brain region important for memory and learning, based on the size and shape of the neurons and the results of colocalization studies, we have shown that most of the cells containing POP in the hippocampus CA1 were glutamatergic pyramidal cells (Van der Zee et al. 1997, Paxinos 2004) rather than GABAergic or cholinergic interneurons. These results substantiate the findings where single or repeated administration of the POP inhibitor, JTP-4819, did not affect the hippocampal ACh levels (Toide et al. 1997). Furthermore, POP was highly colocalized with IP_3R1 in hippocampal CA1 pyramidal cells. In these cells, IP_3 has been associated with LTP and LTD, important phenomena in memory and learning this probably is due to release of Ca^{2+} within the cell (Lynch et al. 1991, Bliss et al. 1993, Khodakhah et al. 1997, Jun et al. 1998, Fujii et al. 2004, Taufiq et al. 2005). Interestingly, LTP is induced by the activation of NMDA-glutamate receptors (Bliss et al. 1993, Fujii et al. 2004) and therefore, based on the proposed involvement of POP in IP_3 signalling (Williams et al. 2000, Schulz et al. 2002, Williams et al. 2002, Cheng et al. 2005) as well as our findings, it appears likely – at least in spatial terms - that POP may have a role in IP_3-mediated LTP regulation in hippocampal CA1. SP is also able to induce LTP in the hippocampus, and NK1Rs are necessary for the mediation of this effect (Langosch et al. 2005). However, the limited coexpression of POP with SP and NK1-receptors points to POP acting in LTP through some other mechanism rather than simple SP hydrolysis. Our studies suggest that the beneficial effects of POP inhibitors on impaired memory and learning (Toide et al. 1995a, Toide et al. 1997, Schneider et al.

2002) may be a consequence of the elevation in the levels of IP_3 and hence enhanced LTP and LTD in the hippocampus.

Furthermore, the expression of IP_3R1 in POP-immunoreactive cells was generally modest in the cerebral cortex and subchronic administration of POP-inhibitors caused no significant increase in the IP_3 levels of cerebral cortex (Jalkanen et al. 2007). This is most likely due to the fact that only a rather minor population of IP_3R1-immunoreactive cells contain also POP, and as a consequence no changes of IP_3 levels could be seen in the whole cortex homogenates after administration of POP-inhibitor

POP was equally present both in inhibitory and excitatory neurons in the thalamus but the coexpression of POP with IP_3R1 in thalamus was striking. IP_3-signalling in the thalamus has been rather poorly characterized, but it may be involved in the regulation of thalamocortical rhythms (de la Vega et al. 1996) and the role of these oscillations in epileptic seizures has been extensively documented (Miller et al. 1990a, Miller et al. 1990b, Gale 1992). VPA evidently modifies these spontaneous rhythms (Nowack et al. 1979, Mares et al. 1992, Zhang et al. 1996), although not via the GABAergic system (Zhang et al. 1996). Interestingly, POP is inhibited by VPA and it is claimed to be a direct target of this drug *in vivo* (Cheng et al. 2005). Collectively, high amounts of POP protein in the thalamus support the involvement of POP in thalamocortical neurotransmission, possibly through the regulation of IP_3-signalling.

6.4. POP in cell proliferation/differentiation and future studies (I-IV)

The variations between the expression of POP in the brain, cell lines and peripheral tissues (see sections 6.2.1 and 6.2.2) may depend on differences in cell proliferation. It is well known that the mature brain tissue is not proliferating, while peripheral tissues and cell lines are continuously dividing. The nuclear colocalization of POP protein and a cell proliferation marker Ki-67 (for review, see Scholzen et al. 2000) was only partial but the colocalization of non-nuclear POP and Ki-67 was rather convincing. Since the Ki-67 is absent from the cells only during the G0-phase, the proposed direct effect of POP to the DNA synthesis *in vitro* (Ohtsuki et al. 1994, Ohtsuki et al. 1997b, Ishino et al. 1998) seems questionable. However, the indirect participation of POP in DNA synthesis may occur since we observed POP to be present in the cytoplasm of the cells during the active phase of proliferation. Furthermore, our studies may support a role in the cell division and/or differentiation since POP activity is also high early in the development (Matsubara et al. 1998, Agirregoitia et al. 2003a, Agirregoitia et al. 2007) and in the cancer cells (Goossens et al. 1996). High POP activities were seen in the

testis where the POP protein was exclusively present in the developing spermatids, supporting the results obtained by Kimura et al. (2002) in mouse testis.

Consequently, in proliferating tissues, such as peripheral tissues and immortal cell cultures, nuclear POP may have some novel roles, such as modification of nuclear transport (Schulz et al. 2005, Puttonen et al. 2006) or other catalytic functions as a prolyl *cis-trans* isomerization (Lu et al. 2007, Ikura et al. 2008). Furthermore, our finding could explain the differences that were noted between neuronal cell culture studies and mature brain tissue, such as POP activity expression in glial cell cultures (see section 6.2.2).

However, more studies are needed to explore the nuclear functions of the POP protein. The localization of POP during ontogenesis should be studied with primary cell cultures and embryonic tissue in order to make conclusions about the physiological role of POP in this period. Moreover, in order to achieve information concerning the POP substrates in the nucleus, a microarray study using POP inhibitors should be executed. To study the role of POP in the cell proliferation/differentiation, cancerous cell lines/tissues should be used. There is also a need for more detailed co-localization study between POP and its putative substrates, such as TRH and AVP, to clarify the spatial possibilities of POP to degrade these substances. Furthermore, our hypothesis of POP as a regulator of thalamocortical signaling should be studied using neuronal tracing and functional studies with POP inhibitors.

Collectively, some conclusions and hypothesis based on our present results and the literature review are collected in Table 6.

Table 6. Different POP locations and suggestions/hypothesis for their functions

Tissue	Cell type	Subcellular location	Suggested functions
Brain	Neurons	Cytoplasm	Polypeptide hydrolysis IP_3 synthesis Microtubulus functions
		Membranes of cell, RER and Golgi	Polypeptide hydrolysis Protein trafficking Protein modification in trans-Golgi network (?) Extracellular hydrolysis (?)
		Axons	Axonal transport
	Oligodendrocytes		(?)
Peripheral tissues	No specific cell type	Cytoplasm	Polypeptide hydrolysis
		Nuclear	Cell differentiation and proliferation Cis-trans isomerization
Plasma			Hydrolysis of polypeptides via POP (?) and ZIP (FAP) activity
Cell lines	Neuronal	Cytoplasm	IP_3 synthesis Protein trafficking (?)
	Non-neuronal	Nuclear	Cell proliferation DNA synthesis (?)
Bacteria			Hydrolysis of full length proteins and polypeptides

7 SUMMARY AND CONCLUSIONS

In this work, the distribution of POP was comprehensively studied in the brain and peripheral tissues both in macroscopic and cellular level. Furthermore, the subcellular localization of POP was determined and its spatial associations with specific neuronal neurotransmitters (GABA, ACh and IP_3) and its proposed substrate, SP, were examined. The following conclusions can be drawn from the results of the present study:

1. POP protein was found in equally high amounts from the brain, testis, thymus and kidney, and POP was distributed extensively in internal organs. Furthermore, POP enzyme activities and relative protein amounts differed significantly, pointing to a strict endogenous regulation of POP activity. At the cellular level, POP was present both in the nuclei and cytoplasm of the peripheral tissues, suggesting a role in the cell cycle and/or cell proliferation.

2. POP was found to be widely but still specifically distributed in the main functional areas in the human and rat brains. The locations of POP protein differed significantly from the enzyme activity measurements and POP mRNA expression, pointing to some kind of endogenous regulation of POP enzyme activity and post-translational modification. Furthermore, the expression of POP in specific brain areas associated with memory and learning and locomotor functions supports a role for POP in these behaviors.

3. It was demonstrated that POP was present in GABAergic, cholinergic and glutamatergic neurons in the brain, suggesting involvement both in excitatory and inhibitory neurotransmission. Furthermore, localizations of POP in the cortex and the corresponding projection areas in the thalamus are pointing to the involvement in the thalamocortical neurotransmission. Subcellularly, POP was found in the cytosol, Golgi apparatus and RER and to a minor extent in the myelin sheathed axons. These findings suggest an involvement of POP in protein processing and the secretory functions of the cells.

4. POP was generally poorly colocalized with SP throughout the brain. This may suggest a non-hydrolyzing role for POP and explains controversial results of POP inhibitors on the neuropeptide levels in the brain. Moreover, practically no colocalization between POP and the SP receptor, NK1R, was seen. Particularly abundant colocalization of POP and IP_3R1 in the hippocampus and thalamus points to a role for POP in IP_3 signalling and in memory and learning functions via the IP_3-mediated LTP.

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9 ORIGINAL PUBLICATIONS

I Timo T. Myöhänen, Jarkko I. Venäläinen, J.Arturo Garcia-Horsman, Marjo Piltonen, Pekka T. Männistö: Distribution of prolyl oligopeptidase in the mouse whole-body sections and peripheral tissues. Submitted

II Timo T. Myöhänen, Jarkko I. Venäläinen, Erkki Tupala, J.Arturo Garcia-Horsman, Riitta Miettinen, Pekka T. Männistö: Distribution of immunoreactive prolyl oligopeptidase in the human and rat brain. Neurochemical Research 32: 1365-1374, 2007

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