Executive summary:

NEUROPRO is a consortium of 8 academic groups and 3 SMEs. This project aimed 1) to validate prolyl oligopeptidases (PREPs) as drug targets, 2) to improve the pharmacological properties of its inhibitors and 3) to use modern techniques to make a substantial progress in the understanding of the physiology of PREPs and their role in disease. In a great scientific effort from all participants, intense research was conducted to discover the actual biological relevance of PREPs in health and disease.

Intracellular, PREP intervenes in the signalling to control axonal transport, secretion and the processing of prohormones and proneuropeptides. Extracellular, it is involved in the activation of immunoactive cells and the control of neural plasticity and migration. Importantly, all these processes are compromised in neurodegeneration and one of the main conclusions derives from the role of PREP in neuroinflammation. Interactions of PREP with alpha-synuclein, GAP43 and structural cytoskeletal proteins have been established, which are respectively related to Parkinson’s disease (PD), nerve outgrowth and vesicle traffic. Structural analysis of inhibitor bound PREP has provided information on the relations with activity structure and protein-protein interactive features. This information leads to a next generation of drugable compounds targeted to specific functions of PREP.

Of medical impact, we have obtained remarkable results which link PREP directly with plaque deposition in neurodegeneration. In cellular models, it was found that PREP is important for the processing of amyloid precursor protein (APP). Disruption of the APP metabolism and/or clearance is one major feature in Alzheimer's disease (AD). In fact, it was discovered that PREP co-localizes with beta-amyloid plaques in human AD brains, but also that its interaction with tau protein was disturbed compared with healthy brains. Furthermore, PREP interacts with alpha-synuclein, modifying its patterns of aggregation. In animal and cellular models of Parkinson’s disease (PD), striking results showed that alpha-synuclein plaque density is decreased upon administration of PREP inhibitors. To support these findings, it was observed that there is indeed co-localization of PREP and alpha-synuclein in actual PD brains.

In the biotechnological and drug development arenas, this project has made significant contributions. A peptide chip technology has been validated for use in diagnostic and clinical research applications. New specific targets for inflammatory disease have been found during the off-target hunting of PREP inhibitors and the underlying mechanism was unravelled. Due to the changes of PREP expression in human serum found in neuroinflammatory conditions, PREP is proposed as a new and reliable marker at least for multiple sclerosis and hepatic encephalopathy. Perhaps the most striking application is based on the discovery that PREP activity inhibition is an adjuvant for plaque clearance in PD and the possibility to extend the same principle for other plaque forming diseases.

The consequences of PREPL deficiency were known based on a small group of patients with the hypotonia-cystinuria syndrome. This metabolic syndrome is characterized by weak muscle tone and dwarfism, but the molecular mechanism(s) causing this phenotype were still unknown at the beginning of NEUROPRO. The research performed within the consortium has unveiled that PREPL is involved in membrane trafficking. These insights, combined with serendipitous observations made during the treatment of patients

have now resulted in the rational design of a therapeutic regimen, which will be tested in a small clinical trial.

From an academic point of view, this project has resulted in several dozens of scientific articles and this high productivity will continue in the aftermaths beyond the end of the funding period. Due to the scientific production of this project, the impact on the scientific community has grown significantly and this growth is now geometrical, counting the number of citations of our work, from an average of 300 citations at the beginning of the project to almost 1000 citations in 2011, a number that will very possibly be surpassed in 2012.

In summary, this project has accomplished all milestones and deliverables. We have considerably advanced the state-of-the-art in PREP and PREPL, opening new and promising research lines. Proof-of-concept on drug targets, compound scaffolds and therapeutic and diagnostic applications for commercial purposes have also been outlined.

Project Context and Objectives:

1. Project context

Neurodegenerative diseases are rapidly exerting an increased pressure for health care in the European ageing population. Current costs linked to dementia alone are estimated at 55 billion EUROS per year in Europe and in the absence of better treatments are expected to rise dramatically in years to come. Efforts to discover new drug targets and improve strategies for diagnosis, treatment and prevention are therefore of utmost importance. Tremendous research efforts in the last two decades have resulted in the identification of several molecular mechanisms that are involved in neurodegeneration. Moreover, it has led to the realization that neurodegenerative disorders as diverse as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease and Creutzfeldt-Jakob disease share common pathologies, like aggregation and deposition of misfolded proteins leading to amyloidosis in the central nervous system. Variations of this scheme are disease specific and concern the identity of the misfolded proteins, their genesis mechanisms, the oligomer, fibril or deposit levels, and the location or neuronal type of these deposits.

Proteolytic cleavage is an essential step not only in the generation and/or degradation of amyloidogenic fragments from precursor proteins, but in essentially all biological processes in the human body, including neuropeptide and peptide hormone metabolism, signal transduction and intracellular transport. PREP is a proline-specific oligopeptidase selective for peptides and protein fragments and is highly enriched in the brain. Several lines of evidence suggest an important role of PREP in neuronal plasticity and that aberrant PREP activity is involved in the progression of neurodegenerative disorders.

There are a number of concepts on which the project is based:

- The high expression level in brain and the cleavage of a large number of neuropeptides and peptide hormones in vitro, suggest an important regulatory role for PREP in many processes in the brain. Several of these peptides, like substance P, arginine-vasopressin, neurotensin, thyrotropin-releasing hormone, and alpha-melanocyte-stimulating hormone (alpha-MSH), have been implicated in learning and memory.
- Detailed analysis of PREP distribution across the brain indicates a role in cells involved in the excitatory and inhibitory neurotransmission through the glutamatergic, GABAergic and cholinergic neurotransmission systems. Furthermore, PREP may be involved in the thalamocortical neurotransmission, memory and learning functions of hippocampal formation and GABAergic regulation of voluntary movements.
- Intracellular PREP is mainly localized in the cytoplasm, probably perinuclear and tightly associated with the cytoskeleton, affecting intracellular transport and secretion. Several other forms of PREP have been observed as well, membrane bound and secreted.
- Growth associated protein 43 (GAP43) was suggested to be a binding partner of PREP. GAP43 is expressed at high levels in neuronal growth cones during development and axonal regeneration. As growth cone dynamics are also affected by PREP inhibition, a functional link between PREP and GAP43 is proposed.
- PREP expression is tightly regulated. Strikingly, the expression of PREP in hippocampus dramatically increases with age. Mice in an enriched

environment showed a considerable decrease in PREP expression, while PREP mRNA levels were significantly increased in the old mice.
- PREP inhibitors are neuroprotective in certain experimental settings, for instance in brain ischemia and T-cell activation-induced cell death. Furthermore, they counteract reactive oxygen species and block nuclear translocation of GAPDH in apoptotic cells.
- PREP cleaves fragments of alpha-synuclein, the major protein component of Lewy bodies. These intracellular deposits are a key feature in Parkinson's disease. Active PREP increased the aggregation rate of alpha-synuclein in vitro.
- Serum levels of PREP are lower in different stages of depression, and higher in patients with mania and schizophrenia. Furthermore, the effect of the mood-stabilizing drugs, lithium, carbamazepine and valproic acid, can be reversed by the inhibition of PREP.
- PREP inhibitors have been shown to reverse scopolamine-induced amnesia, while other inhibitors act as cognitive enhancers, further indicating a role for PREP in memory and learning.
- PREP inhibitors may have additional targets since different classes of inhibitors exert different effects. Other members of the family, like PREPL, acylaminoacyl peptidase, DPP2, and DPP8, are expressed in brain as well and may have similar or complementary activities.

2. Objectives

Prolyl oligopeptidases (PREPs) have been targets for memory deficit and neuroprotection and could hence play an important role in neurodegenerative diseases like AD and PD. The prevalent concepts, at the beginning of the project, were that prolyl oligopeptidases (PREPs) are involved in the regulation of the metabolism of extracellular neuropeptides engaged in memory and learning. PREP was hypothesized to function as a neuropeptide cleaving protein, altering the physiology of the brain by controlling these peptide levels. Therefore it was suggested that by simply modifying the levels of these peptides, learning and memory loss produced by neurodegeneration would be affected.

However, the molecular mechanisms behind the neuroprotective effects were unknown and the relation between PREP, PREP inhibitors and the neuropeptide metabolism had been challenged. In this project, 8 leading academic groups in the field of oligopeptidases with a wide variety of expertise, join forces with 3 small and medium-sized entreprises (SME)'s in order to significantly boost research into peptide metabolism in brain function and dysfunction and to translate basic research into new therapeutic approaches for neurodegenerative diseases.

The overall objectives established in the Grant Agreement of this project were 1) to unravel the mode of action of PREP and PREP-like (PREPL) enzymes in health and disease, 2) to validate prolyl oligopeptidases as drug targets in the treatment of neurodegenerative diseases and 3) to improve the pharmacological properties of PREP inhibitors and to discover new therapeutic targets. Additionally, the goal was to determine the basic physiological relevance of PREP in terms of cellular, tissue and body localization, substrate specificity and expression regulation patterns; subjects that had been previously unresolved. Another main objective was to establish appropriate and controlled biological models of disease to verify and determine the action of PREP and inhibitory compounds. Moreover, PREP inhibitor specificity and determination of actual targets were defined.

The aim of the first part of this project was to establish the relevance of PREP in well controlled biological models of neurodegeneration and to pin-point the actual molecular mechanisms where PREP is involved in. It is obvious that further studies on the extra- and intracellular functions of PREP and the pathways in which they are involved, are essential for the understanding of the roles of PREP in health and disease. The goals for the second period have been 1) to use the results from studying the relevance of PREP in models, to generate new hypotheses 2) to test these hypotheses in improved models and in clinical samples to conclude on the role of PREP in neurodegenerative diseases. During the last leg of the project the gathered information was upgraded to establish firm research avenues on the physiology of PREP in the human being and its pathological neurodegenerative conditions. Milestones have been set to accomplish the proposed goals in time and can be categorised according to several levels of knowledge. The milestones aimed to establish the relevance of PREP in cell death and neuronal growth are at the basic level, while those that were designed to develop specific models are fundamental for testing of existing compounds and those developed within the project.

Since 11 participants collaborate in the NEUROPRO consortium, it was necessary to appoint a coordinator and work package leaders to delegate project management and coordination tasks. One of the main administrative objectives was to set up a public website (see http://www.neuropro.eu online) to support dissemination activities between consortium members, to increase feedback from the research community and stimulate branching with academic and health organizations. In the context of the policy relevance of the research being carried out, it was required to provide standard communication material to the European Commission on a regular basis. Furthermore, a Scientific Advisory Board was appointed to monitor the progress and make suggestions for improvements and follow-up studies. Another goal was the organization of general assembly and steering board meetings twice a year in order to make collaboration between the participants as efficient as possible.

The outcome of this project is expected to lead to proof of concept that PREP inhibition is a valid therapeutic strategy in the treatment of neurodegenerative diseases. Apart from their participation in the research project, the small and medium-sized entreprises (SME)'s - developing peptide technology, animal models and drug candidates - are instrumental in translating the results of the consortium into clinical applications and in disseminating these results to pharmaceutical companies.

The research results have been dissiminated to the scientific community by participation of NEUROPRO members in conferences, national and international scientific meetings. The NEUROPRO grant additionally states that a scientific meeting has to be organized near the end of project, where the results would be presented to a specialist international audience and positioned amongst the latest developments in the field. This has led to the successful conference PSP 2010 - 'Proline Specific Cleavage and Oxoprolyl-Formation – Functions and Therapeutic Strategies' (Halle, Germany, 26-29/05/2010) and mini-symposium new frontiers on neurodegeneration and neuroinflammation (Helsinki, Finland, 26-29/09/2012), where academia and industry met and forged productive relationships.

Project Results:

This section describes and discusses the main results on scientific and technical work performed during the 48-mo project of the NEUROPRO consortium, in relation to the objectives initially proposed. In addition, the conclusions and relevance of the results are included. This description of Science and Technology (S&T) results is based on the last periodic report of the project and presents the outcome of the results from the WP scheme in an integrated form.

In order to offer a concise and logical explanation of the scientific and technical results this section is divided in several parts. In the first part, a description of the results concerning the physiological relevance of PREPs is presented from the molecular level to brain physiology. The second part summarizes the findings indicating the possible roles of PREPs in disease and pathological states. The third part explains the technical contributions reached in the project and the therapeutic potential derived from it. Finally, a conclusion is drawn. This description of the results only includes the most important achievements, minor contributions and their relevance are discussed in the WP report.

1. Physiological role of prolyl oligopeptidases

1.1 Prolyl oligopeptidase (PREP)

PREP, called post-proline cleaving enzyme, prolyl endopeptidase or proline specific endopeptidase, and also abbreviated as POP, PEP or PO, is a prolyl specific peptidase that is able to digest small peptides. It was discovered in the uterus as an oxytocin-cleaving enzyme. Soon after its discovery, it was isolated and shown to digest all short neuroactive peptides with an internal proline in vitro. Several newly synthesized compounds with inhibitory activity showed to be memory enhancers in one set of experiments and neuroprotective in another set. Despite the heterogeneity of the experimental work conditions that gave rise to these results and the diversity of the systems used, the central dogma was that PREP controls the extracellular, synaptic level of relevant neuropeptides in memory and learning, and in anti-apoptotic or growth promoting cellular (neuronal) processes. New findings, which seemed to support these ideas, also emerged: PREP expression levels were detected to rise upon aging and neurodegeneration, and to fall upon growth and regeneration.

Before the NEUROPRO project, the individual participants were sceptical towards the central dogma of PREP, because their previous research as well as the research from other laboratories indicated inconsistencies with the prevalent state-of-the-art. PREP was consistently found intracellularly, which is not in accordance with its putative action on neuroactive peptides in the extracellular space. Moreover, the levels of the putative substrates of PREP did not correlate with the level of its peptidase activity; several instances for example indicated low PREP activity together with low neuropeptide levels. Sometimes the opposite was measured, high levels of PREP activity coexisted with high levels of proline containing neuropeptides, which could not directly be explained. Some experiments still showed the beneficial effect of PREP inhibitors. In fact, several pharmaceutical companies were conducting clinical trials with PREP inhibitors as anti-dementia drugs.

Hence, this project aimed to clarify the role of PREP in memory and learning, to discover the molecular mechanisms behind this role and to validate PREP inhibitors as drug targets for neurodegeneration. This part will present the results obtained during the NEUROPRO project about the physiological role of PREP and discuss how these results practically oppose the initial hypothesis that PREPs are directly involved in neuropeptide regulation.

1.1.1. Intracellular function of PREP

Previous results of NEUROPRO participants indicated that PREP was mainly expressed intracellular with a particular disposition inside the cells. Background research implied an interaction of PREP with the cytoskeleton and a relation with intracellular trafficking and secretion, as well as changes during neuronal development. Indications of direct interaction with other proteins formed the basis for further study. The aims of this part were to identify the relation of PREP with other cellular processes and to identify its actual physiological substrates.

1.1.1.1 Interactions with the cytoskeletal system

The consortium demonstrated that PREP is located in close association with the cytoskeletal component tubulin. This distinct PREP distribution pattern was coincidently altered in response to tubulin dispersion after specific microtubule depolymerization via nocodazole treatment. Additionally, PREP was found to physically interact with the C-terminus of alpha-tubulin in a yeast two-hybrid screen using a HeLa cell line-derived cDNA library. It was proposed that PREP might be involved in microtubule-associated processes, such as intracellular trafficking and vesicle sorting independent of its peptidase function. Additionally, PREP was also detected in axonal and dendritic processes in mouse brain, substantiating a function of PREP in cellular transport processes in neurons in vivo. Investigations during the development of NEUROPRO confirmed these findings and further strengthened the notion that PREP might be involved in intracellular transport along the microtubule associated cytoskeleton. By immunofluorescence double labelling and confocal laser scanning microscopy in a number of cell lines (e.g. HeLa cells) and in mouse primary neurons, it was shown that PREP co-localises with cytoskeletal proteins. These observed associations were substantiated by electron microscopy. In undifferentiated PC12 cells on the other hand, PREP was localized to the Golgi, indicating functions for PREP in protein maturation and secretion.

The effects of PREP inhibitors on tubulin polymerisation and tubulin binding in vitro and on microtubule content measurements in cells were determined. No tight interaction between PREP and tubulin or microtubules was observed. Furthermore, interactions between tubulin or tubulin polymerisation promoting protein 25 (TPPP25) and PREP were not found. However, by employing turbidity measurement approaches it was shown that PREP is able to attenuate microtubule assembly promoted by TPPP. Some degree of PREP binding to microtubules was also observed in sedimentation assays. Glutaraldehyde cross-linking studies suggest a weak and evidently non-specific interaction between the two proteins in vitro.

1.1.1.2 Direct interactions of PREP with other proteins

Besides the interactions found with cytoskeletal proteins, direct contact of PREP with several other proteins was studied and established.

Experiments on the role of PREP in the nucleation phase of the aggregation process were carried out using AEDANS-labelled alpha-synuclein and confocal fluorescence correlation spectroscopy. After addition of PREP a substantial increase in the nucleation rate constant was found, along with a two-fold reduction of the growth rate constant. The AEDANS labelled alpha-synuclein was also used in a FRET experiment, where the AEDANS-label and one or more of the PREP tryptophan residues are in close proximity. The PREP inhibitor KYP-2047 reversed the FRET signal in wild-type (WT) PREP. The S544A mutant has a 10 times higher Kd for AEDANS-labelled alpha-synuclein than the WT, indicating that alpha-synuclein interacts directly with the active site of PREP.

Interaction studies were performed between GAP43 and PREP, GAP43/calmodulin and PREP/TPPP25 by applying isothermal titration calorimetry (ITC) and surface plasmon resonance (SPR) methods. The experimental results showed that besides the partial co-localisation of PREP and GAP43 revealed by immunofluorescence studies, GAP43 only weakly and transiently interacted with PREP. Similarly, only transient and non-specific interactions were observed between PREP and TPPP25 (an important tubulin-binding protein). PREP did not interact with calmodulin. These results indicate that PREP has some potential to modulate the very important GAP43/calmodulin interaction, but its colocalization with GAP43 as well as with the tubulin cytoskeleton in the growth cones are likely mediated by other proteins.

1.1.2 Physiological substrates of PREP

As the evidence of neuropeptide digestion by PREP in vivo was only circumstantial, comprehensive peptidomics studies on PREP inhibited animals in several conditions were carried out. Peptidomic analysis of acute and sub-chronic PREP inhibition in rats was performed. Out of a large number of peptides found to be modified tissue specifically by PREP, the most important (abundant) were: secretogranin-2, somatostatin and thyroliberin in hypothalamus, and fibrinogen alpha chains, beta chains and thymosin-beta-4 (Tbeta4) in cerebellum. A more comprehensive bioinformatics analysis concluded that PREP is involved in the processing and/or degradation of specific proteins, most probably in correlation with other proteases, and depending on the tissue and cellular location. The processed proteins are classified as protein complexes involved in energy metabolism, in gene expression regulation and in cell signalling. While PREP is involved in the regulation of some specific neuroactive peptides, it is also profoundly implicated in the regulation of cell signalling and stability. Analysing the peptidomics data from the hypothalamus, it was found that all the peptides derived from neuroactive peptides were flanked by dibasic sequences (KK, RR, or KR). This indicates that the resulting fragments were generated by the action of cellular convertases. PREP might alter the activity of pro-hormone convertase 1 and hence influence secretion. Laboratories of the consortium demonstrated that PREP also regulates the initial step of Tbeta4 degradation and that is involved in the generation of the pro-angiogenic tetra-peptide Ac-SDKP. PREP also directly alters the levels of fibrinogen B, collagen and Tbeta4 in serum, which is important for diseased states.

1.1.3 Intracellular traffic and association with aggresomes

The partners studied the trafficking and localization of PREP during differentiation, maturation and aging of cerebellar granule cells in

culture and in a model of human neurons (SHSY5Y). It was found that PREP localizes in the nuclei in earlier stages of differentiation and that it traffics out of the nucleus from this stage on. During neuron maturation PREP is located mainly in the cytoplasm and it is disposed perinuclearly upon aging. Additionally, it was found that the cytoplasmic level of PREP activity increases during the logarithmic phase of growth of SHSY5Y cells and that the activity drops with ageing.

Indications about the association of PREP with aggresomes were established. Electron microscopy revealed that alpha-synuclein aggregates are generated in presence of PREP. When production of misfolded proteins exceeds the cellular capacity, the proteins accumulate in the aggresome in a microtubule support ensheathed by a cage of vimentin containing ubiquitin, Hsp70, Hsp90, chaperonins and components of the proteasome. Aggresomes are transported along microtubules and clump together in the perinuclear space where proteasomes are recruited. When alpha-synuclein overexpressing SHSY5Y cells are cultured in conditions of oxidative stress, alpha-synuclein aggregates accumulate in aggresomes. It was shown by double immunolabelling that PREP co-localizes with alpha-synuclein aggregates in the perinuclear space. Moreover, the PREP inhibitor KYP-2047 reduces the number of aggresome containing cells and abolishes co-localization of PREP and alpha-synuclein aggregates without affecting the localization of PREP.

By using immunofluorescence microscopy the localization of PREP with respect to DPP9, alpha-synuclein, the proteasome and stress proteins involved in the intracellular clearance of aggregated proteins, was investigated. The results indicate that PREP and DPP9 do not co-localize within the cell. On the other hand co-localization was apparent with alpha-synuclein, heath shock protein 70 (HSP70) and Rpt5, a subunit of the 26S proteasome. The latter two can theoretically prevent alpha-synuclein aggregation, but at the same time alpha-synuclein protofibrils and oligomers have been shown to inhibit the 26S proteasome and HSP70 respectively. These results provide new clues on PREP's involvement in alpha-synuclein aggregation and valuable insights for identifying interaction partners of PREP in neuronal cells.

1.1.4 Interaction between PREP and specific central neurotransmitter systems

The participants of the consortium studied the localization of PREP within neurotransmitter systems. PREP was found in the GABAergic and cholinergic interneurons of the thalamus and cortex, but not in the nigrostriatal dopaminergic neurons. Further studies showed that PREP is partially co-localized with IP3 receptors in the cortex, but that this co-localization was higher in thalamus, hippocampal CA1 field and in cerebellar Purkinje cells. PREP also co-localized with dopaminergic neurons and with alpha-synuclein in the substantia nigra of the human brain. In the hippocampus, co-localization of PREP with beta-amyloid was weak but detectable. This distribution was disturbed in Alzheimer's (AD) and Parkinson's disease (PD), as discussed in part 2.

Although rodent experiments have shown that most of the PREP immuno-reactivity localizes in hippocampal neurons, an increase in the level of immuno-detectable PREP was found in astroglia of aged human brain.

The localization of PREP in thalamocortical and corticothalamic projection neurons in the ventrobasal complex, medial geniculate nucleus

of thalamus and somatosensory/motor and auditory cortices was established by using a retrograde neurotracer. Over 50% of the thalamic and cortical projection neurons contained PREP. These results support the hypothesis that PREP is involved in thalamocortical and corticothalamic signal processing.

Neurotransmitter-specific lesions in rat brain were studied as a collaborative effort. The neuronal and possible glial induction of PREP was followed after depletion of cholinergic, dopaminergic, serotonergic and substance P neurons at different surviving times post lesion. Immunohistochemistry was performed on brain slices of control and immunotoxin-treated rats. PREP immunoreactivity was co-localized with astroglia and microglia in some cases. This further indicated that specific neuronal lesions result in the expression of PREP in glial cells.

The brain enzymatic activity of PREP in rodents was the highest at embryonic day 18, while the protein amounts reach their peak at birth. PREP is located in the nucleus early in development, but is already transferred to the cytosol before parturition. These results confirm the previous cell culture data and support a role of PREP in neurogenesis. A discordance of antenatal protein amounts and enzymatic activities suggests a non-hydrolytic role and a tight regulation of PREP activity at this stage.

1.1.5. Structure-function relationship in PREP and inhibitors

The covalently binding transition state analogue Z-Pro-Prolinal (ZPP) was the first compound to be co-crystallized with PREP and it yielded the first protein-ligand interaction information for this enzyme. At commencement of NEUROPRO, most of the compounds tested as PREP inhibitors were based on substrate-like structures similar to ZPP, the first PREP inhibitor published. In this project we have developed several PREP inhibitors possessing chemical scaffolds that differ significantly from ZPP. These compounds have been kinetically characterized and crystal structures have been determined in order to deliver structural information to facilitate structure-function analyses and ultimately to develop novel drug candidates and research tools.

A number of compounds based on the established inhibitor JTP-4819 have been synthesized and kinetically characterized by the University of Eastern Finland and Helsinki. Four of these inhibitors have been successfully co-crystallized with PREP. These compounds are structurally very similar to ZPP and they share a very similar binding mode, as was expected. Three of the most promising inhibitors developed by the University of Antwerp have been co-crystallized with PREP. The overall binding mode closely resembles that of ZPP. A number of compounds have also been synthesized and kinetically characterized by Probiodrug. Eight of the most promising inhibitors have been co-crystallized with PREP.

In order to understand the role of surface loops facing the active site in the structural dynamics of PREP catalysis, a number of variant PREP enzymes were prepared by site-directed mutagenesis and characterized using a range of biochemical and biophysical techniques. The structure of one of these variants, namely His680Ala, has been determined. In this structure PREP is in the closed conformation. Despite this, loop A is disordered, as is the loop containing the mutated His680. These loops are also disordered in the open state of bacterial PREP crystal structures.

This signifies the importance of movement coordination between these loops for catalysis.

1.1.6 Physiological relevance of PREP

At the beginning of the NEUROPRO project, the idea that PREP was a modulator of extracellular levels of neuropeptides became challenged. Here we describe the most important mechanisms where PREP is involved in. All these results constitute novel findings resulting from efforts by all partners.

PREP and secretion and transport - Detailed analysis of PREP localization and dynamics within the cell, as well as its interaction with the cytoskeletal structure, have established a novel role for PREP in transport and have opened the path towards a further definition of the molecular mechanisms. The finding that PREP is specially co-localized within Golgi has been the strongest evidence of its role in secretion, but also in protein turnover. Furthermore, PREP was shown to co-localize with alpha-synuclein and the addition of inhibitors does not only break the interaction, but also seems to increase its clearance. This suggests involvement of PREP in secretory pathways. PREP expression within the cell is also differentially compartmentalized during growth and differentiation, which is another evidence of a role in transport, not only along the neural terminals, but also from or to the nuclei during the cell cycle.

The results of the peptidomic analysis in the hypothalamus contribute substantially to the idea that PREP is involved in secretory pathways. The altered peptide levels derived from secreted pro-hormones and pro-neuropeptides are products of pro-protein convertases suggesting that PREP controls the activity of these enzymes, which are part of the secretion Golgi machinery.

PREP and cell signalling and inflammation - We have shown that PREP is substantially modified in an in vitro model of neuroinflammation based on lipopolysaccharide (LPS) treated cells. After administration of LPS to mice, their cognitive function, locomotor activity and ability to cope with stress were evaluated. Impairment in contextual fear memory extinction and increased immobility time were found in LPS treated animals. PREP protein and activity levels in the hippocampi were significantly increased in comparison with control animals. Furthermore, an increased reactive microgliosis was detected. It is worth to mention that this model exhibits important similarities with multiple sclerosis (MS), a neurodegenerative disease characterized by neuronal death due to axonal degeneration and acute inflammatory attacks. Encouraged by these results, PREP levels were evaluated in patients suffering from diseases with a (neuro)inflammatory component. These results are discussed below in the paragraph of PREP and disease. In general, significant changes in circulating PREP as well as a decrease in alpha-2-microglobulin (A2M) were found. A2M inhibits proteases including PREP and is the major ligand of lipoprotein receptor-related protein 1 (LRP1). Stimulation of LRP1 receptors, mediated by A2M and measured by ERK1/2 phosphorylation, is altered by PREP inhibitors. Surprisingly, exogenous PREP also had a substantial impact on the levels of ERK 1/2 in a process mediated by a medium factor. This factor originates from the serum added to the medium, since pre-treatment of the serum with PREP has the same effects on kinase activation as PREP directly added to the cell cultures. Fractionation of the treated serum indicates that this factor consists of one or several

peptides including peptides from the fibrinogen beta-chain, collagen and Tbeta4. These proteins are cleaved by PREP in a synergistic process involving unidentified peptidases in the serum, which in turn provoke ERK 1/2 signalling into the cell.

PREP in neuron plasticity – In studying neuron differentiation, it was found that PREP is regulated by retinoic acid (RA), which is one of the routes involved in neurogenesis and migration. On the other hand, in a series of experiments in neuroblastoma cells, a decreased expression of neural cell adhesion molecule (NCAM) and polysialylated (PSA-)NCAM was found in response to PREP overexpression. NCAM and PSA-NCAM play important roles in neuronal migration, neurite outgrowth and synaptogenesis. In order to assess the effect of PREP inhibitors on NCAM-mediated neuronal plasticity, recombinant PREP was added to PSA-NCAM expressing primary cultures of cortical neurons. Addition of PREP significantly reduced the levels of PSA-NCAM and fibroblast growth factor receptor 1 (FGFR1) expression. PSA-conjugated NCAM potentiates signal transduction by the FGFR pathway and thereby regulates cell migration and cell adhesion capability. Addition of the PREP inhibitors ZPP or KYP-2047 to cell culture media counteracted PSA-NCAM reduction without affecting FGFR1 expression. The data show that PREP is able to affect neuronal plasticity via reduction of PSA-NCAM/NCAM levels on the cell surface and that inhibition of excessive PREP by ZPP or KYP-2047 at least partially restores altered plasticity.

PREP in differentiation - The cytoplasmic PREP activity levels in neuroblastoma cells were increased during the differentiation period (upon RA addition) and augmented further to higher levels compared to control proliferating cells of the same age. If RA was removed from the medium, PREP levels gradually decreased to control proliferation levels. Furthermore, in the presence of RA PREP activity is low in the nucleus, in comparison to the very high levels during proliferation at the early growth phase. The levels of PREP mRNA increased after RA-induced differentiation, which suggested that PREP activity is transcriptionally and also post-transcriptionally regulated by endogenous modulators.

During the assessment of neuron differentiation upon RA addition, PREP inhibition seemed to delay the maturation of neurons, neurite outgrowth and neuron connectivity. The link between PREP and neural growth cones was studied to find a mechanism to explain these findings. Therefore, the dose-response and time-course dependent nerve growth factor (NGF)-induced neurite outgrowth in PC12 cells was investigated and different components of the system were measured (MAPK, PI3-K, PLC-gamma, etc.). The resulting data, however, did not show any interference of PREP inhibition on the NGF-induced neuronal differentiation and survival in PC12 cells.

Pharmacology, toxicology and pharmacokinetics of PREP inhibitors - During the development of the project around 30 compounds were used and tested. Most of them were used to study the structure relationship on computer models or in structural x-ray studies. A few selected compounds were characterized and their pharmacological properties were studied, such as specificity, secondary effects, pharmacokinetics and brain penetration. The results described here correspond mainly to the compound KYP-2047, which was the most potent and specific compound.

Pharmacokinetic study revealed that KYP-2047 showed the highest brain/blood ratio and the best ability to reach intraneuronal brain PREP. No difference in concentrations between brain areas was detected. It also

inhibited brain PREP at high degree after a single dose. PREP inhibition did not produce any visible changes in health status of mice and rats, nor a decrease in the viability of cells in culture from different sources (neuroblastoma, HEK, neuronal primary cultures, etc.).

In vivo microdialysis studies assessed the effect of PREP inhibitors on neurotransmitter turnover in the rat brain. It was found that the metabolite levels of dopamine, serotonin and acetylcholine remained unchanged. However, direct administration of inhibitors into the brain by retro-dialysis decreased acetylcholine levels maximally by 50% in a concentration dependent manner. The mechanism governing the decrease in acetylcholine levels elicited by local administration (supra-inhibitory concentrations) of PREP inhibitors is not known, but it represents a very interesting new line of research worth following. In general, inhibition of PREP by KYP-2047 did not have a significant effect on neurotensin- and substance P-like immuno-reactivity in brain microdialysate. In summary, systemic administration of KYP-2047 had no significant effect on the levels of selected neurotransmitters and their metabolites, which indicates absence of the compound’s undesirable effects in the brain.

A new pharmacological effect of PREP inhibitors was discovered during this project. Both in SHSY5Y cells as in murine macrophage J774A1 cells, PREP inhibitors modulate the response of MAP kinase phosphorylation during stimulation of LRP1 signalling through A2M stimulation. This effect, initially unlinked to PREP activity, might be associated to the controlled leak of PREP to the media, which would represent a novel aspect on PREP physiology.

A comprehensive analysis of the effects of PREP inhibitors on the activity of related hydrolases was performed in the search for off-targets. KYP-2047 was by far the most specific, showing no effects of any of the related enzymes tested. Several methods to fish PREP inhibitor off-targets were developed and no proteins with affinity for PREP inhibitors were found. Furthermore, computer models and simulations along with bioinformatics analysis were applied in the search for off-targets. This resulted in, at the most, few targets with low binding scores, which included G-protein couple receptors. Based on these results, binding experiments were performed on a large set of dopamine, NMDA, and acetylcholine-receptors. However, the effects of PREP inhibitor tested were near to background levels.

1.1.7 Regulation of Prep gene expression

Before the start of the project no information was available on the regulation of PREP levels at the genetic level. An increase of PREP expression was previously reported during aging and PREP mRNA levels were decreased in laboratory animals as a response to an enriched environment. Studies within NEUROPRO demonstrated that PREP levels are tightly regulated during neuron differentiation and that RA stimulates mRNA transcription. At the chromosomal level, mammalian sequences of the PREP promoter region were analysed. Phylogenetic trees showed a high conservation of the proximal promoter between mammalian species. Predictions of transcription factor binding sites (TFBS) were performed and validated. State-of-the-art programs were used synergistically to predict conserved TFBS. This analysis suggests that the gene expression of PREP is controlled by cMyc, YY1, CEBPB and NF-kappaB transcription factors.

1.1.8 Effects of PREP deficiency

Several models where PREP expression was chemically (PREP inhibition) or genetically manipulated were developed and comprehensively studied in this project. A large number of conditions and paradigms were tested.

As low PREP activity had been reported to be neuroprotective, a large set of experiments was designed to test this hypothesis. However, most of the experiments using PREP inhibitors suggested no PREP involvement in the apoptotic or necrotic mechanisms elicited by stressors, such as 6-hydroxydopamine (6-OHDA), potassium deprivation, staurosporin, amyloid-beta-peptides and hydrogen peroxide, among others. However, a HEK-293 PREP knock down cell line was significantly more sensitive to serum deprivation than the parent cell line, which implicates a role for PREP in growth at least in this particular system.

While in PC12 cells no effect of PREP inhibition on NGF-induced differentiation was found, PREP inhibition caused a delayed neurite outgrowth during the assessment of neuron differentiation by RA. This suggests a possible action for PREP during development.

A genetically PREP deficient mouse line was obtained and tested. Phenotypical screening, magnetic resonance imaging (MRI) and magnetic resonance spectroscopy (MRS) showed that the body weight of PREP knockout (KO) mice was significantly smaller. Although their neurological index was not affected, PREP KO mice have smaller whole brain and cortical volumes. In the Y-maze test male PREP KO mice showed decreased spontaneous alternation scores. In the open field test, PREP KO mice were more active compared to WT mice, while there were no differences in the time spent in the open arm of the elevated plus-maze. PREP KO mice showed decreased freezing scores for memory of context and cue in the contextual fear-conditioning test, indicating memory deficits. Finally, PREP KO mice showed increased depressive behaviour in the tail suspension test compared with WT littermates.

1.2 Prolyl oligopeptidase-like (PREPL)

Prolyl oligopeptidase-like (PREPL) is also a member of the S9a subfamily of serine proteases and shares an overall similarity of 29% with PREP. PREPL is expressed highest in brain while intermediate levels are found in heart, kidney and skeletal muscle. Low but detectable levels are found in all other tissues. Deletion of part of the PREPL gene has been discovered in a group of patients with a recessive syndrome that we refer to as the Hypotonia-Cystinuria syndrome (HCS) and that gives rise to growth hormone deficiency and weak muscle tone. At the beginning of NEUROPRO, the only information available from PREPL was essentially based on its DNA sequence and its similarities with PREP. The results of this project significantly increased the knowledge on PREPL from its function to its relevance in disease.

1.2.1 Interactions with the cytoskeletal system

Studies on the subcellular localization and co-localization of PREPL using immunofluorescence double labelling and confocal laser scanning microscopy in a number of cell lines and in mouse primary neurons revealed interactions with cytoskeletal proteins (tubulin and F-actin) and a clear localization in Golgi (syntaxin and membrin), indicating

functions for PREPL in protein maturation and secretion. The interactions with the cytoskeletal system are supported by two dimensional-differential gel electrophoresis (2D-DiGE) analysis which compared differentially expressed proteins in human PREPL deficient skin fibroblast compared to control skin fibroblasts.

1.2.2 PREPL enzymatic activity

Based on homology studies with PREP and oligopeptidase B (opdB), PREPL contains all the sequence attributes of a peptidase. PREPL has the catalytic triad, in the order of Ser, Asp and His at topologically equivalent positions as PREP. Therefore, a proteolytic activity is expected, although at the start of the project no substrates had been found. None of the substrates cleaved by PREP or Oligopeptidase B (OpdB) were cleaved by PREPL. Functionality of the catalytic machinery could be confirmed though, using the activity based probe fluorophosphonate-biotin that binds covalently to serine residues within an active catalytic triad.

The NEUROPRO consortium has used differential peptidomics on HEK293T cells to test whether PREPL cleaves cytosolic peptides. After transfection with a knock down or overexpression construct, peptides were extracted and analysed with liquid chromatography mass spectrometry (LC-MS) in combination with DeCyder MS software. No obvious differential peptides were found. In a second approach, a database comprising of more than six hundred endogenous peptides was screened for PREPL peptidase activity with MALDI-TOF MS. The processed data did not reveal any substrates. Therefore it can be concluded that based on the enzymatic activity, PREP and PREPL show little similarity. PREP will cleave most prolyl bonds in peptides smaller than 30 amino acids whereas no cleavage of peptides, ester, or thio-ester substrates could be demonstrated for PREPL despite intensive research. Considering the involvement of PREPL in HCS, it is interesting to put more effort in the investigation of the non-catalytic role of PREPL, rather than to only focus on its proteolytic activity.

1.2.3 Physiological relevance of PREPL

The first studies on the localization of PREPL in the mouse brain were done in this project. Complete mapping using immunohistochemical staining revealed a widespread presence of PREPL throughout the brain, including basal forebrain, hypothalamus, brain stem, neocortex and hippocampus. In some of these populations there was a prominent, punctuate staining most likely resembling compact protein assemblies in peptidergic neurons. This indicates a function of PREPL in the brain.

The above described co-localization of PREPL with components of the cytoskeletal system and Golgi apparatus point towards a role in protein maturation and secretion, which might correspond to the observed growth hormone deficiency in HCS patients. Since HCS patients suffer from neonatal hypotonia, PREPL might also be involved at the neuromuscular junction.

1.2.4 PREPL structure

Using native gel electrophoresis and gel filtration chromatography it was shown that PREPL exhibits a dimer-monomer transformation upon dilution. A Kd of 0.4 µM was calculated at physiological ionic strength and pH. The

dissociation constant was found to be much lower at higher ionic strength, but was relatively independent on pH. DTNB titration detected the highest number of accessible thiols at the concentration of half dissociation (Kd), which is reduced in the monomeric and even more reduced in the dimeric state of the protein. This indicates a slow dimerization process and the need for some structure opening preceding oligomer formation. ITC also suggests an uncommon dissociation comprising of at least two simultaneous processes. Higher oligomeric forms appear upon slow concentration of PREPL and these oligomers finally transform into an aggregate or polymeric form upon incubation or slowly dissociate into monomers during high dilution.

In this project several PREPL expression systems were developed for crystallography. The experimental design for bacterial expression and purification of WT PREPL was set and a novel on-column digestion assay for GST-tag removal was worked out, together with a delicate concentrating method in order to circumvent protein aggregation. This resulted in more concentrated material, required for crystallisation. Efforts to obtain PREPL crystals were carried out to improve diffraction by using different salt concentrations and the hanging drop method. Co-crystallization of PREPL with compound 8, an inhibitor that was recently documented in the literature, has been performed. Furthermore, 30 Probiodrug compounds have been screened for their potential inhibitory activity using the activity-based probe fluorophosphonate-biotin, which binds covalently to serine residues within an active catalytic triad. The most potent inhibitors will be co-crystallized with PREPL.

2. Role of Prolyl oligopeptidases in disease

2.1 PREP and Alzheimer's disease

Several AD mouse models were developed and characterized during the NEUROPRO project.
Tg6799 mice with APP/presenilin (PS)1 mutations showed no changes in behavioural tests after PREP inhibitor treatment. PREP inhibition, however, induced a slight suppression of cortical GFAP and total APP mRNA in 15-month-old Tg2576 mice that overexpress human APP (hAPP). These mice also exhibited an apparent up-regulation of cortical IL-6 and hippocampal APP mRNA. There were no obvious effects of PREP inhibitors on Abeta-plaque pathology, on the accompanying gliosis or on the expression of pro-inflammatory cytokines in this experimental setup.

The 47 C-terminal APP fragment (CTF47) was cleaved by PREP at a proline located 10 residues before the C-terminal within the YENPRY motive. Additional evidence suggests that the C-terminal APP fragment of 31 amino acids, derived from caspase cleavage, may be hydrolysed by PREP in physiological conditions.

Immunohistochemistry of PREP was performed on human AD brains. A strong PREP labelling of layer III and V pyramidal neurons was observed in control brain, but in a number of AD cases this PREP labelling was significantly reduced. In AD, furthermore, the expression of PREP was found to be higher in temporal cortex – known to be severely affected by AD pathology – than in cerebellar cortex, which was virtually spared. In AD the total amount of PREP was reduced in temporal cortex as compared to control tissue.

In other experiments, PREP was found to co-localize with beta-amyloid aggregates in the hippocampus/entorhinal cortex, where it is probably situated at the endoplasmic reticulum/Golgi network. PREP was also found to co-localize with tau protein in normal brains, but this interaction was disturbed in AD. A massive activation of astroglial cells in AD hippocampus was observed. PREP is present in these cells and is possibly secreted in the extracellular space.

2.2 PREP and Parkinson's disease

By using purified proteins in a cell free system, it was established that PREP facilitates the aggregation of alpha-synuclein in a PREP inhibitor sensitive manner. This effect is dependent on the active enzyme and alpha-synuclein is not degraded by PREP. Preliminary experiments showed that in cell culture PREP inhibitors reduced the plaque deposition by about 50%. An effect of PREP inhibitors on alpha-synuclein deposition in SHSY5Y cells (overexpressing alpha-synuclein) was confirmed in additional experiments using several PREP inhibitors. New data indicate that PREP also accelerates the formation of beta-fibrils, which can be reversed by KYP-2047. Experiments on the role of PREP in the molecular mechanisms of these effects were done and they indicate that alpha-synuclein interacts directly with the active site of PREP. The synuclein region responsible for this interaction has been narrowed through C-termini truncated forms of alpha-synuclein.

The distribution of soluble aggregates and insoluble forms of alpha-synuclein in SHSY5Y cells overexpressing WT and A30P mutant alpha-synuclein was analyzed in different conditions of (oxidative) stress and in the presence or absence of KYP-2047. A co-localization of PREP with alpha-synuclein was confirmed in non-stimulated WT SHSY5Y cells.

PREP expression in PD brain was also analyzed. Human brain tissue was characterized with regard to sub-regions and individual neuronal populations affected or spared by neuropathology. On the basis of a number of previous studies it is known that neurons ensheathed by aggrecan-containing perineuronal nets are frequently spared from degenerative events. On the other hand, neurons lacking such specialized forms of extracellular matrix are often marked by presynaptic axonal coats containing link protein and CRTL-1. Double immuno-histochemical labelling of the substantia nigra pars compacta, pars reticulata and lateralis with calbindin, linking protein CRTL-1 and aggrecan immuno-reactive perineuronal nets on PD material, revealed striking differences in the vulnerability of defined dopaminergic neuronal subpopulations. In particular, a neuronal subpopulation unaffected by neurodegeneration was found to be ensheathed by perineuronal nets, whereas neurons in the same area lacking such perineuronal nets and contacted by multiple presynaptic axonal coats (link protein, CRTL-1) were highly vulnerable. Further analysis showed clear co-localization of PREP with alpha-synuclein in the substantia nigra of PD human brains. This is in agreement to the fact that PREP is observed on alpha-synuclein aggregates in cells and transgenic human A30P-synuclein mice, which further supports the beneficial effect of PREP inhibitors. PREP was co-localized in dopaminergic neurons and also in the substantia nigra, especially in healthy brains.

2.3 PREP and neuroinflammatory diseases

A significant decrease in PREP activity was observed in clinical forms of MS with a high inflammatory component [i.e. clinical isolated syndrome (CIS), remitting-relapsing (RR) and primary progressive (PP) MS], except for the secondary progressive (SP)MS form, where only a trend to decreased activity was observed. A significant reduction of PREP with age was noted. This reduction correlated to disease severity. On the contrary, PPMS showed a significant increase of PREP activity in correlation with the severity of the disease. In the experimental autoimmune encephalomyelitis MS mouse model, PREP inhibition increased the sensitivity to the clinical effects of auto-antibodies against myelin oligodendriocyt.

Further confirmation on changes of PREP levels was found in other neuroinflammatory models. In induced hepatic encephalopathy in rats, induced by a high ammonia diet or via ammonia administration through a porta-cava shunt, a minor decrease of PREP in the cerebellum of ammonium loaded animals and a decrease of protein content in the cortex of porta-cava shunt animals was found. Furthermore, a strong increase of immuno-histochemically labelled PREP in hippocampus (CA1 layer), cortex, cerebellum and striatum was observed. Plasma from cirrhotic patients showed a reduction in PREP levels, although this decrease did not correlate with the diagnosed encephalopathy. The decrease in PREP, however, correlated with IL-6 and cGMP levels, which further points to a tight relation of PREP with inflammatory processes.

2.4 PREPL in Hypotonia Cystinuria Syndrome

The Hypotonia-Cystinuria Syndrome (HCS) is caused by deletions of the prolyl endopeptidase-like (PREPL) and SLC3A1 genes. This recessive metabolic syndrome is characterized by cystinuria, neonatal hypotonia with spontaneous improvement and growth hormone deficiency. Since it is well established that inactivating mutations or deletions in the SLC3A1 gene cause isolated cystinuria, all other symptoms can be attributed to the deletion of PREPL. How PREPL deficiency causes these clinical manifestations is currently unknown.

Before the start of the NEUROPRO project five deletions, involving PREPL and SLC3A1 and resulting in HCS, had already been reported. Two other deletions involve C2orf34 in addition, which causes the more severe atypical HCS (deletions E and F). The 2p21 deletion syndrome in addition involves a fourth gene PPM1B and is clinically the most severe. During the project two HCS patients homozygous for two novel deletions of the PREPL and SLC3A1 genes have been described by the NEUROPRO consortium. Following the nomenclature these deletions were named H and I. Both patients exhibited several HCS symptoms, such as cystinuria and neonatal hypotonia. As the diagnosis of HCS currently is often delayed, an increased awareness for HCS is needed.

2.5 PREPL and neurodegeneration

During the analysis of AD brains, it was found that the amount of PREPL protein did not change significantly compared to healthy brain. However, the dimer-monomer equilibrium of PREPL was shifted towards the monomeric state. This reveals a completely new avenue of research on the pathology of AD.

3. Technological contributions

3.1 New method to deliver small peptides into living cells

A reproducible method for significant transfection of neuronal and glial cells with small peptides was developed. This method is based on the DeliverX technology by Panomics and uses virus-derived amphipathic peptides to form non-covalent nanopores. These nanopores allow receptor-independent diffusion through the cellular membrane. To establish the method, known cleavage products of PREP enzymatic activity (namely substance P and neurotensin fragments) were fluorescent-labelled and used for transfection. The analyses of (i) dose-dependency, (ii) time course, (iii) transfection rate and (iv) intracellular localization in different cell types (neurons and glia) revealed the following: (a) doubling the transfectant concentration is optimal for transfection efficiency, (b) the peptides are detectable within cells at 60 minutes after initiation of the transfection and the intracellular label is stable for at least 72 hours, (c) the transfection rate differs between cell types and reaches 10% in mouse primary astrocytes, 40% in mouse hypothalamic GT1-7 neurons and 80% in mouse primary neurons and (d) the delivered peptides are not targeted to endosomes and lysosomes but mostly are present in the cell soma.

3.2 PepChip analysis and development of an in vitro assay for peptidases

This project has described the lack of specificity of random 15-mer peptide microarrays upon digestion with different proteases. Accordingly, the peptide microarray platform was studied in detail in order to find critical parameters for protease analysis. The level of saturation of the microarray surface with covalently coupled peptides was substantially improved by varying the concentration of peptides in the spotting solution and the amount of solution deposited. It was concluded that the peptide concentration and not the total amount of peptide is rate limiting for the coupling reaction to the microarray surface and that our highest concentration does not fully saturate the surface binding capacity. Subsequently, three fluorescent dyes, Fluorescein, R-Phycoerythrin and Cy3, all conjugated to streptavidin, were validated for sensitivity and signal-to-noise ratio for the detection of biotinylated peptides on microarrays. Cy3 was the most sensitive, followed by Fluorescein. R-Phycoerythrin failed to detect a significant number of peptides. Cy3 gave slightly elevated background signals, possibly due to the overall higher quantum yield of this dye. We conclude that Cy3 is the best dye tested for detection of peptides on microarrays, but Fluorescein gives satisfactory results as well.

In order to validate the results on an independent detection platform, called the minicard, experiments using ultra performance liquid chromatography (UPLC) on peptides in solution were performed. The surface modification was optimized to get a high yield covalently linked peptide to the glass-slide. The read-out was done by binding Streptavidin-Cy3 to biotinylated peptides. Digested peptides miss the N-terminal synthesized biotin and should not result in a positive signal after Streptavidin-Cy3 incubation. As an alternative to the biotin-streptavidin system, labelled peptides can also be used to measure cleavage, for example by using a series of 6 peptides directly conjugated to 5,6-FAM. To optimize the PREP activity in the minicard system different surface modifications were made. A thin hydrogel layer with a low amount of functional groups and a low density of peptides resulted in specific digestion with a good standard deviation. Optimal results were obtained with a relative low affinity antibody whose binding is very sensitive to a small loss of

peptide due to specific digestion. With high affinity antibodies no difference was observed.
Based on the results discussed above a full library of approx. 800 different solid-phase bound peptides was selected and is synthesized on mini-cards. To find an optimal peptide surface density the peptides were synthesized in a low, medium and high density. In addition, the peptides were synthesized as linear or as constrained (clipped). PREP substrates coupled to a minicard with low density functional groups gave a read-out improvement of PREP activity compared to a minicard with high density functional groups. With these tests it also became clear that PREP digestion is not only proline specific, since the negative control which had its proline substituted by a serine was also digested by PREP. Linear peptides gave better digestion results than constrained peptides.
Further studies with PREP and the inhibitor KYP-2047 were done. The digestion of YQDYEPEA was inhibited using a 1:10 PREP:KYP-2047 ratio. The termination of YQDYEPEA digestion in solution takes 10 times less KYP-2047 than to stop PNA substrate digestion.

In summary, specific PREP digestion can be measured on minicards through specifically selected antibodies that are sensitive to a relative small loss of peptides.

The read-out of the PREP digestion is now performed by Fourier Transform Mass Spectrometry (FTMS). To detect the digestion of a substrate in the minicard platform only 2% of digested substrate is necessary. An extra step is the removal of the remaining part of the digested peptide, using a special linker. The benefit is that it is possible to detect where the digestion of the peptide had taken place, which is not only interesting for PREP, but also for other proteases with unknown digestion sites. High throughput analysis is also possible using this method.

Peptides derived from proteins that might interact with PREP were also synthesized on a minicard. Minicards with a library of 15 mer long peptides with overlapping sequences derived from alpha-synuclein, GAP43, MARE and BASP were screened for their interaction with PREP. Binding of His-tagged PREP was quantified with an anti-His antibody. The results with the alpha-synuclein based peptides demonstrate an increased affinity of PREP towards peptides that are derived from the C-terminus of alpha-synuclein. The insertion of single and double loop conformations into peptides also had an effect on the accessibility of certain peptides. In the case of the alpha-synuclein-peptide set, the binding of PREP is significantly increased after the introduction of a single loop conformation into the peptide GKNEEGAPQEGILED (101-115). Introduction of a double loop conformation, in which Pro108 is replaced by a cysteine, is accompanied by a weaker binding. The results from the other peptide sets are less straightforward to interpret and still need to be evaluated.

3.3 High-throughput protease inhibitor screening

A set of soluble random bicycle peptides was synthesized, which are clipsed and as a consequence are shaped with 2 loops. Therefore, they are also called ‘2-CLIPS’ peptides. Over 600 random soluble 2-CLIPS peptides were screened for their inhibiting capacity on PREP. From this large set of 2-CLIPS peptides 0.9 % reduced the PREP activity with greater than 90%. In order to validate the results and determine the IC50, the top 5 of the most potent 2-CLIPS peptides were resynthesized and purified. These 5 peptides were also used as a scaffold for further optimization of the inhibitory capacity via G-scan analysis and substitution of any

present proline and/or methionine residues by glycine or isoleucine respectively. Some of the modified 2-CLIPS had an improved inhibiting activity and will be purified to determine the IC50. At first glance the inhibitory potency of the 2-CLIPS peptides is in accordance with the substrate specificity of PREP.

3.4 Development of biological models

3.4.1 Cellular models

A large number of cellular models for PREP and disease research in general were developed in this project.
PREP silencing RNA plasmid clones were established, which have been multiplied by transformation in E. coli and transfected in a PREP positive HEK-293 cell line. The most effective silencing clone, which was able to silence ~85% of PREP at the mRNA level, was selected. PREP silencing was further confirmed at the protein level by SDS-PAGE and western blotting, and at the activity level by using the chromogenic substrate Z-gly-pro-pNA. Around 50% reduction was detected. Further experiments demonstrated that reduced PREP expression induces activity of caspase-3 and 9 and that this effect was counteracted by the co-transfection with a PREP overexpressing plasmid. This model is useful for cell death research.

In order to investigate the possible role of PREP in AD a protocol was developed for culturing primary cortical neurons from 1-day old pups of transgenic Tg6799 mice with APP/presenilin (PS)1 mutations (5xFAD). In cultures prepared from transgenic and wild type animals no differences in neuronal death were observed. A PREP activity assay in these cultures is in progress.

A model for neuro-inflammation was established in microglial cells in culture. Cells were isolated from 1-3 days old rats and the activation of microglia was induced by the addition of LPS. The level of activation was measured by the expression levels of Iba-1 and iNOS. Addition of LPS induced increased levels of Iba-1, as was measured by western blot. Increased levels of iNOS, as well as Iba-1, were also detected in immunohistochemical assays. Based on these findings it is proposed that during inflammation activated microglia might induce PREP expression in other cells e.g. neurons.

NEUROPRO established models of neuronal death using beta-Amyloid (25-35) or colchicine and developed cellular models to investigate the efficacy of compounds on IL-6 secretion. Pathological up-regulation of astrocytic IL-6 expression is known to play a pivotal role in the onset and progression of neurological diseases including AD, MS, PD and traumatic brain injury. Oncostatin M (OSM)-treated human glioma U343 cells were used as a model for astrocyte-derived elevated IL-6 expression. This model is used to screen low molecular weight compound libraries for IL-6-lowering effects.

Partners also validated the rat E14 primary midbrain culture system in Wistar rats (enriched culture with dopaminergic neurons), where tyrosine hydroxylase positive neuronal cell death is induced by 6-OHDA. A consistent 35-45% dopaminergic neuronal death has been achieved upon treatment. This model is validated for the use on toxicity elicited by human amyloid beta 1-42, glutamate and oxygen glucose deprivation.

Rat embryonic stem cells were developed and validated as a tool for testing maintenance and differentiation of neural progenitors. Stem cells were isolated from hippocampi of one-day-old mouse pups. Neural progenitors were able to divide and could be maintained in the undifferentiated state during up to 10 passages. Differentiation was induced by RA in the presence of bFGF. The differentiation state was controlled using antibodies against neuronal markers. This culture of undifferentiated neural progenitors can be further used for the evaluation of the roles of enzymes or proteins in differentiation and survival.

3.4.2 Development of genetic animal models

A PREPL KO mouse model was generated in which exon 10 of PREPL is excised resulting in a deletion of the catalytic site. The phenotype of adult PREPL KO mice shows similarities to the HCS symptoms caused by PREPL deficiency. Analysis of secretion and neuromuscular junction in this animal model is planned to investigate the role of PREPL and to explain the pathophysiology of HCS. Since HCS symptoms are mainly expressed during childhood, it is necessary to study younger mice from one to three weeks old. Mouse embryonic fibroblasts (MEFs) have been generated from WT and KO mice for differential peptidomics analysis and immunofluorescence microscopy.

Attempts were undertaken to create PREP/PREPL double KO mice. This mouse model would be instrumental to study the (partial) redundancy of both proteins. If both proteins participate in the same pathways, the phenotype of the dKO is expected to be more severe than the single KOs. Heterozygous PREP KO mice were crossed with homozygous PREPL KO mice and re-derivation procedures were undertaken. Despite several attempts, no mice survived.

A mouse strain lacking the PREP gene was constructed and characterized. This model can be used for research on MS, as well as on neuroinflammation. Furthermore a transgenic animal model that carries the human A30P alpha-synuclein (hA30PSyncA) mutation and a deletion of PREP was also constructed. This will be a valuable tool for the study of alpha-synuclein clearance.

Based on the Alzheimer’s disease model Tg6799, the project has produced a mouse model which contains APP/presenilin (PS)1 mutations. These double-mutants provide a useful murine model to investigate the mechanisms and therapeutic interventions for multiple synaptic and memory dysfunctions associated with AD.

A neuroinflammation mouse model was also developed. Bilateral i.c.v. administration of LPS or saline induced signs of inflammation (increased microgliosis, astrocytosis and iNOS levels) in comparison to naive mice. A new murine model with impaired cognition due to deficiency in the NCAM gene has been established for the evaluation of the enhancing memory properties of compounds.

A battery of neurodegenerative disease models has been created, such as (1) a unilateral 6-OHDA lesion model of PD, (2) a scopolamine induced mouse model of amnesia and cognitive impairment, (3) a global ischemia model of delayed neurodegeneration and cognitive decline, (4) aged rats and mice to study aging and mild cognitive impairment and (5) a Tg2576 transgenic mouse model of AD, which overexpresses a mutant form of

amyloid precursor protein (APP), APPK670/671L. For unilateral 6-OHDA as a model for PD (1), both striatal and MFB lesioning have been validated in Wistar rats. Biochemical/histological end-points validated at 45 days include 75% depletion of striatal dopamine and metabolites analysed by HPLC and 50-70% neuronal death using tyrosine hydroxylase positive neuronal counts in the substantia nigra. In the mouse model for scopolamine induced amnesia (2), nicotine and Donepezil were used as positive controls. Scopolamine consistently causes impairment in the fear-conditioning assay, which can be reversed by acute nicotine and Donepezil treatment. To produce global cerebral ischemia (3) a bilateral common carotid artery occlusion was used in hypotensive male Sprague-Dawley rats. This model was optimized for delayed neuronal death in CA-1 pyramidal neurons of the hippocampus and for related cognitive decline. 24-28 months old male Wistar rats and 20-24 months old male C57/BL6 mice (4) were used to study aged rodent related dementia and neuronal dysfunction. Consistent learning and memory decline could be observed. In addition an in vivo proton magnetic resonance spectroscopy (1H-MRS) for the pre-frontal cortex showed a 20% decline of N-Acetyl-Aspartate in aged animals versus young controls. For the Tg2576 APP transgenic mouse model of AD (5), two versions of the model have been validated. 5-month-old mice are used to investigate cognitive decline and to perform rapid screening of therapeutic compounds, while 13-month-old Tg2576 mice are suitable for insoluble Abeta and plaque load analysis. In these mice increased levels of diffuse Abeta plaques were detected. Furthermore Tg2576 mice were characterized by in vivo MRI, 1H-MRS and arterial spin labelled perfusion MRI for brain volumetry, metabolic profiling and cerebral blood flow. However, no pathology was detected by any of these MRI methods.

Finally, the CVN Alzheimer’s mouse model was characterized. These APPSwDI/NOS2-/- bigenic mice harbour the APPSwDI transgene and a targeted null-mutation at the nitric oxide synthase 2 (NOS2) locus. They develop early amyloidosis, tau pathology, inflammation, vascular pathology, cognitive decline and neuronal death. This transgenic line was received from the DUKE and Stony Brook Universities and purified by embryo transfer. A new breeding colony was established and currently approximately 20 male and 20 female homozygous breeders and corresponding littermates have been obtained. Breeding is being expanded and mice for PREP inhibitor study will soon be available.

3.4.3 Development of assay methods

This project developed and validated a PREP intracellular activity assay using a nitroblue tetrazolium (NBT) dye and the substrate UAMC-00682 in primary cultures of rat cortical neurons. Three known PREP inhibitors, including KYP-2047, showed inhibition of intracellular PREP activity in this assay. This was also confirmed by the kinetic PREP activity assay using cell homogenates and the chromogenic substrate Z-Gly-Pro-pNA in primary rat cortical neurons. Therefore, it was concluded that the NBT assay is a very powerful tool to study PREP activity in situ in both cultured cells and tissue slices.

4. Conclusions

NEUROPRO, a consortium of 8 academic groups and 3 small and medium-sized entreprises (SME)s aimed to 1) validate prolyl oligopeptidases as drug targets, 2) improve the pharmacological properties of its inhibitors and 3) use modern techniques in biochemistry, molecular biology, structural

biology and pharmacology to make a substantial progress in the understanding of the physiology of oligopeptidases and their role in disease. Despite the scientific obstacles these tasks represented and the initial conceptual drawbacks, the goals of this project were reached. Furthermore, new avenues for research and product/patent development were created to the point that provocative proposals for further technical, scientific and medical development have been set.

We have considerably advanced the state-of-the-art in PREP and PREPL, produced new insights into their role in health and disease and opened new promising research lines. The proof-of concept for new drug targets, compound scaffolds and therapeutic and diagnostic applications for commercial purposes has also been outlined.

Potential Impact:

1) Potential impact

The NEUROPRO project aimed to develop new avenues for the detection and treatment of neurodegenerative diseases, as well as to unveil the physiological role of the PREP family of enzymes.

The prevalent concepts, at the beginning of the project, that prolyl oligopeptidases (PREPs) were involved in the regulation of the metabolism of extracellular neuropeptides engaged in memory and learning, and that simply modifying the levels of these peptides would affect the learning and memory loss produced by neurodegeneration, were soon proved to be erroneous. Despite this shortcoming, in a great scientific effort from all participants of the project, intense research was conducted to discover the actual biological relevance of PREPs in physiology and disease. The results obtained in this project contribute substantially to the knowledge of the biological mechanisms where PREP and PREPL are implicated.

In contrast with the simplistic original idea of PREP controlling neuropeptide levels, we have established that it is involved in the control of intracrine and endocrine processes. Intracellular, it intervenes in the signalling to control axonal transport, secretion and the processing of prohormones and proneuropeptides. Extracellular, it is involved in the control of neural plasticity, migration and activation of immunoactive cells. Importantly, all these processes are compromised in neurodegeneration and one of the relevant conclusions derives from the role of PREP in neuroinflammation.

During the development of this research, it has been found that peptidase activity of PREP is only one of the features of the protein. Interactions of PREP with alpha-synuclein, GAP43 and structural cytoskeletal proteins have been established, which are respectively related to Parkinson’s disease (PD), nerve outgrowth and subcellular vesicle traffic. Structural analysis of inhibitor bound PREP has provided strong information on the relations with activity structure and protein-protein interactive features of the peptidase. This information leads to a next generation of drugable compounds targeted to specific functions of PREP.

Of medical impact, we have obtained remarkable results which link PREP directly with plaque deposition in neurodegeneration. In cellular models, it was established that PREP is important for the processing of amyloid precursor protein (APP). Disruption of the APP metabolism and/or clearance is one major feature in Alzheimer's disease (AD). In fact, it was discovered that PREP co-localizes with beta-amyloid plaques in human AD brains, but its interaction with tau protein (another relevant AD protein) was disturbed compared with healthy brains. Furthermore, it has been shown that PREP interacts with alpha-synuclein, modifying its patterns of aggregation. In animal and cellular models of Parkinson’s disease (PD), striking results showed that alpha-synuclein plaque density is substantially decreased upon administration of PREP inhibitors. To support these findings, it was observed that indeed there is co-localization of PREP and alpha-synuclein in actual PD brains.

In the biotechnological and drug development arenas, this project has made significant contributions. A peptide chip technology has been validated to the point that peptide microarray analysis can be used for

diagnostic and clinical research applications. New specific targets for inflammatory disease have been found during the off-target hunting of PREP inhibitors and the underlying mechanism was unravelled. Due to the changes of PREP expression in human serum due to neuroinflammation, PREP is now proposed as a new and reliable marker at least for multiple sclerosis (MS) and hepatic encephalopathy. It can therefore be used as a disease marker and/or an indicator of disease progression. Perhaps the most striking application is based on the discovery that PREP activity inhibition is an adjuvant for the plaque clearance in PD and the possibility to extend the same principle for other plaque forming diseases.

The consequences of congenital PREPL deficiency were known at the start of this project, based on a small group of patients with the hypotonia-cystinuria syndrome (HCS). This metabolic syndrome is characterized by neonatal hypotonia and dwarfism, but the molecular mechanism(s) causing this phenotype were still unknown at the beginning of NEUROPRO. The research performed within the consortium has unveiled that PREPL is involved in the regulation of membrane trafficking. These insights, combined with observations made during the treatment of patients have now resulted in the rational design of a therapeutic regimen, which will soon be tested in a small clinical trial.

In summary, this project has accomplished all milestones and deliverables. Deviations from deliverables were minimal and led to gainful outcomes. We have considerably advanced the state-of-the-art in PREP and PREPL, opening new and promising research lines. Proof-of-concept on drug targets, compound scaffolds and therapeutic and diagnostic applications for commercial purposes have also been outlined.

2) Main dissemination activities

From an academic point of view, this project has resulted in over forty scientific articles and this high productivity will continue in the aftermaths beyond the end of the funding period. Considering all members of the consortium and according to the Web of KnowledgeSM database, the average number of publications on the specific area of PREP has grown from 3 per year before the consortium assembly (10 year average) to 10 per year during NEUROPRO life. An impressive production of 15 publications was made in 2011, which was almost 30% of the world production on the field. The number of worldwide publications in all fields, which are citing PREP related papers, rose from around 1500 per year before the start of NEUROPRO, to more than 2500 in 2011. Counting the number of citations of our work, the number has climbed from an average of 300 citations at the beginning of the project to almost 1000 citations in 2011, a number that will very possibly be surpassed in 2012. These numbers reflect a geometrical growth of the consortium’s impact on the scientific community worldwide and especially in Europe, where most of the production on the field is performed (Web of KnowledgeSM). An overview of the NEUROPRO publications is listed in 'template A1: List of scientific peer reviewed publications' of the final report.

The research results have further been disseminated to the scientific community by participation of NEUROPRO members at conferences, national and international scientific meetings. Two scientific meetings have been organized to present our results to a specialist international audience and position it amongst the latest developments in the field. The

successful conference 'PSP 2010 - Proline Specific Cleavage and Oxoprolyl-Formation – Functions and Therapeutic Strategies', which was partially sponsored by NEUROPRO was held in Halle, Germany in 2010. This meeting was organized by Probiodrug, participant 9 of NEUROPRO, and was attended by more than 150 scientists from Europe, the US, Australia and Japan. Top scientists of the field, amongst which several members of the NEUROPRO consortium, have presented the frontiers on the research of prolyl-specific enzymes, including PREP and PREPL. Besides its scientific attractiveness, the meeting has also tightened collaborations in the area and provided new opportunities for cooperation. At the end of the project the mini-symposium 'New frontiers on neurodegeneration and neuroinflammation' was held in Helsinki in association with the Finnish Pharmacological Society. During the meeting, the final results from the consortium members were exhibited. Invited speakers broadened the focus of the meeting and made it possible to put the achievements of the project into perspective against the wider research field of neurodegeneration and inflammation.

NEUROPRO has also contributed to student training as reflected by the approximately 20 academic dissertations at all levels. Additionally, the participants have attended multiple universities worldwide to give lectures, doctoral training and departmental seminars related to the role of PREP and PREPL in health and disease.

In the dissemination arena, the project specific website (see http://www.neuropro.eu online) has been attractive to the public. Besides containing general information, the Internet page is also updated with the latest news and publications. The site was essential to increase feedback from the research community and stimulate professional contacts with academic and health organizations. Through a password-protected section, the web page was able to support communication between participants. Finally, it has also been a valuable tool in exchanging information to the scientific community, policy makers and general public.

A more detailed description of the main external dissemination activities of the NEUROPRO consortium can be found in 'template A2: List of dissemination activities of the final report'.

The internal dissemination activities that were held between consortium members and the Scientific Advisory Board to make collaboration as efficient as possible. These include general assembly and steering board meetings twice a year to exchange the latest research results, report on the progress and undertake corrective actions if necessary.

3) Exploitation of results

Details on the main exploitable results are summarised in 'template B2: Overview table with exploitable foreground'. During the development of this project several outcomes were generated. Most of the general advancements on scientific knowledge on PREP and PREPL in physiology and disease have already been published in peer-reviewed journals or communicated at scientific meetings (see templates A1 and A2 of the final report). The most prominent features of these advancements deal with the roles of PREP and PREPL in protein clearance and secretion or with the role of PREP in inflammatory processes, especially in the brain. Indications of the role of PREPL in HCS, and of PREP in AD and PD have

also been established, which all contain a large potential for commercial exploitation.

At the end of the project no patent applications have been filed yet, although the NEUROPRO project has potential to result in five applications for clinical use. First, PREP can be used as a bio-marker for the prognosis, development and progression of neurodegeneration, for example in MS and PD, where PREP plasma or brain levels are changed. Non-invasive methods for PREP detection based on PREP inhibitors and imaging are being developed to substantiate the outcome from PD research. Second, a novel neurodegenerative disease diagnosis method, based on the peptide chip technology developed in this project, has been established and its validation and commercial viability are being worked out. Third, the use of PREP inhibitors as adjuvants for plaque clearance in PD is being validated at pre-clinical stages and seems to be a promising therapeutic alternative. Fourth, new principles for the development of a non-steroidal anti-inflammatory drug have been set, which brings up an innovative and potential profitable project. And fifth, the studies on PREPL have shed a light on the pathophysiology of HCS. Efforts to materialize this information into a therapeutic regime are being undertaken and might result in a patent application in the future. The protocols that were validated to serve as background for these potential patent applications, as well as details on specific technical procedures or chemical entities will temporarily be kept confidential. All these applications are based on the new status of the state-of-the-art on the knowledge of prolyl oligopeptidases in physiology and pathology, which is diametrically different from the one prevalent to the start of the project.

List of Websites:

http://www.neuropro.eu