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Neuropeptide Y (NPY) cleaving enzymes: Structural and functional homologues of dipeptidyl peptidase 4

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ABSTRACT

N-terminal truncation of NPY has important physiological consequences, because the truncated peptides lose their capability to activate the Y1-receptor. The sources of N-terminally truncated NPY and related peptides are unknown and several proline specific peptidases may be involved. First, we therefore provide an overview on the peptidases, belonging to structural and functional homologues of dipeptidyl peptidase 4 (DP4) as well as aminopeptidase P (APP) and thus, represent potential candidates of NPY cleavage *in vivo*. Second, applying selective inhibitors against DP4, DP8/9 and DP2, respectively, the enzymatic distribution was analyzed in brain extracts from wild type and DP4 deficient F344 rat substrains and human plasma samples in activity studies as well as by matrix assisted laser desorption/ionisation-time of flight (MALDI-TOF)-mass spectrometry. Third, co-transfection of Cos-1 cells with *Dpp4* and *Npy* followed by confocal lasermicroscopy illustrated that hNPY-dsRed1-N1 was transported in large dense core vesicles towards the membrane while rDP4-GFP-C1 was transported primarily in different vesicles thereby providing no clear evidence for co-localization of NPY and DP4. Nevertheless, the review and experimental results of activity and mass spectrometry studies support the notion that at least five peptidases (DP4, DP8, DP9, XPNPEP1, XPNPEP2) are potentially involved in NPY cleavage while the serine protease DP4 (CD26) could be the principal peptidase involved in the N-terminal truncation of NPY. However, DP8 and DP9 are also capable of cleaving NPY, whereas no cleavage could be demonstrated for DP2.

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1. Introduction

N-terminally truncated derivates of NPY and PYY are involved in several physiological functions. In contrast to the full-length peptide, truncated forms starting at position 2 or 3 lose their efficacy at the Y1-receptor but they are active especially at the Y2-receptor. For a better understanding of this differential regulation detailed knowledge on the sources as well as on the local and systemic regulation of $NPY_{3–36}$ and $PYY_{3–36}$ levels is indispensable. In fact, $NPY_{3–36}$ and $PYY_{3–36}$ have been shown to play a role in energy metabolism via inhibition of exocrine pancreas function [32] or other feeding associated processes [36,58,74] and probably are involved in several other as yet to be discovered physiological functions. These regulatory processes are closely dependent on the expression and function of DP4-like peptidases due to their capability in hydrolyzing the post-proline bond between positions 2 and 3 of NPY and PYY.

In a series of studies we characterized F344 rat substrains, which are deficient for DP4 and which exhibit improved glucose tolerance, differential weight gain, as well as differential immune functions [46]. In addition, these DP4 deficient substrains exhibit a phenotype of reduced stress-responsiveness and anxiety [49], and were used to demonstrate that central application of NPY results in more potent anxiolytic-like and sedative-like effects when injected intracerebroventricularly (icv) in deficient animals [48]. Recently, we also found an increased potency of icv NPY with regard to pain perception in DP4 deficient substrains [47]. These findings are most likely mediated by prolonged activation of central NPY Y1-receptors, which is the predominant anxiolytic-like acting receptor type of NPY [50].

DP4 (CD26) is presumably the peptidase most frequently involved in N-terminal truncation of NPY [64]. From a theoretical point of view, however, other DP4-like peptidases may also be involved in NPY cleavage. In this review we summarize recent developments in the field of DP4 functional homologues and – in addition – present initial data investigating intracellular localization of DP4 and NPY using confocal analysis of *Npy/Pyy* and *Dpp4* co-transfected COS-1 cells as well as cleavage of NPY by functional homologues of DP4.

2. Review on DP4-like structural and functional homologues

2.1. Classification of peptidases

Enzymes in general are classified into six enzyme classes due to their catalytic reaction by the code system IUPAC and IUBMB. Peptidases are also named peptide hydrolases, based on their capability to hydrolyse peptide bonds and belong to the subclass 3.4, which is further sub-divided into 14 sub-subclasses, depending on the type of active site of the enzymes and/or the kind of the preferred substrate. Furthermore, a new structure-based classification system, called MEROPS, was introduced by Neil D. Rawlings und Alan J. Barett in 1993, assigning peptidases with statistically significant similarities in amino acids to a family, whereas homologous families are grouped together into a clan [78].

In the following, we especially focus on the potential NPY degrading aminopeptidases, the members of the DP4 gene family, the functional homologues of DP4, DP2 and the X-prolyl aminopeptidases. The classification of these enzymes within the different systems, their expression, and potential physiological role is summarized in Table 1.

2.1.1. Dipeptidyl peptidase 4 (DP4)

Dipeptidyl peptidase 4 (DP4) as representative member of the DP4 gene family is the best understood proteinase with the rare capability of hydrolysing post-proline bonds [29]. DP4 comprises 766 amino acids and is a type II transmembrane glycoprotein that has also a soluble shedded form [65]. The multifunctional peptidase has a molecular weight of 110 kDa and is active as a homodimer. It is known to cleave peptide hormones such as GLP-1, GLP-2, GIP, glucagons; neuropeptides including NPY, substance P, endomorphin 1 and 2 as well as various chemokines. Thus it is involved in glucose homeostasis, food up-take, anxiety, stress, cardiovascular, nociception and chemotaxis. Furthermore, it functions as an extracellular adhesion molecule by binding to collagen, fibronectin and plasminogen. In addition, it is implicated in various immune responses via its interaction with several immunological molecules such as ADA or CD45 and acts as a marker for activated T-cells [11,39,53]. It is ubiquitously distributed with the highest expression in kidney, lung, liver and small intestine whereas low expression is found in brain, heart and skeletal muscle. According to kinetic analysis, DP4 has the highest selectivity for NPY and PYY [9,30,37,40]. The human gene location of DP4 is 2q24.2.

2.1.2. Fibroblast activation protein α (FAPα)

Fibroblast activation protein α (FAPα) alias seprase is a type II transmembrane protein. It consists of 760 amino acids and forms a 170 kDa homodimer [27]. Like DP4, the monomeric, *N*-glycosylated 97 kDa subunits are proteolytically inactive, thus their proteolytic activities are dependent upon subunit association [75]. Furthermore, FAPα can form a heterodimeric membrane bound proteinase complex with DP4 [83]. In comparison to DP4, FAPα displays only a hundredth of post-proline dipeptidyl aminopeptidase activity [25]. However, in addition to its DP4-like activity, it exhibits also post-proline endoproteolytic activity specific for $\ldots X_{aa}$-Gly-Pro-$Y_{aa}\ldots$ sequences [24]. Thus, it has been described as a gelatinase and collagenase type I, involved in wound healing and metastasis. Unlike DP4, protein expression of FAPα is found on pathological tissue such as epithelial cancer, wounds and stellate cells in liver cirrhosis [28]. A soluble form has recently been found in serum where it was shown to cleave alpha2-antiplasmin [55,56]. So far, there have been no reports on the cleavage of NPY by FAPα, though its post-proline dipeptidyl aminopeptidase activity is expected to be minor and no endoproteolytic hydrolysis should occur. The human gene localization is 2q23, close to the *Dpp4* gene and therefore gene duplication has been suggested [1].

2.1.3. Dipeptidyl peptidase 8 (DP8)

Dipeptidyl peptidase 8 (DP8) consists of 882 amino acids and has a molecular weight of 100 kDa. Although DP8 has previously been reported to be monomeric, recent data gave

Table 1 – Structural and functional homologues of NPY cleaving peptidases

Name/synonymous/EC	Expression	Function	Structural/functional relationship	Reference
Peptidases known to cleave NPY				
DP4, dipeptidyl peptidase IV, CD26; EC 3.4.14.5	Ubiquitously, high expression in kidney and lungs	Major role in physiological processes including endocrine and immune functions	Clan SC; family S9B; post-proline dipeptidyl aminopeptidase activity	[18,64,45,11,79]
DP8, dipeptidyl peptidase VIII, DPRP-1, prolyl dipeptidase	Ubiquitously, highest levels in testis, prostate, muscle, and brain	Non-lysosomal function suggested, not yet been associated with any biological process, however, NPY cleavage reported	Clan SC; family S9B; about 27% AAS identity with DP4 / FAP; post-proline DP-like activity	[2,9,77]
DP9, dipeptidyl peptidase IX, DPRP-2, DPLP9	Ubiquitously, predominantly in muscle, liver and leucocytes	Has not yet been associated with any particular biological process, however, NPY cleavage reported	Clan SC; family S9B shares 19% AAS identity with DP4; post-proline DP-like activity	[77,4,9,68]
XPNPEP1, X-prolyl aminopeptidase 1, aminopeptidase P1; soluble form	Ubiquitously, highest expression in pancreas, heart and muscle; expression in brain	Suggested to be involved in the maturation and degradation of peptide hormones, neuropeptides and tachykinins; cleaves NPY and bradykinin	Clan MG, family M24	[60,61,88]
XPNPEP2, X-prolyl aminopeptidase 2, aminopeptidase P; membrane form EC 3.4.11.9	Ubiquitously, predominantly in kidney, lung and heart; no expression in brain	Cleavage of NPY, bradykinin, involved in cardiovascular disease	Clan MG, family M24	[43,70,89,43,70,77,89]
Peptidases most likely to be involved in NPY cleavage				
FAPα, fibroblast activation protein α, seprase, 170-kDa melanoma gelatinase; EC 3.4.21	Integral membrane serine protease on activated fibroblasts and myofibroblasts (i.e. sites of tissue remodeling)	Collagen type I-specific gelatinase activity, suggested role in tissue remodeling during development and wound healing, contributes to invasiveness of certain cancers	Clan SC; subfamily S9B; FAP-α shows 48% AAS identity with DP4; protease activity similar to DP4	[83,80,72,28]
Peptidases not being able to cleave NPY_{1-36} due to size restrictions				
DP2, dipeptidyl peptidase II, QPP/DP7 EC 3.4.14.2	Ubiquitously, quiescent lymphocytes	Related to cell death of quiescent lymphocytes, degradation of proline containing tripeptides	Clan SC; family S28 no homology with S9; family post-proline DP-like activity; substrates are oligo-/tripeptides	[57,59,87,16]

DP4: dipeptidyl peptidase IV; AAS: amino acid sequence.

strong evidence for a dimeric structure with a suggested molecular weight above 200 kDa [9]. So far, it has been suggested to be located in the cytoplasm as a soluble protein and up to now, there has been no evidence for any secretion [2,15]. Using several chromogenic substrates [77], DP8 was shown to display post-proline dipeptidyl aminopeptidase activity similar to that of DP4. The well-known natural substrates of DP4, NPY and PYY, are both also cleaved by DP8, however with lower efficiency compared to DP4. In fact, while NPY was demonstrated to be the best substrate for DP8, PYY had a very long half life [9]. This would imply that the specificity is extended to P'_1, which differ in serine and isoleucine for NPY and PYY, respectively. DP8 is distributed ubiquitously with its highest expression in testis and brain. Furthermore, it is up regulated in activated lymphocytes [2]. However, its physiological function is presently unknown and still awaits further studies. The human gene localization is 15q22.

2.1.4. *Dipeptidyl peptidase 9 (DP9)*

Dipeptidyl peptidase 9 (DP9) has previously been reported to be active as a cytosolic monomer comprised of 863 amino acids with a molecular weight of approximately 100 kDa [4]. Further ORFs of 2913 bp [28] and 3006 bp [4] have been described. Recently, a new DP9 variant with another start site in a prolonged ORF leading to an enzymatically active protein of 892 amino acids has been published by Bjelke et al. [9]. This variant was shown to be active as homodimer with an estimated molecular weight above 200 kDa, whereas no activity could be detected for the 863 amino acid variant [9]. Using several chromogenic substrates, Qi et al. [77] and Ajami et al. [4] illustrated post-proline dipeptidyl aminopeptidase activity for DP9 similar to that of DP4. Like DP8, DP9 is able to cleave NPY and PYY, though with a lower efficiency compared to DP4. Likewise, NPY, was shown to be the best natural substrate for DP9, whereas PYY exhibited the longest half life of the investigated substrates [9]. DP9 is ubiquitously distributed, with its highest expression in liver, heart and skeletal muscle [4,68,77]. Its physiogical function is not known so far. The localization of the human gene is 19p13.3. Due to their shortest gene size, lowest number of exons and the active site being located in one exon in comparison to DP4 and FAPα, DP8 and DP9 have been suggested to be the most ancient DP4-like enzymes [1,2]. It should be mentioned that side effects obtained during the course of toxicological studies of a non-selective inhibitor were due the inhibition of DP8 and/or DP9 [54].

The two other members of the DP4 gene family are not involved in NPY processing, because they lack any DP4 activity due to the absence of the catalytic serine and are therefore designated with dipeptidyl peptidase like protein 1 (DPL1) and 2 (DPL2). Both of them are type II membrane-bound glycoproteins, suggested to interact with the voltage-gated potassium channel Kv4. While DPL1 is exclusively expressed in the brain, DPL2 is found in brain, pancreas and adrenal gland [1,14,15,28,77,86,91].

2.1.5. *Dipeptidyl peptidase II (DP2)*

Dipeptidyl peptidase II (DP2) alias quiescent cell proline dipeptidase (QPP), belongs to the family S28. The soluble serine protease possesses a proform and has a length of 492 amino acids [16,87] with a molecular weight of 58 kDa. Dimerization is required for the catalytic activity and occurs via a leucine zipper motif, which is novel for proteases. The homodimer is located in cellular vesicles that are distinct from lysosomes [57]. Using chromogenic substrates, DP2 displays post-proline dipeptidyl aminopeptidase activity similar to DP4, however with an acidic pH optimum of 5.5 [59]. Hydrolysis of peptides is highly restricted to size. While DP2 readily hydrolyses tripeptides, its activity decreases rapidly with increasing chain length of peptide. Thus, it was shown to cleave only fragments of substance $P_{1–4}$, bradykinin$_{1–3}$ or bradykinin$_{1–5}$ [13,67]. DP2 is ubiquitously distributed with high expression in kidney, brain, testis and heart [21,31]. Since it was previously thought to be a lysosomal enzyme, its physiological function to date is unknown. The human gene localization is 9q34.3.

Four enzymes have previously been acclaimed to exhibit DP4-like activity, including attractin (DPPT-L) and *N*-acetylated alpha-linked acidic dipeptidases I, II and L (NAALADase I, II and L) [20–22,71,84]. However, this is controversially discussed and there are also several hints from a thorough analysis of serum DP4 activity for attractin [23] and kinetics of purified recombinant NAALADase II, respectively [7] that these proteins exibit no DP4-like activity.

Furthermore, NPY is also truncated to $NPY_{2–36}$ by prolyl aminopeptidases, belonging to family M24 [43,60,62,63]. There are two X-prolyl aminopeptidases, located on different genes.

2.1.6. *X-prolyl aminopeptidase 1 (XPNPEP1)*

X-prolyl aminopeptidase 1 (XPNPEP1) is a soluble cytosolic protein, lacking the hydrophobic signal sequence at the N-terminus and the GPI-anchor at the C-terminus [17]. It is a homodimer, comprised of 623 amino acids with a molecular weight of 71 kDa per subunit [88]. The enzyme contains a putative proton shuttle 5 and divalent metal ligands [85]. Due to its proline specificity, it is suggested to hydrolyse peptide hormones, neuropeptides and tachykinins. Unlike DP4, it is able to hydrolyse peptides containing two consecutive prolines in penultimative N-terminal position (X_{aa}-Pro-Pro-Y_{aa}...), such as bradykinin [33,34,60]. XPNPEP1 is ubiquitously distributed, with its highest expression in pancreas, followed by heart and muscle. Only XPNPEP1 but not XPNPEP2 is found in the brain [88]. The human gene location is 10q25.3.

2.1.7. *X-prolyl aminopeptidase 2 (XPNPEP2)*

X-prolyl aminopeptidase 2 (XPNPEP2) is a GPI-anchored membrane-bound aminopeptidase encoding for 673 amino acids with a molecular mass of 75.5 kDa. XPNPEP2 is expressed in kidney, lung, heart, placenta, liver, small intestine, and colon, but not in brain, skeletal muscle, pancreas, spleen, thymus, prostate, testis, ovary, or leukocytes [89]. It hydrolyses NPY and bradykinin [69,70] and is suggested to be involved in cardiovascular diseases [3,10]. The human gene localization is Xq25.

Based on this compelling theoretical evidence, and the fact that vesicular localization of soluble DP4 has already been observed in α-cells of islets of Langerhans [35,76], we started to investigate a possible co-localization of DP4 and NPY/PYY intracellularly.

3. Materials and methods

3.1. Animals

While the F344/Crl(Por/98) and F344/Ztm rat substrains exhibit a DP4 wild type-like phenotype, the substrain F344/Crl(Wiga)SvH-*Dpp4* is deficient for DP4. F344/Crl(Por/98) and F344/Crl(Wiga)SvH-*Dpp4* substrains were originally obtained from Charles River in 1998 and are now further inbred.

3.2. Tissue extraction

Brains, obtained from F344/Crl and F344/Crl(Wiga)SvH-*Dpp4* rats, were extracted with 20 mM Tris(hydroxymethyl)aminomethane (Tris)/HCl, pH 7.6 by homogenisation, sonification and subsequent centrifugation at 13,000 × *g*. The extracts were further fractionated into cytosolic and membrane by ultra-centrifugation at 100,000 × *g* for 1 h. The resulting pellet was resupended with equal volumes of 20 mM Tris–HCl, pH 7.6, containing 0.1% β-octylglucopyranoside. Human EDTA plasma was obtained from healthy volunteers.

3.3. Cloning and purification of DP2

The DP2 gene was cloned into the plasmid pcDNA3.1(+) and COS-7 cells were transiently transfected with the plasmid. Expression was examined by Western blot analysis. The cells were lysed and the soluble fraction was applied to an affinity resin nickel–nitrilotriacetic acid (Ni–NTA) (Pharmacia, Uppsala, Sweden). Active fractions were eluted by 0.3 M imidazole and pooled fractions were subsequently applied onto a size exclusion chromatography. The active fractions were pooled and used for kinetic investigations.

3.4. Activity and inhibition studies

Activity was determined with 0.125 mM alanyl-prolyl-7-amido-4-methylcoumarin (Ala-Pro-AMC) in 40 mM *N*-2-Hydroxyethylpiperazine-*N*′-ethane-sulfonic acid (HEPES), pH 7.6 and 0.25 mM Ala-Pro-AMC in 74 mM NaAcetate, pH 5.5 for DP4-like and DP2 activity, respectively. Activity was measured at excitation 380 nm and emission 470 nm with microplate reader Fluorostar Optima (BMG LabTech GmbH, Offenburg, Germany). Protein concentration was determined by Bradford method, using BSA as standard [12]. The selective inhibitors UG 92, UG 93 and DAB were used against DP4, DP8/9 and DP2, respectively. P32/98 was a non-selective inhibitor which inhibits all DP4-like enzymes. Inhibitor mix was composed of all the selective inhibitors. Except for L-2,4-diaminobutyrylpiperidinamide (DAB) (Merck Bioscience, Darmstadt, Germany), all inhibitors were synthesized by probiodrug.

3.5. MALDI-TOF-mass spectrometry

A 25 μM NPY (probiodrug AG, Halle, Germany) was incubated with 30 mU of recombinant enzyme or tissue extract in absence/presence of selective and non-selective inhibitors. Analysis of DP4-like enzymes in tissue extracts was performed in 20 mM Tris/HCl, pH 7.6, while DP2 was assayed 10 mM 2-(*N*-morpholino)-ethanesulfonic acid (MES), pH 5.5. Several aliquots were taken between 2 min and 24 h and the reaction was stopped with equal amounts of 0.1% trifluoroacetic acid (TFA). Afterwards, samples were purified with ZipTip C18 (Millipore GmbH, Schalbach, Germany) according to the instructor's manual, mixed with the matrix sinapinic acid at a ratio 1:1, and analysed with MALDI-TOF mass spectrometry (Voyager-DE Pro Biospectrometry workstation from Applied Biosystem). For blood analysis, 100 μM NPY was applied in the same assay system described above.

3.6. Cell culture

COS-1 cells (American Type Culture Collection; Rockeville, USA) were grown in Dulbecco's modified Eagle's medium (DMEM) medium with 10% fetal calf serum (BioWest, Essen, Germany) at 37 °C. Plasmid transfection of COS-1 cells was performed with diethylaminoethyl (DEAE) dextran [5].

3.7. Confocal fluorescence microscopy

Confocal images of living cells were acquired on a Leica TCS SP2 microscope using a 63 water planachromat lens (Leica Microsystems) essentially as described before [44].

3.8. Construction of cDNA clones

3.8.1. DP4 cDNA

DP4 cDNA–mRNA was isolated from the prepared small intestine of F344/Crl(Por/98) rats with Qiagen tissue kit. cDNA was synthesized with the First Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Uppsala, Sweden). PCR reactions were performed with primers (MWG-Biotech GmbH) designed after the published mRNA sequence of Rattus norvegicus dipeptidyl peptidase 4 [gi:6978772]. For further cloning the chosen sense-primer 5′-AAAA**AAGCTT**T GAAGACACCGTGGAAGGTT-3′ introduced a HindIII site (bold) and the antisense-primer 5′-AAA**GGATCC**GAGAGCCTTGCCATGCTA-3′ a BamHI site (bold) into the PCR product. r*Dpp4* was cloned into pEGFP-C1 (Invitrogen/Clontech Laboratories, Inc., Heidelberg, Germany). Constructs were restriction-mapped and sequenced to verify correctness of the fragments.

3.8.2. NPY cDNA

NPY cDNA—hNPY(-CPON)eGFP-N1 was kindly provided by Richard E. Mains (Department of Neuroscience; The University of Connecticut Health Center). GFP was replaced by dsRed1 from pcdsRed1-N1 (Invitrogen/Clontech Laboratories, Inc., Heidelberg, Germany) by vector digestion with *Not*I and BamHI (MBI Fermentas, St. Leon-Rot, Germany).

3.8.3. PYY cDNA

PYY cDNA—rat PYY fragment cloned into pGEM3 encoding a portion of the C-terminal extension were kindly provided by Dr. Greeley (University of Texas Medical Branch) [92]. The PYY fragment was purified and completed by PCR choosing the following primers: thereby the sense-primer 5′-AGAATTCATGGTGGCGGTACGCAGGCCTTGGCCCGTTATGGTC-3′ introduced an *Eco*RI site and the anti-sense primer 5′-TTT**GGATCC**GCCCCACTGGTCCACACCTTC-3′ a *Bam*HI restriction site at the end of the construct. The purified PCR product was

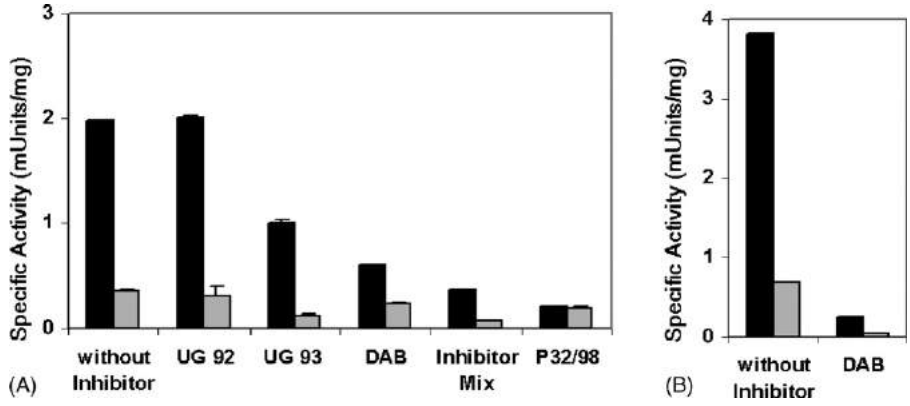

Fig. 1 – Activity and inhibition studies of crude brain extracts, obtained from F344/Crl (dark columns) and F344/Crl(Wiga)SvH-*Dpp4* (bright columns) rats, applying selective inhibitors against DP4-like enzymes. (A) Brain extract assayed with 0.125 mM Ala-Pro-AMC, pH 7.6 at 37 °C; (B) brain extract assayed 0.25 mM Ala-Pro-AMC, pH 5.5 at 37 °C, UG92, DP4 selective; UG93, DP8/DP9 selective; DAB, DP2 selective; mix, UG92 + UG93 + DAB; P32/98, non-selective inhibitor.

cloned into the *Bam*HI and *Eco*RI restriction sites of pcdsRed1-N1.

4. Results

Fig. 1 clearly depicts that most of the DP4-like activity in brain extracts, determined with the chromogenic substrate Ala-Pro-AMC and selective inhibitors, was contributed by DP2, followed by DP8/9, whereas only low levels of DP4 could be detected. Interestingly, there seemed to be no compensation by the other DP4-like enzymes in DP4 deficient F344/Crl(Wiga)SvH-*Dpp4* rat substrain (Fig. 1). However, using recombinant human DP2, no hydrolysis of NPY could be observed (Fig. 2) in contrast to human recombinant DP4 that degraded NPY completely after 30 min (data not shown). Furthermore, MALDI-TOF-mass spectrometry of NPY hydrolysis by brain extract from

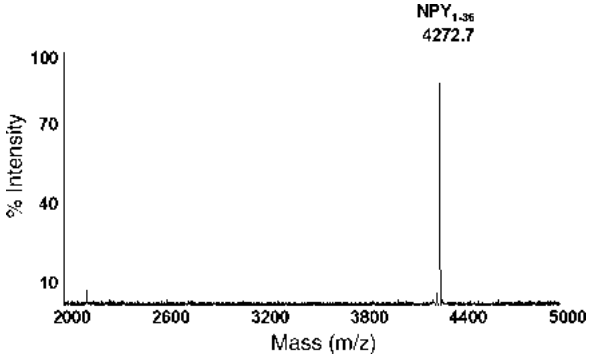

Fig. 2 – MALDI-TOF-MS analysis of NPY cleavage by recombinant human dipeptidyl peptidase 2 (rh-DP 2), showing no cleavage after 6 h incubation at 37 °C. 30 mU rhDP 2 were incubated with 25 mM NPY in 10 mM MES buffer, pH 5.5 for 6 h at 37 °C. Afterwards, the reaction was stopped with 0.1% TFA, samples were purified with ZipTip (Millipore GmbH, Germany), mixed with the matrix sinapinic acid at a ratio 1:1 and analysed with MALDI-TOF-MS (Voyager-DE Pro Biospectrometry workstation from Applied Biosystems).

F344/Crl rats in presence/absence of selective inhibitors of DP4-like enzymes showed the existence of DP4 and DP8/9 as illustrated in Fig. 3A–D. On the contrary, NPY hydrolysis of human plasma revealed a major contribution by DP4, and a minor one by DP8/9 (Fig. 3E–H). In addition, longer incubation of NPY and cytosolic fraction of brain extract confirmed its cleavage to NPY_{2-36} by cytosolic prolyl-aminopeptidase. A dominant fragment of NPY found in all assays with brain extracts, was NPY_{1-30} (Figs. 3 and 4).

Fig. 5A–C show the expression of NPY-dsRed1-N1 and DP4-GFP-C1 in the same transfected Cos-1 cell being screened at different wavelengths, thereby measuring emission of DP4-GFP-C1 (Fig. 5A) and NPY-dsRed1-N1 (Fig. 5B) fluorescent constructs that are transformed to a single image (Fig. 5C) (overlay). The Golgi apparatus, in Fig. 5A-C located approximately in the center of the picture, is detectable by transported NPY-dsRed1-N1 and DP4-GFP-C1 constructs. NPY-dsRed1-N1 containing vesicles leave the trans-Golgi network (TGN) and are further transported along cytoskeleton tracks that are clearly indicated in Fig. 5B and C. These span over the nucleus, visible below the Golgi apparatus. DP4-GFP-C1 associated vesicles, are transported through the cytoplasm towards the cell membrane as well, where vesicle fusion and integration of DP4-GFP-C1 takes place. Fusion and integration at the cell membrane are indicated by non-vesicular appearance of DP4-GFP-C1. Furthermore, transport of DP4-GFP-C1 containing vesicles along the cell membrane could be observed. The overlay in Fig. 5C suggests that DP4-GFP-C1 and NPY-dsRed1-N1 are transported in distinct vesicles although sometimes seeming to co-localize in the Golgi apparatus or in the cytoplasm due to an overlap or slack flow of different vesicles. Further observations were made in Cos-1 cells, transfected with PYY-dsRed1-N1 and DP4-GFP-C1 (data not shown), where also no clear evidence for a co-localization of DP4-GFP-C1 and PYY-dsRed1-N1 could be demonstrated so far.

5. Discussion

In the present paper, the members of the DP4 gene family as well as structural and functional homologues are discussed as

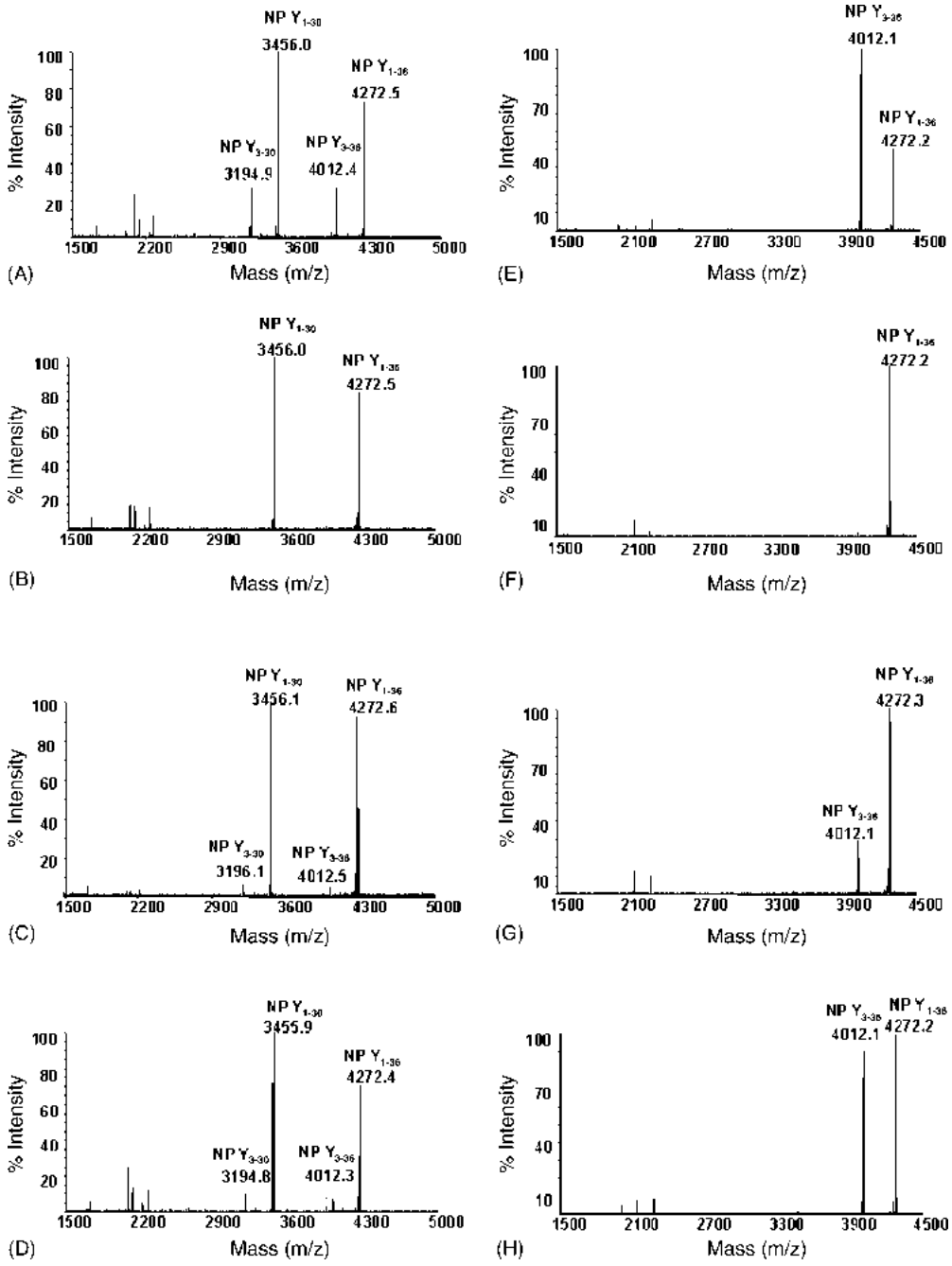

Fig. 3 – NPY hydrolysis in crude brain extract from F344/Crl rats or in EDTA-Plasma determined by MALDI-TOF-MS after 30 min incubation at 37 °C in absence or presence of different inhibitors. The reaction was stopped with 0.1% TFA, samples were purified with ZipTip (Millipore GmbH, Germany), mixed with the matrix sinapinic acid at a ratio 1:1 and analysed with MALDI-TOF-MS (Voyager-DE Pro Biospectrometry workstation from Applied Biosystems). (A–D) crude brain extract. (A) without inhibitor; (B) with non-selective inhibitor P32/98; (C) with DP4 selective inhibitor UG 92; (D) with DP-8/9 selective inhibitor UG 93. (E–H) EDTA-plasma. (E) without inhibitor; (F) with non-selective inhibitor P32/98; (G) with DP4 selective inhibitor UG 92; (H) with DP8/9 selective inhibitor UG 93.

candidates for N-terminal NPY hydrolysis. While FAPα may cleave dipeptides from the N-terminus [25], though at a much lower rate, it is highly unlikely to hydrolyse NPY endoproteolytically due to its lack of the – Gly-Pro-sequence in – P2-P1-position [24]. Furthermore, as FAPα, is exclusively found in pathogenic tissue [1,19,26,28,52,72] except for serum [55,56], it can be ruled out as a NPY cleaving enzyme. Although, DP2 was shown to be the most dominant DP4-like enzyme in rat brain (Fig. 1), it was unable to cleave NPY due to its peptide length (Fig. 2) [66,67]. This is in agreement with recent findings of Brandt et al. that investigated the hydrolysis of several potential natural substrates by DP2, thereby obtaining no

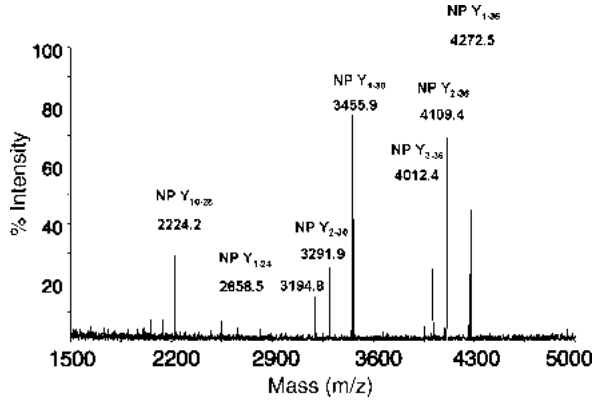

Fig. 4 – MALDI-TOF MS analysis of NPY hydrolysis by cytosolic brain fraction from F344/Ztm rats after 30 min incubation at 37 °C. The reaction was stopped with 0.1% TFA, samples were purified with ZipTip (Millipore GmbH, Germany), mixed with the matrix sinapinic acid at a ratio 1:1 and analysed with MALDI-TOF-MS (Voyager-DE Pro Biospectrometry workstation from Applied Biosystems).

cleavage [13]. Thus, the remaining DP4-like enzymes potentially cleaving NPY are DP4, DP8 and DP9. Using chromogenic substrate and selective inhibitors, higher levels of DP8/DP9 than DP4 could be clearly demonstrated in the brain as illustrated in Fig. 1. Nonetheless, MALDI-TOF-mass spectrometry showed similar cleavage of NPY by DP4 and DP8/9 respectively, confirming the higher catalytic efficiency of DP4 on the one hand [9] and larger representation of DP8/9 on the other hand. In addtition, analysis by MALDI-TOF-mass spectrometry could also detect $NPY_{2–36}$ truncation by soluble prolyl aminopeptidase (Fig. 4) [61,62,66]. Preliminary results of enzymatic histochemistry revealed that DP4 is found predominantly in the meninges and blood vessels, whereas DP8/9 seemed to be more ubiquitously distributed in the brain (data not shown). Conversely, NPY is mainly hydrolyzed by DP4 in human plasma and only partially by DP8/9. As there is already strong evidence that serum NPY crosses the blood brain border (BBB), one can conclude that peripheral NPY is primarily truncated by soluble serum DP4 or during crossing of the BBB by membrane-bound DP4 at the meninges and/or blood vessels [51]. Alternatively, NPY from neurons in the brain [6] may most likely be cleaved by DP8/9. Thus, neither the histology nor the cytology of NPY cleavage are sufficiently understood at this time. Peptides of the NPY family are synthesized as large precursor molecules in the endoplasmic reticulum. Following post-translational modification, precursor molecules are translocated to the Golgi apparatus, sorted in the trans-Golgi network, and guided in vesicles towards the secretory pathway. After exocytotic release of NPY-like peptides, their local action relies on various circumstances such as their concentration, receptor selectivity and expression of Y-receptors. However, their half lives and receptor selectivity is strongly modulated by specific peptidases such as the DP4-like enzymes and prolyl-aminopeptidase [61,62,66]. Therefore, the action of NPY and NPY-like peptides is also influenced by the local distribution and concentration of its degrading peptidases either intracellularly or extracellularly [90].

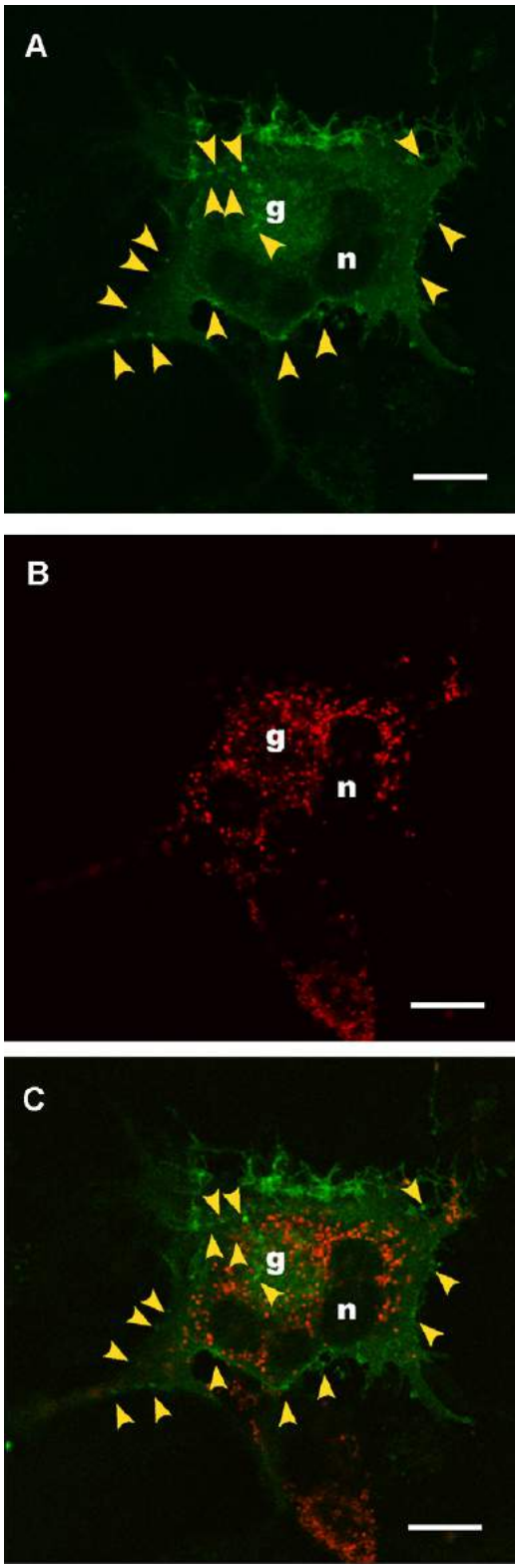

Fig. 5 – Confocal analysis of NPY and DP4 in transiently transfected COS-1 cells. Live cell image of Cos-1 cell transfected with (A) DP4-GFP-C1 and (B) NPY-dsRed1-N1 48 h post-transfection. (C) Overlay image of DP4-GFP-C1 and NPY-dsRed1-N1. Yellow arrowheads indicate DP4-GFP-C1 containing vesicles (A) that do not co-localize with NPY-dsRed1-N1 (C). Scale bars, 10 μm; n, nucleus; g, Golgi apparatus.

Confocal microscopy of COS-7 cells transfected with DP8 or DP9 have shown cytosolic localization close to the Golgi apparatus [2,4]. Analysis by electron microscopy in turn, demonstrated granular localization of soluble DP4 in α-cells of porcine islets of Langerhans [35,76]. Furthermore, there has been a number of reports on internalization of DP4, partially depending on post-translational modification [8,38,41,42,73,81,82].

Hence, while it is very likely that extracellular peptidases with DP4-like functional homology cleave NPY and PYY, there also might be intracellular cleavage resulting in release of N-terminally truncated peptides. For this reason we performed transfection and confocal lasermicroscopy studies in Cos-1 cells and investigated whether DP4 and NPY get into contact in the cell and co-localize within vesicles thus enabling a more fine-tuning mechanism via a possible cleavage within a vesicle.

The possibility of a shared transport path from the trans-Golgi network in collective transport vesicles or via internalization of DP4 into the cell and thus the theoretical ability to modify NPY could not clearly be demonstrated and confirmed by the present confocal approach. The techniques used so far do neither exclude nor undoubtedly illustrate a co-localization and challenge further studies.

Although these results provide no direct evidence for an intracellular N-terminal truncation of NPY by DP4, in general, an intracellular cleavage cannot be excluded. If NPY is not hydrolyzed by DP4 within the cell several other proteases such as DP8 or DP9 remain potential candidates for peptide cleavage intra- and extracellular.

On the basis of this summary, we can conclude that at least up to five enzymatically active peptidases (DP4, DP8, DP9, XPNPEP1, XPNPEP2) as shown in Table 1, are potentially involved in NPY cleavage.

Although DP4 still shows the highest selectivity, each single role of these peptidases should be thoroughly investigated in the future. The intracellular and extracellular cleavage of NPY by peptidases distinct from DP4 is not only an additional mechanism in the regulation of this neuropeptide. It also requires taking the hydrolyzing activities of those peptidases into consideration when analyzing DP4 enzyme activities and their associated functions downstream in physiology and immunity. Thus, many functions previously ascribed to DP4 and its inhibition may actually be derived from the activity and inhibition of DP8, DP9, and other peptidases that are listed in Table 1. Furthermore, some of the peptidases might be able to compensate functions of DP4 after chronic inhibition by selective DP4 inhibitors.

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