Functional and genetic analyses of dipeptidyl peptidase 4 deficiency (*Dpp4*/*Cd26*) in a rat model

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Abstract

Dipeptidyl peptidase 4 (DP4/CD26) is a multifunctional serine exopeptidase. As dipeptidyl peptidase, DP4 modulates the functions of its substrates by specific truncation especially of Xaa-Pro dipeptides from the N-terminus. Neuropeptide Y (NPY) is of special interest as it represents one of the best substrates for DP4, and as it is involved in various physiological, psychological, and immune functions. Here, the NPY-DP4-axis was studied on three different levels: (1) cleavage of NPY by DP4/DP4-like peptidases, (2) its role in a newly developed congenic animal model of DP4 deficiency, and (3) within central processing of pain perception.

Firstly, enzyme activity studies on DP4-like peptidases, with respect to their potential to truncate NPY, revealed that peripheral NPY is mainly truncated by serum and endothelial DP4 whereas the intracellular dipeptidyl peptidase 8 and 9 possibly cleave NPY within the central nervous system (CNS). Initial co-transfection studies of DP4 and NPY in Cos-1 cells provided however no evidence for a vesicular co-localization of the peptidase and its substrate – thereby "opposing" the possibility of an intracellular cleavage of NPY by DP4.

Secondly, in-depth characterization of congenic DP4 deficient DA rats revealed a number of beneficial metabolic effects including improved glucose tolerance – associated with increased levels of glucagons-like peptide 1 – and reduced diet-induced body weight gain. Furthermore, DP4 deficient rats exhibited diminished anxiety and stress-like responses in behavioural and endocrinal tests, probably due to a reduced N-terminal truncation of NPY (see third approach). In contrast to these "beneficial" effects, however, several immune alterations, e.g. differential leukocyte subset composition at baseline, blunted NK-cell and T-cell functions, were observed. These considerable consequences of DP4 deficiency point to potential adverse effects and question the concept of chronic use of DP4 inhibitors in a clinical setting, such as treatment of diabetes type II. Thirdly, the DP4-NPY axis was investigated with regard to its potential role in stress-induced analgesia. We showed that, depending on the contextual stress (habituated vs non-habitiuated), DP4 deficiency ameliorates stress-induced analgesia, thereby providing direct evidence for its stress-protective action within the CNS.

In conclusion these studies illustrate that DP4 functions are highly important in various processes and that it will be hard to separate one aspect as a therapeutic target without taking others into account. Due to multiple actions of further DP4-like peptidases at various levels and sites, the upcoming DP4 inhibitors will have to act short-term, highly target- and site-specific in order to minimize the risk of unwanted side effects.

Keywords: Dipeptidyl peptidase 4 – neuropeptide Y – comprehensive phenotyping

Kurzzusammenfassung

Die Serin-Exopeptidase Dipeptidyl Peptidase 4 (DP4/CD26) ist in zahlreichen biologischen Prozessen durch spezifische N-terminale Trunkierung von Substraten, speziell von Xaa-Pro Dipeptiden, involviert. Neuropeptid Y (NPY) ist als eines der besten Substrate von DP4 aufgrund seiner umfangreichen Funktionen von besonderem Interesse. Die NPY-DP4-Achse wurde hier hinsichtlich dreier Aspekte untersucht: (1) Spaltung durch DP4-ähnliche Peptidasen, (2) Rolle im kongenen DP4-defizienten Tiermodell und (3) Rolle während der zentralen Verarbeitung von Schmerz.

Zunächst zeigten Enzymaktivitätsstudien mit DP4-ähnlichen Peptidasen in Bezug auf deren Potential, NPY zu trunkieren, dass peripheres NPY hauptsächlich durch Serum- und endotheliale DP4 gespalten wird, während die intrazellulären Dipeptidyl Peptidasen 8 und 9 von größerer Bedeutung bei der NPY-Spaltung im zentralen Nervensystem (ZNS) sind. Es ergaben sich jedoch in Kotransfektionsstudien keine Hinweise für eine intrazelluläre Spaltung von NPY durch DP4. Im zweiten Ansatz zeigte der DP4-defiziente kongene DA Ratten-Substamm DA.F344-*Dpp4*^{m}/SvH verbesserte metabolische Effekte, unter anderem verbesserte Glukosetoleranz im Zusammenhang mit erhöhten Spiegeln an Glucagon-ähnliches Peptid 1, sowie eine reduzierte diätinduzierte Gewichtszunahme. Interessanterweise zeigten Verhaltenstests und endokrine Untersuchungen reduzierte Angst und stressprotektive Effekte, die vermutlich durch die reduzierte N-terminale NPY-Trunkierung in DP4-defizienten Ratten verursacht wurden. Neben diesen scheinbar positiven Effekten der DP4-Defizienz wurden jedoch auch verschiedene Alterationen im Immunsystem, wie z.B. ein verändertes Leukozytenblutbild und eingeschränkte NK- und T-Zell-Funktionen festgestellt. Mit diesen Beobachtungen verweisen wir auf Nebeneffekte, welche wiederum das Konzept des chronischen Einsatzes von DP4-Inhibitoren in der Klinik in Frage stellen. In einem dritten Ansatz wurde die DP4-NPY-Achse im Hinblick auf ihre Rolle bei stressinduzierter Analgesie untersucht. Dabei konnten wir zeigen, dass DP4-Defizienz in Abhängigkeit vom Test-Stress-Niveau (habituiert vs. non-habituiert), eine stressprotektive Wirkung im ZNS aufweist.

Zusammenfassend zeigen die drei Publikationen, dass DP4 an einer Vielzahl wichtiger Prozesse beteiligt ist. Die Phänotypisierungsergebnisse der DA.F344-*Dpp4*^{m}/SvH-Ratten machen deutlich, dass einzelne Aspekte der DP4 nicht isoliert betrachtet werden sollten, verweisen jedoch auch gleichzeitig auf neue therapeutische Ansatzpunkte wie z.B. Angst, Ernährung und Schmerz. Aufgrund der multiplen Aktionen weiterer DP4-ähnlicher Peptidasen müssen DP4-Inhibitoren höchst spezifisch und reversibel wirken, um das Risiko von ungewollten Nebenwirkungen zu minimieren.

Schlagwörter: Dipeptidyl Peptidase 4 – Neuropeptid Y – umfassende Phänotypisierung

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List of abbreviations:

3 D	three-dimensional
3 V	third ventricle
aa	amino acids
ABC	avidin-biotin-peroxidase complex
ACTH	adrenocorticotropic hormone
ADA	adenosin deaminase
AgRP	agouti-related peptide
AMC	7-amino-4-methylcoumarin
ALAT	alanine aminotransferases
ANOVA	analysis of variance
AP	alkaline phosphatase
APAAP	alkaline phosphatase-anti-alkaline phosphatase
APC	antigen presenting cell
APP	aminopeptidase P
ARC	arcuate hypothalamic nucleus
ASAT	aspartate aminotransferases
BBB	blood-brain barrier
BCM	β-casomorphin
CNS	central nervous system
CRH	corticotropin-releasing hormone
CSF	cerebrospinal fluid
CVO	circumventricular organ
DA	Dark Agouti
DAB	L-2,4-diaminbutyrylpiperidinamide
DAPI	4', 6-diamidino-2-phenylindole
DASH	DP4 activity and/or structure homologs
DMEM	Dulbecco's modified Eagle's medium
DP4	dipeptidyl peptidase 4
DP6	dipeptidyl peptidase 6
DP8	dipeptidyl peptidase 8
DP9	dipeptidyl peptidase 9
DP10	dipeptidyl peptidase 10

ECM	extracellular matrix
EPM	elevated plus maze
FAP-α	fibroblast activation protein α
GFCF	gluten-free, casein-free
GFP	green fluorescent protein
GIP	glucose dependent insuliotropic polypeptide
GLP-1	glucagon like petide 1
GLP-2	glucagon like petide 2
Gly-Pro-pNA	glycyl-prolyl-4-nitroaniline
GPR	G protein-coupled receptor
HCD	high calorie diet
HEPES	N-2-Hydroxyethylpiperazine-N-ethane-sulfonic acid
HPA	hypothalamic-pituary-adrenocortical
i.c.v.	intracerebroventricular
Ile-Thia	isoleucyl-thiazolidide
ir	immunoreactivity
kDa	kilo Dalton
KO	knockout
LU	lytic units
mAb	monoclonal antibody
MALDI-TOF	matrix assisted laser desorption/ionisation-time of flight
MEE	medial eminence
MES	2-(N-Morpholino)-ethanesulfonic acid
MHC	major histocompatibility complex
NALADase	N-acetylated α–linked acidic dipeptidase
neg	negative
Ni-NTA	nickel-nitrilotriacetic acid
NPY	neuropeptide Y
OGC	oral glucose challenge
OGTT	oral glucose tolerance test
ORF	open reading frames
PBMC	peripheral blood mononuclear cells
PFA	paraformaldehyde
PLT	platelets

pNA	4-nitroaniline
PNS	peripheral nervous system
POP	prolyl oligopeptidase
pos	positive
PP	pancreatic polypeptide
PPI	prepulse inhibition
PVN	paraventricular hypothalamic nucleus
PYY	peptide YY
QPP	quiescent cell proline peptidase, DP2
RBC	red blood cell
RIA	radioimmunoassay
RNO	*Rattus norvegicus*
SDF-1	stromal cell derived factor-1
SEM	standard error of the mean
SI	social interaction
SIA	stress-induced analgesia
SP	substance P
SNS	sympathetic nervous system
TCR	T cell receptor
TFA	trifluoroacetic acid
TG	triglyceride
Treg	regulatory T cell
Tris	tris(hydroxymethyl)aminomethane
XPNPEP1	X-prolyl-aminopeptidase 1
XPNPEP2	X-prolyl-aminopeptidase 2
VMH	ventromedial hypothalamic nucleus
WBC	white blood cell

1. Introduction

Dipeptidyl peptidase 4 (DP4, EC 3.4.14.5) is a multifunctional serine exopeptidase that is also known as leukocyte differentiation marker CD26 [1, 2]. DP4 has been referred to in the literature by a wide variety of abbreviations including DPP IV, DP-IV, and DPP4. We chose the abbreviation DP4 in analogy to the more recently cloned dipeptidyl peptidases 8 and 9, which were abbreviated by DP8 and DP9. The membrane-bound form of DP4 is a type II transmembrane glycoprotein [3]. Due to the secondary structure of its catalytic domain, DP4 is classified as alpha/beta-hydrolase fold enzyme. According to its sequence homology around the seryl residue in the active site, the arrangement of the catalytic triad and the homologic structure in the C-terminus, DP4 is classified together amongst others with prolyl oligopeptidase (POP, EC 3.4.21.26) and acylaminoacyl peptidase (EC 3.4.19.1) into the family of prolyl oligopeptidases (S9) [4]. DP4 was discovered in rat liver homogenates by Hopsu-Havu and Glenner in 1966 [5].

1.1. Structure and structural properties of DP4

The cDNA of rat DP4 codes for a sequence of 767 amino acids (aa) that can be divided into five structural regions (Fig. 1). The highly conserved N-terminal cytosolic region, composed of six aa, is followed by a hydrophobic region of 22 aa – the transmembrane domain – which serves as signal peptide and membrane anchor [3]. The extracellular part of DP4 consists of 739 aa and can further be divided into three regions. These are the glycosylated region, containing five of eight potential N-glycosylation sites of the extracellular region [6, 7], the cysteine-rich region, expressing ten of twelve cysteine residues [8], and the C-terminal catalytic region. The C-terminus harbors the active site of this enzyme, which comprises a triad of the catalytically active amino acids Ser631, Asp709 and His741 [9, 10]. The active Ser631 of DP4 is situated in a so called ”nucleophile elbow“ in the sequence Gly-Trp-Ser-Tyr-Gly which is in agreement with the conserved sequence Gly-X-Ser-X-Gly for the α/β hydrolase family.

There is a homology of 84,5% between human and rat DP4 cDNA [11]. Human DP4 is a 766 aa sized molecule, has an additional N-glycosylation site on N281 (according to human numbering) [12] and the amino acids of the catalytic triad are in positions 630, 708 and 740. In contrast to rat DP4, human DP4 is able to bind adenosine deaminase (ADA) [12, 13]. Oligosaccharide side chains are reported to modulate biophysical and biological properties of glycoproteins such as protein folding, stability, translocation and cell adhesion processes.

However, the precise role of the glycosylations of DP4 is unknown. Fan et al. [14] demonstrated that N-glycans are essential for folding and biological stability of this molecule. O-glycosylation has been controversially discussed. Whereas no O-glycosylation could be detected in membrane-bound DP4 from rat liver [15-17], Naim et al. and Matter et al. reported on O-glycosylation of DP4 on human intestinal brush-border cells and Caco-2 cells (human adenocarcinoma cells of colorectal origin), suggesting that O-glycans were important for apical targeting [18, 19]. Glycosylation is not a prerequisite for catalytic activity or dimerization [12]. However, the stability of the cysteine-rich region of DP4 is proposed to be of importance for dimer formation [8].

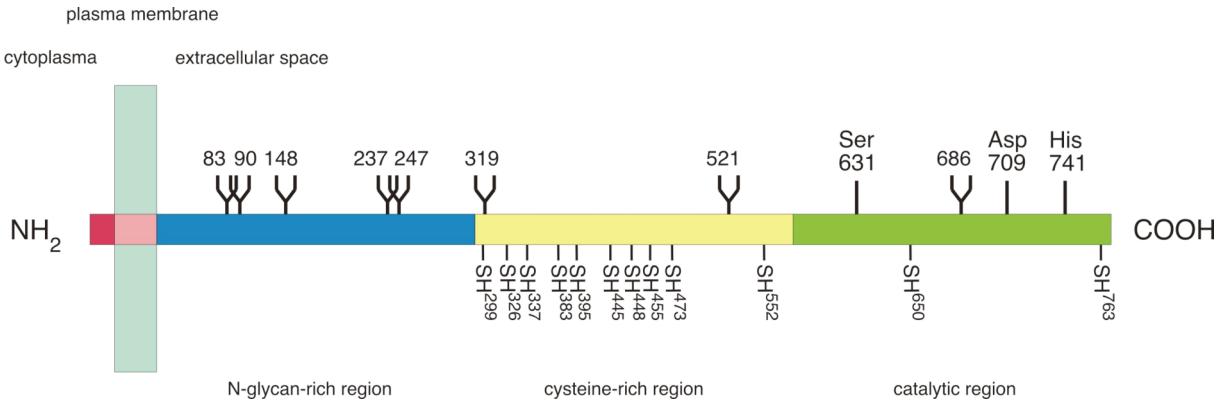

Figure 1: Schematic representation of wild type rat DP4 deduced from its primary structure. Modified after Fan et al. [14]; Y-shaped structures represent N-glycans; SH, cysteine residue; catalytic triad: Ser, Asp, His; numbers represent the amino acid position; magenta, cytoplasmic tail; pink, transmembrane domain

In humans, the cysteine-rich domain is involved in ADA binding [20]. Moreover, Dobers et al. [8] showed via cysteine point mutations in rat DP4 that the cysteine residues 326, 337, 445, 448, 455, 473, and 552 are essential for correct folding and intracellular trafficking of DP4. Two non-covalently connected independent monomers form the functional DP4 homodimer, in which again the active sites of both monomers are connected via electrostatic interactions [21]. Homodimerization is essential for the enzyme activity of DP4, whereas the monomeric form on its own has no enzyme activity [22-26]. The molecular weight of the DP4 monomer depends on the species, the origin/type of tissue, and the level of glycosylation. It differs around 110 kDa to 150 kDa in mammals [27-29]. There is an initially synthesized precursor form with a molecular weight of 103 kDa in rat hepatocytes that is further processed to the mature form of 109 kDa during the intracellular transport [30].

While the extracellular part of DP4 has been approximately divided into the three regions on basis of the amino acid sequence (Fig. 1), cryo-transmission electron microscopy and crystal structures revealed a new three-dimensional (3D) structure for human DP4. This 3D structure gives precise details about the amino acids that compose functionally different domains. Thus, analysis of the crystal structure of DP4 revealed that the extracellular part of each DP4 monomer consists of two domains (Fig. 2), the catalytically active α/β-hydrolase domain (amino acids 39-51 and 506-766) – that is located closest to the membrane – and an eight-bladed β-propeller domain (55-497) [31, 32]. The catalytic site is located in a large cavity formed between the α/β-hydrolase domain and the propeller domain. Both domains are reported to participate in inhibitor binding [32].

1.2. Distribution of DP4

DP4 is highly conserved among different species [31] and DP4 expression in rat tissues does not significantly differ from expression in humans. The membrane-bound form of DP4 is ubiquitously expressed with different levels in tissues like kidney, lung, adrenal gland, intestine, liver, spleen, testis, and brain. Endothelial and epithelial DP4 is localized on the apical cell membrane [33]. The abundant expression on endothelial cells, especially in blood vessels and capillaries [34], enables a close contact to hormones, chemokines, and cytokines that are circulating in the blood. There is a soluble form of DP4 that lacks the transmembrane domain [35, 36] and which can be found in plasma and at low levels in the cerebrospinal fluid (CSF) [35], urine, and seminal fluid [37, 38]. The origin of soluble DP4 so far is unknown. However, shedding of the membrane-bound form via other proteases is a proposed mechanism. Furthermore, DPPIV/CD26 is expressed on cells of the immune system and here, especially on activated T-helper cells and B lymphocytes [39-41], activated NK cells [42], and subsets of macrophages [43]. In the adult central nervous system (CNS) DP4 has contact with neuropeptides mainly in the CSF but also via the blood-brain barrier (BBB). DP4 is primarily found in the circumventricular organs (CVOs) and on leptomeningeal cells, in brain capillaries and ependymal cells [44, 45]. DP4 positive neurons are only found in the fetal brain; they decrease during development [44, 46-49]. In the peripheral nervous system (PNS), DP4 is located in the perineurium and Schwann-cells [50].

The overall presence of DP4 enables the peptidase to act on substrates and immune cells in body fluids underlining its relevance in physiological, psychological and immunological processes [51]. Aberrant DP4 levels have been reported as clinically relevant for various diseases [24, 52-54]. For example, patients with major depression [55] or schizophrenia [56]

show a decrease in serum DP4-like activity, whereas an increase in DP4-like serum activity was reported in patients with hyporectic eating disorders like anorexia and bulimia nervosa [54, 57]. Furthermore, altered DP4 levels are reported in autoimmune diseases like rheumatoid arthritis [58-60] or HIV/AIDS [61] as well as in allograft rejection [62] or pregnancy [63].

1.3. Functional role of DP4 – enzymatic activity, extracellular matrix binding, and T cell activation

The importance of DP4 is derived from various biological processes and the multifunctional role in mammalian organisms is not only a result of enzymatic activity but also of cell adhesion and T cell activation processes (Fig. 2).

Independently from its enzymatic activity, DP4 shows high affinity for the matrix proteins collagen and fibronectin. It could be shown that DP4 initiates the adhesion of rat hepatocytes on a collagen matrix [64, 65]. Furthermore, interactions of rat DP4 with fibronectin could be demonstrated [66]. The cysteine-rich domain of DP4 is reported to interact with collagen and fibronectin of the extracellular matrix (ECM) and two separate binding sites for collagen and fibronectin were proven in this domain [2, 67, 68].

DP4 acts as costimulatory surface molecule, influences T cell activity, and modulates chemotaxis [69]. Due to its enhanced expression on activated lymphocytes, DP4/CD26 is originally described as a T cell activation marker, but is now regarded as a general marker of cellular activation. DP4/CD26 is considered to play a role in T cell activation [20, 70, 71] and proliferation via costimulation [72-76]. Maximal T cell activation requires an antigen-specific stimulation of the TCR/CD3 complex, provided by a MHC/peptide complex, and a costimulatory signal. In this context, caveolin-1 is actually discussed to act as costimulatory ligand for DP4/CD26. Ligation of CD26 by caveolin-1 induces upregulation of CD86 – the ligand of CD28 – via NF-κB activation. CD28 then mediates costimulation and potentiation of T cell proliferation in a TCR/CD3-dependent manner [77, 78].

DP4 disposes of major influence in body functions via its protease activity. As serine exopeptidase, DP4 regulates various physiological processes by cleaving Xaa-Pro dipeptides from the N-terminus of oligo- and polypeptides. According to Schechter and Berger [79], residues in a peptide substrate are called P_1, P_2, P_3.... P_n counting from the scissile bond toward the N-terminus and P_1', P_2', P_3'... P_n' counting toward the C-terminus. Originally, DP4 was considered to cleave specifically after proline, or with less efficiency after alanine, at P_1 position. Meanwhile, the substrate spectrum has been enlarged, thus, other amino acids like

valine, glycine, and serine for example are accepted in penultimate (here, P_1') position – however, with strongly reduced catalytic efficiency [80, 81]. Numerous biologically active peptides like neuropeptides, peptide hormones and chemokines possess evolutionary conserved proline-rich regions in their sequences [82, 83]. These proline residues serve as proteolytic processing regulatory element and prevent from unspecific proteolysis.

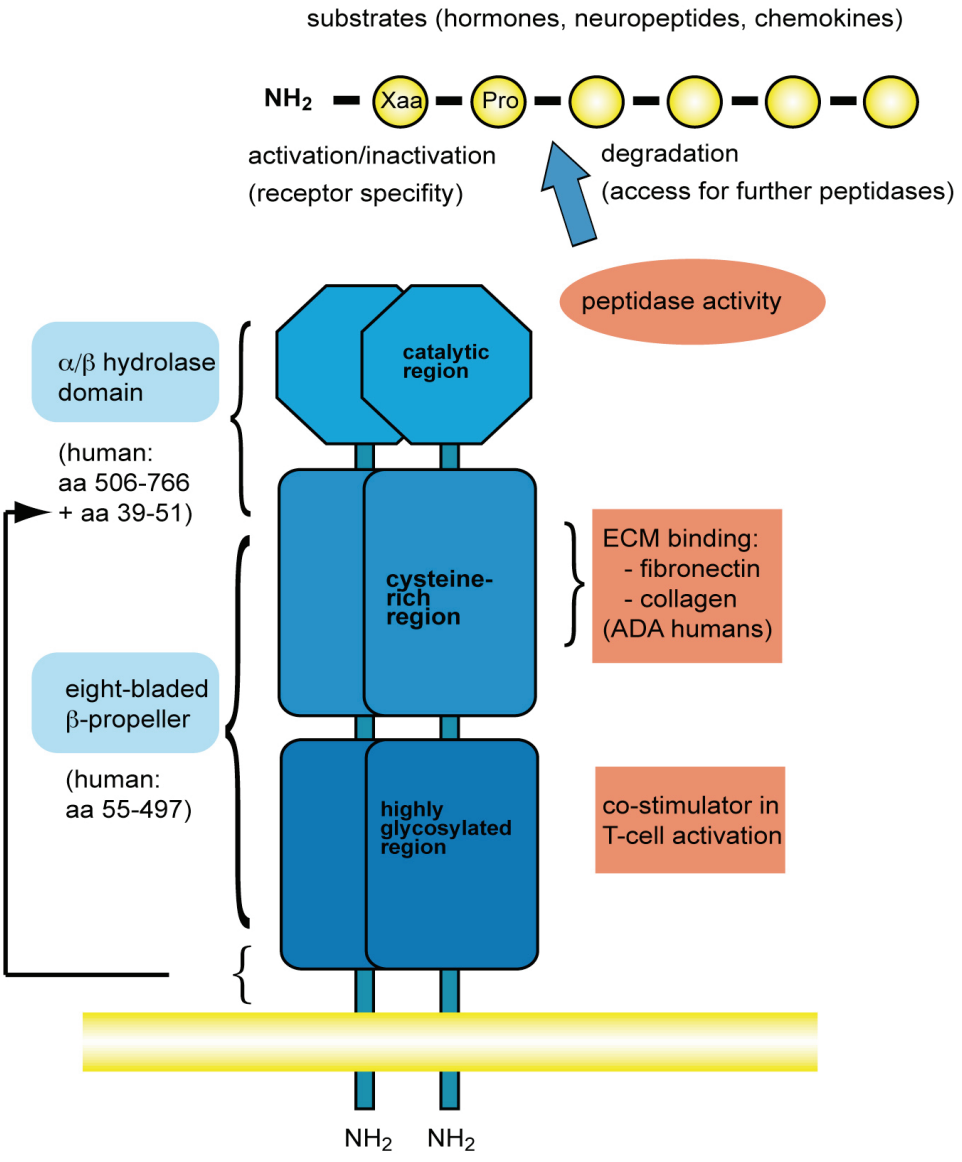

Figure 2: Schematic representation of the structure of the DP4 homodimer with respect to its 3D composition and to its main functional role: α/β hydrolase domain and the eight-bladed β-propeller domain with corresponding numbering of amino acids (aa) (according to human DP4); yellow, plasma membrane; red, functions of DP4; Xaa, variable amino acid

As sole natural imino acid, proline connotatively influences secondary and tertiary structures of peptides and thus controls their biological activity [84]. Regulation of this activity can take

place via proline specific peptidases. In case of many regulatory peptides, it could be shown that they are substrates of DP4 [2, 51, 69]. Cleavage of N-terminal dipeptides leads to activation or inactivation of the substrates and thus prevents the binding at specific receptors and enables the regulatory intervention in biological cascades. Furthermore, cleavage by DP4 also makes substrates accessible for further or for total degradation by other peptidases (Fig. 2).

1.4. Structural and functional homologues of DP4

For many years DP4 was believed to be a unique cell membrane protease cleaving Xaa-Pro dipeptides from the N-terminal end of peptides. Subsequently, other "DP4 activity and/or structure homologues" termed DASH have been discovered. These proteins are grouped on the basis of having an associated DP4-like enzymatic activity with or without much structural homology, or they reveal structural similarity but are enzymatically inactive (dipeptidyl peptidase 6 (DP6) and 10 (DP10)). The DASH protein family comprises, for example, fibroblast activation protein α (FAP-α) alias seprase, quiescent cell proline dipeptidase (QPP) alias dipeptidyl peptidase 2 (DP2), dipeptidyl peptidase 8 (DP8), dipeptidyl peptidase (DP9), attractin, N-acetylated α-linked acidic dipeptidases (NAALADases) and thymus-specific serine protease [85, 86]. These peptidases should be taken into account when conducting tests based on DP4-like activity and inhibitor studies.

1.5. Substrates of DP4 and their physiological impact

Whereas former experiments concentrated on the question which peptide hormones, chemokines, and neuropeptides are cleaved by DP4, later experiments focused on its biological function *in vivo*. Therefore, DP4 specific inhibitors and DP4 negative animal models were useful. Exogenously administered inhibitors of DP4 prolong the biological half-life of DP4 substrates, with several of them being highly important clinical and pharmaceutical targets for drug development.

In vivo and *in vitro* experiments demonstrated the truncation of many substrates by DP4. N-terminal cleavage of the peptide hormones glucose dependent insulinotropic polypeptide (GIP), glucagon like peptide 1 and 2 (GLP-1, GLP-2) leads to their inactivation. GIP and the active form of GLP-1 – GLP-1(7-36) – stimulate, in response to blood glucose increment, the release of insulin from pancreatic beta-cells. Inhibition of DP4 increases the amount of intact incretins like GLP-1 and GIP, improves their insulinotropic effect, and has been proposed as being a

valid therapeutic approach for lowering glucose levels in type 2 diabetes or other disorders involving glucose intolerance [51]. Treatment of diabetes type 2 is of major interest and a present field of research. Actually, new pharmaceuticals acting as DP4 inhibitors are introduced to the market.

Endomorphin-2 is an endogenous opioid that is abundantly present in the cortex of the human brain. It has a high affinity for the μ (morphine) opioid receptors that produce analgesia. The *in vivo* inactivation of endomorphin-2 by DP4 was demonstrated in rats and central inhibition of DP4 leads to enhanced analgesic actions of exogenous endomorphin-2 [87]. Another substrate is β-casomorphin (BCM), which is derived from the milk protein casein from mammals. It is converted by DP4 into further β-casomorphin fragments [88-91]. β-casomorphins have potent opioid activity and therefore are referred to as exorphins or formones (food hormones). Casomorphins can influence the digestion and intestinal absorption processes. They extend the storage period of food in the gastrointestinal tract and influence the postprandial metabolism via stimulation of insulin and somatostatin secretion [92].

Substance P (SP) is a widespread neuropeptide that originates from spinal ganglia. This peptide has a well-established role in immunity and induces the release of inflammatory mediators from mast cells and causes an increase in vascular permeability. SP is – together with other tachykinins – responsible for nociceptive transmission from the peripheral to the central nervous system [93, 94]. Additionally, DP4 is supposed to enhance nociception by processing SP to a more potent derivate (SP_{3-11}; SP_{5-11}) [95] in combination with inactivating potent analgesic μ-opiate receptor agonists and other DP4 substrates [53]. Another substrate is the gut hormone peptide YY (PYY) [96, 97] which is secreted from the endocrine L cells of the gut [98]. It is found at low levels in the small intestine with concentrations increasing distally throughout the gut to reach maximum concentration in the rectum. Circulating PYY levels are low in the fasting state and rapidly increase postprandially. N-terminal truncation of PYY_{1-36} by DP4 results in PYY_{3-36} which is a potent physiological regulator of satiety.

1.6. NPY – a DP4 substrate of high relevance

NPY was discovered and isolated in 1982 by Tatemoto [99]. Together with the pancreatic polypeptide (PP) and PYY, NPY belongs to the pancreatic polypeptide (PP-fold) family. Common characteristics of this family are a tyrosine-rich sequence of 36 aa and a C-terminal tyrosineamide residue that is essential for biological activity. The name of NPY and PYY is due to the presence of a large number of tyrosine residues – abbreviated by the letter Y in the single letter amino acid code – including both ends of the molecule.

The evolutionary high conservation of NPY (the sequence of human and rat NPY is identical) supports the idea of being a key mediator in the regulation of physiological processes. As neuropeptide, NPY acts in the CNS and PNS and displays a large array of functions [100-102]. NPY is the most abundant neuropeptide in the brain [103] and it is preferentially expressed in interneurons [104, 105]. Particularly high levels of NPY-like immunoreactive material are found in the hypothalamus, the septum, the nucleus accumbens, the periaqueductal gray, and the locus coeruleus [103, 105, 106] whereas moderate levels can be detected in the amygdala, the hippocampus, cerebral cortex, basal ganglia, and the thalamus [103, 107]. Furthermore, NPY is broadly distributed via NPY-containing cell bodies and is found in fibres of organs like pancreas, intestinal tract, heart, thyroids, kidney, lung, and gonads [103]. The localization of nerve terminals and cell bodies suggests a wide-ranging role for NPY in behaviour and physiology.

NPY influences feeding behaviour and thus the body weight regulation [108, 109]. The influence of NPY on the inhibition of adipolysis and on increased lipoproteinase activity leads to an increased storage of fat [110]. Based on this, an important role in pathophysiology of obesity and diabetes is attributed to NPY.

1.6.1. NPY and NPY receptor subtypes

In rat, the peptides of the PP family activate a heterogeneous population of at least four G protein-coupled receptors (GPRs; Y_1, Y_2, Y_4, and Y_5) that differ in selectivity [111-113]. NPY and PYY are both processed by DP4 via N-terminal dipeptide cleavage from a molecule of 36 aa length (NPY_{1-36}/PYY_{1-36}) to NPY_{3-36} /PYY_{3-36} and thus exhibit changed receptor specificity. The (3-36) derivates show a reduction in Y_1 receptor affinity, whereas their affinities are not or scarcely reduced at the Y_2 and Y_5 receptors which makes them selective for Y_2 and Y_5 receptors relative to Y_1 receptors [114]. The different NPY receptors that are involved in activating processes in rat and human are presented in table 1.

Further discovered and discussed receptor subtypes are the Y_3 receptor – which is pharmacological detectable, however, not yet cloned – the Y_6 receptor that is non-existent in rats and non-functional in humans and other primates, and the Y_7 receptor which is not found in mammals. A very recently discovered potential NPY receptor is the G protein-coupled receptor (GPR)83 [115].

Originally, the subdivision of NPY receptors comes from the observation that C-terminal fragments of NPY or PYY, e.g. NPY_{13-36}, can mimic some NPY responses while some others

cannot [116]. This has led to the proposal that receptors that are only activated by the holopeptides are designated Y_1, whereas those that are activated by the holopeptides and the C-terminal fragments are designated Y_2. Despite the synthesis of numerous C-terminal fragments, the (3-36), (13-36), and (18-36) fragments of NPY and PYY are most frequently used without apparent advantages between them [113]. C-terminal NPY fragments are still useful to discriminate Y_1 and Y_2 receptors, but they are not selective for Y_2 receptors since they can also activate Y_5 receptors at similar concentrations [114]. Therefore, synthesized forms of NPY and PYY exist in which different amino acids are substituted (e.g. [Pro^{34}]NPY).

Table 1: Functional NPY receptor subtypes in rat/human

Receptor	**Y_1**	**Y_2**	**Y_4**	**Y_5**	**GPR83/GIR**
Major occurence	periphery hypothalamus	brain hippocampus	intestine colon	hypothalamus	brain, thymus; selectively up-regulated surface molecule by Treg cells
Related action	anxiolysis, food intake, vasoconstriction, nociception	memory, epilepsy, secretion, food intake?	gastro-intestinal regulation	food intake?, epilepsy?	orphan G protein-coupled receptor
Agonist order of potency	NPY ≥ PYY >> PP	NPY ≥ PYY >> PP	PP > NPY = PYY	NPY ≥ PYY ≥ PP	NPY ≥ PYY >> PP
Selective agonists	[Leu^{31}, Pro^{34}]NPY[a], [Pro^{34}]NPY[a], [Leu^{31}, Pro^{34}]PYY[a], [Pro^{34}]PYY[a]	NPY_{3-36}[b], PYY_{3-36}[b], NPY_{13-36}[b], PYY_{13-36}[b]	PP	[Ala^{31}, Aib^{32}]NPY	NPY_{3-36}[b], PYY_{3-36}[b]

[a] selective relative to Y2 receptors

[b] selective relative to Y1 receptors

modified after Cabrele et al., 2000 [111], and Alexander et al. 2007 [117]; Treg cells, regulatory T cells

NPY is involved in various physiological and behavioural processes that are mediated via DP4 cleavage [1, 51, 118]. Activation of the Y_1 receptor induces a long-lasting vasoconstriction and enhances the effects of vasoconstrictive substances like angiotensin II or noradrenaline – influenced by NPY [119-121]. Anxiolysis and sedation are mediated within the CNS and are modulated via the Y_1 receptor [112, 122-124]. In this context, NPY has a sedative [124-126] and an anxiolytic effect [112, 127, 128]. Furthermore, mainly the Y_1 receptor seems to be involved in regulation of food up-take, although the Y_5 receptor is discussed as appetite

receptor [114, 129-131]. The Y_2 receptor has also been focussed in regard to its food intake regulation [132-134]. NPY has an antinociceptive effect that is mediated via Y_1 receptors. Various effects that are mediated via the Y_2 receptor are based on the inhibition of the release of neurotransmitters. Thus, NPY inhibits the release of glutamate in the CNS as well as the release of noradrenaline and acetylcholine in the PNS via presynaptical activation of the Y_2 receptor. NPY enhances the memory retention by activation of the Y_2 receptor [135-137] and regulates the circadian rhythm of rodents [138-143].

1.6.2. Role and sources of N-terminally truncated NPY_{3-36} and PYY_{3-36} peptides

The power of DP4, to act as modulator of important regulatory processes via N-terminal truncation, gives rise to think about the precise localization where these actions may take place. Different factors that determine these regulatory processes are amongst others the amount and localization of the corresponding substrate receptors, substrate concentration, kinetic parameters, and the consequence of action (e.g. activation, inactivation, or degradation). In line with this, sources of N-terminally truncated NPY, PYY, and further substrates are of highest interest. An important role for N-terminally truncated PYY has been suggested when Batterham et al. [144] reported on the potent feeding regulatory effects of PYY_{3-36}. However, controversies arose when a number of laboratories could not repeat the core observation that PYY_{3-36} suppressed food intake in rodents.

Although the detailed comparison of methodologies and protocols has led to several hypotheses, the reasons for these discrepancies are unknown. Nonetheless, publications of positive and negative results on pharmacological effects and endogenous roles of PYY in energy balance have triggered new studies with increased attention to these details and experimental conditions [145]. Unfortunately, the source of this truncated peptide remained unknown. Despite the reported release of PYY_{1-36} into the circulation, it seems to be unlikely that such important effects that are ascribed to PYY_{3-36}, are primarily due to „non-specif“ cleavage in the plasma. We therefore hypothetized that PYY_{1-36} and also NPY_{1-36} are truncated intracellularly by DP4 and investigated this in co-transfection experiments.

1.7. Animal models of DP4 deficiency

The overall importance of the ubiquitously distributed DP4 is not only due to its involvement in various diseases and immune functions but is reflected by its broad range of substrates –

each being individually involved in biological cascades. Animal models serve for a better understanding of the role and function of D4. Therefore, former investigations concentrated on the charaterization of the DP4 deficient model of F344 rats – including the two DP4 deficient substrains F344/DuCrjSvH-*Dpp4*^{m} and F344/Crl(Wiga)SvH-*Dpp4*^{m}, previously named F344/DuCrj(DPPIV-) and F344/Crl(Ger/DPPIV-) – that were compared to wild type rats F344/Crl(USA) or F344/Ztm [146-148].

It has been shown that in rat *Dpp4* is located on rat chromosome 3 (RNO3) and is inherited in a semi-domant mode [146]. Furthermore, these rats are DP4 deficient due to a point mutation in the *Dpp4* gene – a transition from G to A at nucleotide 1897 that leads to substitution from Gly to Arg in the catalytic center of the enzyme at amino acid position 633 [30]. While normal levels of DP4 mRNA are produced [149], the mutation causes a conformational change, leading to a rapid degradation in the endoplasmic reticulum [30] and thus, leading to a total loss of DP4 activity [30]. DP4 deficiency leads to a prolonged half-life of DP4 substrates.

Consequently, animals of the former model were phenotyped by our group mainly with respect to behavioural and physiological aspects that were attributed to differential degradation processes of various substrates. Although both DP4 deficient F344 substrains bear the identical mutation, they differ in some results of phenotyping [146, 147]. Due to this fact and the point that the different rat substrains were originally obtained from different Charles River facilities, it is very likely that differences in the genetic background might have an influence on those differential outcome measures in F344/DuCrjSvH-*Dpp4*^{m} and F344/Crl(Wiga)SvH-*Dpp4*^{m} rats. In addition, microsatellite analysis revealed differences in the genetic background between the mutant and the various non-deficient F344 substrains apart from *Dpp4* (unpublished data). To yield a homogenous genetic background for precise investigation and to enhance behavioural and physiological differences that were found between the DP4 deficient F344 substrains and the F344 wild type substrain, the new DP4 deficient congenic DA substrain DA.F344-*Dpp4*^{m}/SvH was developed. Thereby, the mutation of the F344/Crl(Wiga)SvH-*Dpp4*^{m} substrain was transferred onto the genetic background of DA/Ztm rats. The development was speeded-up with help of marker-assisted selection. Wild type DA/Ztm rats show a nearly two-fold expression of DP4-like activity in comparison to wild type F344/Crl(USA) rats. Thus, we expect differences between DP4 deficient and DP4 wild type rats to be more distinct.

A further aspect that requires and justifies this new congenic animal model next to the above mentioned advantages of high DP4-like activity and the homogenous genetic background, is for example, the possibility of testing the specificity of DP4 inhibitors – wherefore the homogenous genetic background is essential. In addition, the species-specific contribution of

Rattus norvegicus can back-up findings of the existing *Dpp4*^{tm1Nwa} mouse model (CD26 knockout (KO)) but importantly, it can also reveal discrepancies. Interestingly in this context are also studies with NPY transgenic rats [150, 151] that over-express prepro NPY mRNA or NPY KO mice. The latter revealed contradictory results to effects that were ascribed to NPY [152-154]. However, as these "classical" KO mice represent germ-line KO mice, a life-long compensation for the lack of NPY might explain why the phenotype is less pronounced. This suggests that there is a need for inducible NPY and NPY receptor KO mice. Furthermore, this clearly demonstrates that any kind of approach gives its own answer and reminds that various approaches help to clarify regulatory functions and networks.

1.8. Aims of the present thesis

The aim of the present study is to further investigate and understand the role of DP4 *in vitro* and *in vivo* with special emphasis on its interaction with NPY. Therefore, we focused in a first attempt on the intracellular transport of DP4 and its substrate NPY to search for possibilities of fine-tuning mechanisms in intracellular regulation of NPY_{1-36}/NPY_{3-36} levels, caused by DP4. Additionally, the involvement of structural and functional homologues of DP4 was discussed with respect to their potency to control endogenous NPY levels [155].

A second step was the investigation of DP4 deficiency in a new animal model on the homogeneous genetic background of DA rats. We conducted a comprehensive phenotyping strategy based on endocrine and immunological as well as on behavioural and physiological aspects. We focused on the latter, since behaviour and physiology are likely to be regulated by DP4 substrates, especially by NPY. These studies were made to detect fields of impact and to appraise possible consequences of the nowadays actually discussed DP4 inhibitors. Furthermore, our data serve to support theories and hypotheses of the function of DP4 from findings in the previously used animal model of DP4 deficient F344 rats and will be helpful for the further use of the novel animal model for DP4 deficiency.

The last field of our studies focused on the differential pain perception/processing in DP4 deficient and wild type F344 and DA substrains, which relates to the differential stress-responsiveness in DP4 deficient animals. These variable findings in stress responsiveness result in the phenomenon of differential stress-induced analgesia. Here, DP4 deficient congenic DA rats were compared with spontaneous mutant DP4 deficient F344 rats in a genetically and pharmacological based approach. The modulation of stress-induced analgesia in DP4 deficient animals was compared with effects resulting from pharmacological inhibition of DP4. This additional pharmacological approach comprises the analysis of DP4 deficient and wild type

F344 rat substrains after i.c.v. application of either NPY or the DP4 inhibitor isoleucyl-thiazolidide (*Ile-Thia*).

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.

2.1. 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 position 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 (i.c.v.) in deficient animals [48]. Recently, we also found an increased potency of i.c.v. 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.

2.2. Review on DP4-like structural and functional homologues

2.2.1. 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 and 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 *Dpp4* 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.

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.

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 …X_{aa}-Gly-Pro-Y_{aa}… 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].

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 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.

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. (2003) and Ajami et al. (2004) 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 on 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].

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 $P_{1\text{-}4}$, bradykinin$_{1\text{-}3}$ or bradykinin$_{1\text{-}5}$ [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.

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.

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.

2.3. Materials and methods

Animals

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

Tissue extraction

Brains, obtained from F344/Crl(Por/98) and F344/Crl(Wiga)SvH-*Dpp4*^{m} 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.

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.

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.

MALDI-TOF-Mass Spectrometry

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.

Cell culture

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

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].

Construction of cDNA clones

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.

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 NotI and BamHI (MBI Fermentas, St. Leon-Rot, Germany).

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'-AGAATTCATGGTGGCGGTACGCAGGCCTTGGCCCG TTATGGTC-3' introduced an EcoRI site and the anti-sense primer 5'-TTT**GGATCC**G CCCCACTGGTCCACACCTTC-3' a BamHI restriction site at the end of the construct. The purified PCR product was cloned into the BamHI and EcoRI restriction sites of pcdsRed1-N1.

2.4. Results

Figure 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*^{m} 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 F344/Crl(Por/98) rats in presence/absence of selective inhibitors of DP4-like enzymes showed the existence of DP4 and DP8/9 as illustrated in Figure 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} (Fig. 3 and 4).

Figures 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 Figure 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 Figure 5B and 5C. 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 Figure 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.

2.5. Discussion

In the present paper, the members of the DP4 gene family as well as structural and functional homologues are discussed as 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 (Figure 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 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 Figure 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]. 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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2.8. Tables and Figures

Name/ Synonymous/ EC	Expression	Function	Structural/functional relationship	Ref.
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; subfamily 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; subfamily 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 leukocytes	Has not yet been associated with any particular biological process, however, NPY cleavage reported	Clan SC; subfamily S9B; shares 19% AAS identity with DP4; post-proline DP-like activity	[4], [77], [9], [68]
XPNPEP1, X-prolyl aminopeptidase 1, aminopeptidase P 1; 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], [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	[16], [57], [59], [87],

Table 1: Structural and functional homologues of NPY cleaving peptidases

DP4 = dipeptidyl peptidase IV; AAS = amino acid sequence

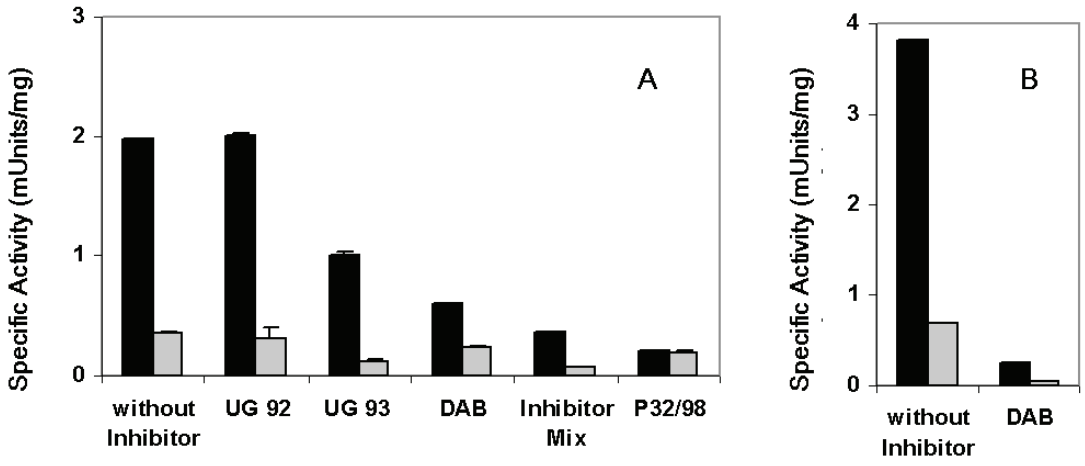

Figure 1: Activity and inhibition studies of crude brain extracts, obtained from F344/Crl(Por/98) (dark columns) and F344/Crl(Wiga)SvH-*Dpp4* (bright columns) rats, applying selective inhibitors against DP4-like enzymes. (A) Brain extract assayed with 0.125 mM Ala-Pro-AMC, pH 7.6 at 37°C; (B) brain extract 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.

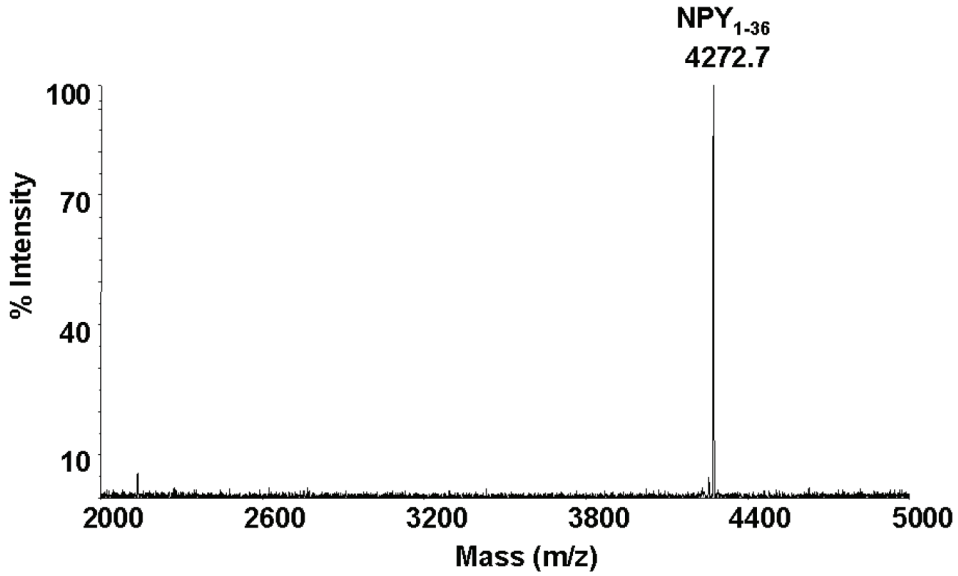

Figure 2: MALDI-TOF-MS analysis of NPY cleavage by recombinant human dipeptidyl peptidase 2 (rh-DP 2), showing no cleavage after 6 h incubation at 37°C. 30 mUnits rhDP 2 were incubated with 25 mM NPY in 10 mM MES buffer, pH 5.5 for 6 h at 37°C. Afterwards, the reaction was stopped with 0.1% TFA, samples 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).

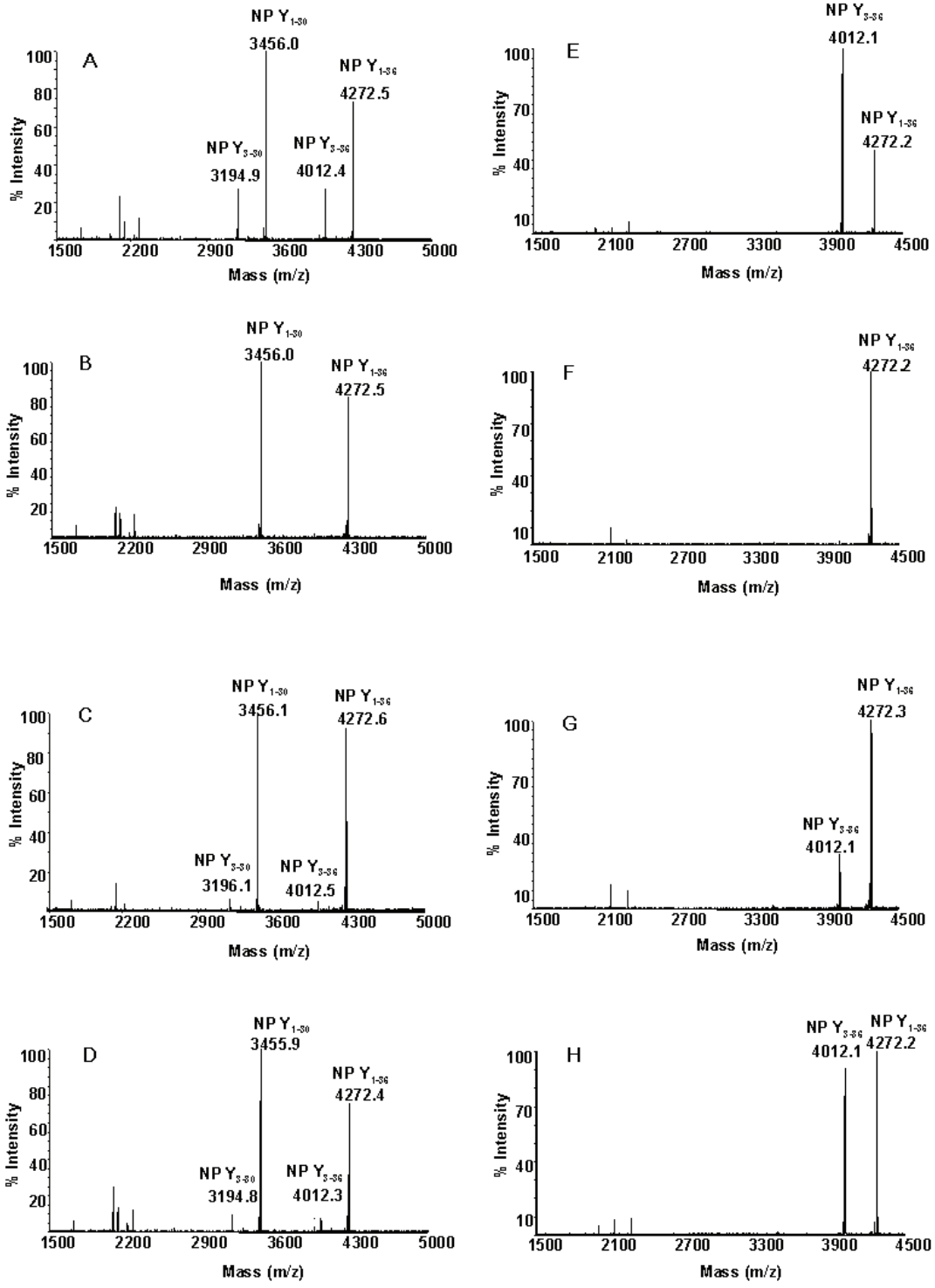

Figure 3: NPY hydrolysis in crude brain extract from F344/Crl(Por/98) rats or in EDTA-Plasma determined by MALDI-TOF-MS after 30 min incubation at 37°C in absence or presence of different inhibitors. The reaction was stopped with 0.1% TFA, samples were

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.

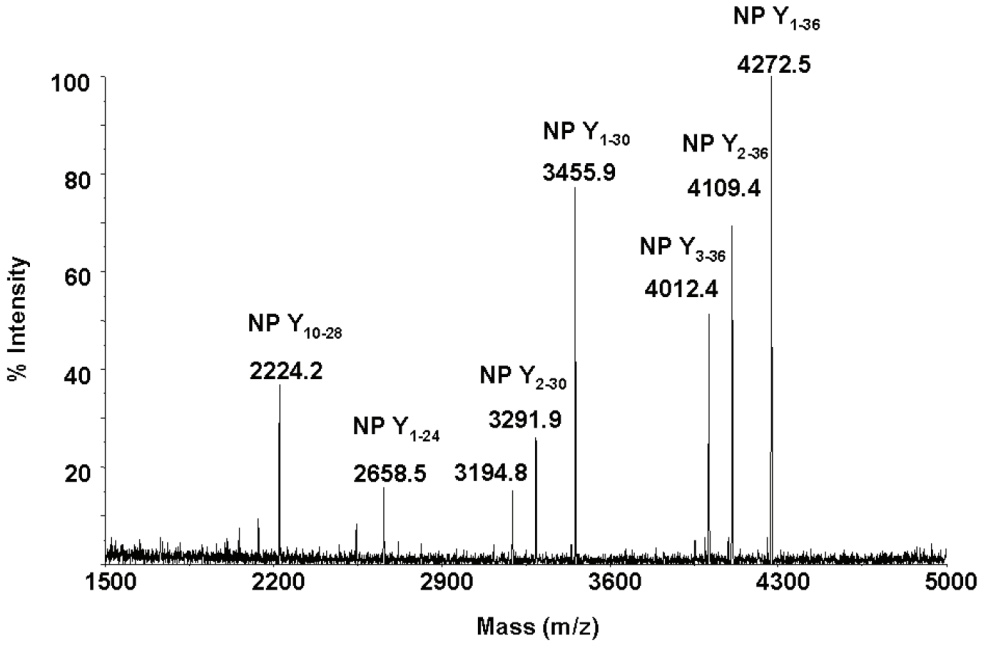

Figure 4: MALDI-TOF MS analysis of NPY hydrolysis by cytosolic brain fraction from F344/Ztm rats after 30 min incubation at 37°C. The reaction was stopped with 0.1% TFA, samples were purified with ZipTip (Millipore GmbH, Germany), mixed with the matrix sinapinic acid at a ratio 1:1 and analysed with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

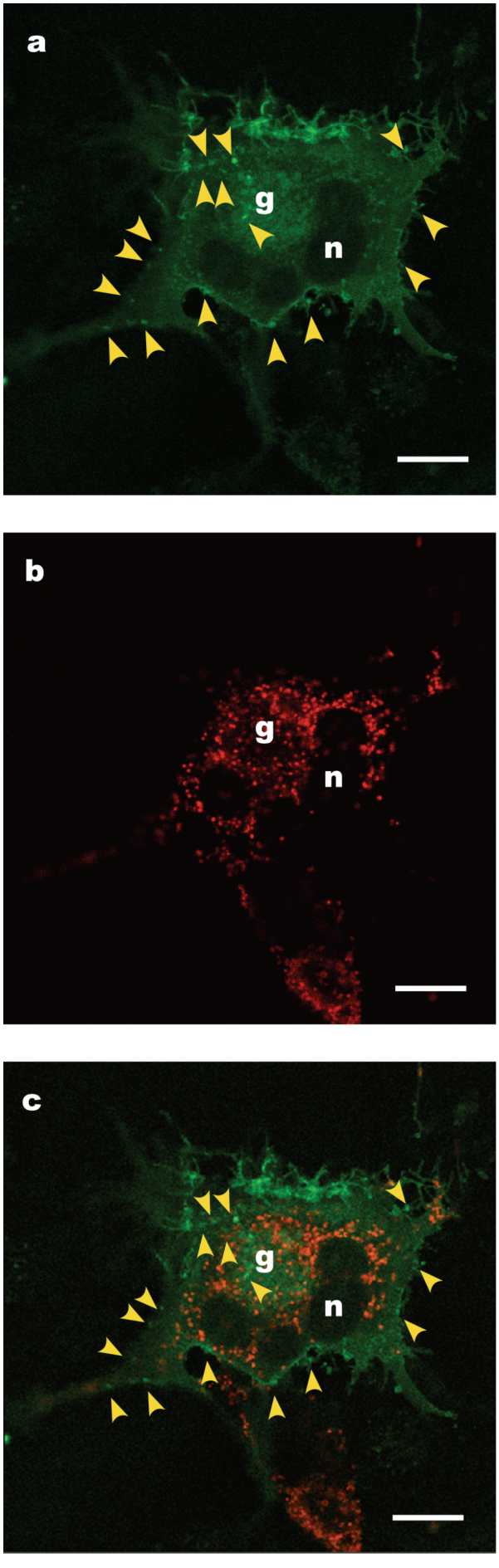
a
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Figure 5: Confocal analysis of NPY and DP4 in transiently transfected COS-1 cells. Live cell image of Cos-1 cell transfected with (A) DP4-GFP-C1 and (B) NPY-dsRed1-N1 48 hours post-transfection. (C) Overlay image of DP4-GFP-C1 and NPY-dsRed1-N1. 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.

Protection against obesity in speed congenic DA rats lacking dipeptidyl peptidase 4 is associated with behavioral and immune alterations

Short running title: Phenotype of congenic DP4 deficient DA rats

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Abstract

Objective. Treatment of diabetes type 2 using chronic pharmacological inhibition of dipeptidyl peptidase 4 (DP4) still requires an in-depth analysis of models for chronic DP4 deficiency, because adverse reactions induced by some DP4 inhibitors were recently described.

Research Design and Methods. In the present study, a novel congenic rat model of DP4 deficiency on a "DP4-high" DA rat genetic background was generated (DA.F344-*Dpp4*^{m}/SvH rats) and comprehensively phenotyped by a combination of different neurobehavioral, endocrine, hematological, metabolic, and immune assays.

Results. Similar to chronic pharmacological inhibition of DP4, DP4 deficient rats exhibited a phenotype involving reduced diet-induced body weight gain and improved glucose tolerance associated with increased levels of GLP-1 and bound leptin as well as decreased aminotransferases and triglycerides. Additionally, DA.F344-*Dpp4*^{m}/SvH rats showed anxiolytic-like and reduced stress-like responses, a phenomenon presently not targeted by DP4 inhibitors. Whereas all these aspects in the phenotype of this novel model of DP4 deficiency may be considered as "beneficial", several immune alterations such as differential leukocyte subset composition (eosinophils, NK cells, B cells) at baseline, blunted NK cell and T cell functions, and altered cytokine levels were observed, which may be problematic if appearing under conditions of chronic pharmacological inhibition of DP4.

Conclusions. While this animal model confirms a critical role of DP4 in GLP-1-dependent glucose regulation, genetically induced chronic DP4 deficiency apparently also affects stress-regulatory and immune-regulatory systems, indicating that the use of chronic DP4 inhibitors might have the potential to interfere with CNS and immune functions *in vivo*.

3.1. Introduction

Inhibition of the serine protease dipeptidyl peptidase 4 (DP4) reduces the N-terminal cleavage of dipeptides of the insulinotropic peptide-hormone glucagon-like peptide-1 (GLP-1) and opens new alternatives for the treatment of diabetes type 2 (1). Within the clinical trials performed, these compounds have been demonstrated to be safe and efficacious. However, clinical trials including phase III have certain limitations and sometimes, adverse reactions are only observed after market introduction. As DP4 is not specific for insulinotropic hormones, having also modulating effects on a broad range of other substrates, unwanted effects cannot be excluded at this stage. In line with this assumption, recently two compounds were put on hold in phase III, in one case for undisclosed toxicity issues and in the other case due to adverse skin reactions (www.novartis.com/downloads_new/investors/2006.11.13%20Galvus%20US%20NDA%20Review.pdf). Obviously, further preclinical research on potential adverse effects caused by chronic inhibition of DP4 is needed.

We therefore generated a novel congenic DP4 deficient animal model on a defined Dark Agouti (DA) rat genetic background with pronounced differences in DP4 activity between congenic and wild type animals and studied the effects of this genetically induced DP4 deficiency in a comprehensive phenotyping approach, following the general rules and modifications for rats as previously described (2; 3). For the development of congenic DA.F344-*Dpp4*^{m}/SvH rats, the previously characterized spontaneous point mutation in the *Dpp4* gene of the F344 rat substrains (F344/DuCrjSvH-*Dpp4*^{m} and F344/Crl(Wiga)SvH-*Dpp4*^{m}) (2; 4-7) was used. This strategy resulted in DP4 deficient animals, being comparable to *Dpp4*^{tm1Nwa} mice (CD26 knockout mice) (8), but providing the advantages of the species *Rattus norvegicus*, being in metabolism, toxicology, and neurobiology more comparable to humans (9-12). DP4 is ubiquitously expressed on leukocytes, epithelia and endothelia of most vertebrate tissues and is involved in T cell activation and cell adhesion processes as well as in the degradation of hormones, chemokines and neuropeptides. Known substrates for DP4 are for example substance P (SP), neuropeptide Y (NPY), peptide YY, growth-hormone-releasing-factor, GLP-1, enterostatin or β-casomorphins (13). Relevant substrates in immune reactions are for example chemokines (eotaxin, RANTES) (14; 15). These facts strongly suggest that new animal models of DP4 deficiency and their comprehensive phenotyping are necessary, as DP4 activity modulates numerous psychological and physiological processes affecting nervous, endocrine, and immune functions. A priori, no prediction of the resulting effects can be made thereof.

Here, we characterized this new DP4 deficient congenic DA strain and report on a reduced body weight gain, both under standard rat chow and high-calorie diet, on improved glucose tolerance being associated with increased GLP-1 and bound leptin levels as well as decreased aminotransferases and triglycerides. While these findings basically represent the targets of pharmacotherapy using DP4 inhibitors, in addition, a reduction in stress-hormone levels (ACTH and corticosterone) of the HPA axis associated with anxiolytic-like responses in several behavioral assays were observed, which also can be considered beneficial. The probably most important findings of this screen were blunted immune functions of natural killer (NK) and T cells, altered interleukin-6 (IL-6) and interleukin-10 (IL-10) levels and differential leukocyte subset compositions, thus pointing to potential adverse effects as a result of a chronic blockade of DP4. We therefore propose to use this mosaic of observations to more closely monitor patients, who are presently receiving gliptins (16) as a novel therapy inducing chronic DP4 inhibition.

3.2 Research Design and Methods

Animals

DA/Ztm and DA.F344-*Dpp4*^{m}/SvH rats were housed and bred at the Central Animal Facility of the Hannover Medical School (Ztm) under conditions as described previously (4). All research and animal care procedures were approved by the Review Board for the Care of Animal Subjects of the district government, Hannover, Germany, and performed according to international guidelines for the use of laboratory animals.

Development of congenic animals

Development of the congenic strain was started with an initial cross between F344/Crl(Wiga)SvH-*Dpp4*^{m} females, homozygous for the loss-of-function-mutation in the *Dpp4* gene on RNO3 (4) and a DA/Ztm wild type male rat, to fix the Y chromosome of the DA background. Male F1 rats were then backcrossed to DA/Ztm females. Heterozygosity of the *Dpp4* locus of the resulting N2 males was tested via (a) DP4 expression on T cells using Fluorescene activated cell sorting (FACS) analysis of T cell receptor (mAb R73) and DP4 (mAb Ox61) immunopositive events and via (b) *Dpp4* genotyping by means of two gene specific microsatellite markers. D3cd26-7 (forward: GGAACTGTTGAATTAGCTCTCTGC; reverse: CTCTGGACTGCCATCTCCTACTTC) is localized within *Dpp4* and D3cd26-10 (forward: GCAATCTGGCGCAGAGTAATTAC; reverse: GTCATCTGTCTCCGCTCCCAT) is closely linked to *Dpp4* on RNO3. The genetic background of N2 DA.F344-*Dpp4*^{m}/SvH males, heterozygous for both *Dpp4* alleles, was genotyped using 100 polymorphic microsatellite markers with an intermarker distance of about 20 cM covering all chromosomes. The N2 male with the highest proportion of DA background was selected for the next cross. This scheme was used at each generation until N5. A N5 male and a N5 female, homozygous for the DA background, were then intermated to produce DA.F344-*Dpp4*^{m}/SvH founders. The DP4 deficient congenic DA strain is maintained via brother × sister mating. We used several age and sex matched sets of DA.F344-*Dpp4*^{m}/SvH N5F2 rats for the experiments.

Experimental sets of animals

To avoid major influences from the high number of different test paradigms applied to the animals on the one hand and to confirm certain test results on the other hand, several independent sets of age-matched male DA rats – DA/Ztm and DA.F344-*Dpp4*^{m}/SvH – were used for the behavioral studies following rules as previously described (2).

Spontaneous feeding behavior, gliadin feeding, high fat diet and body weight gain

For studying feeding behavior on standard diet (Altromin Standard diet 1320: Altromin GmbH, Lage, Germany), the animals were housed either in or kept singly in a cage and the observation periods lasted 72 h each (2). For examination of body weight gain under high calorie diet animals were fed with a high calorie diet (Altromin, 157p/c1057) and weighed once a week. Furthermore, the calorie up-take was determined. For analysis of body weight gain under gliadin-rich diet – providing insight into the metabolism with respect to DP4 activity (17) – DP4 deficient and wild type DA rats were fed a modified rat diet (modified Altromin Standard Diet 1320) for a three week period whereas control rats were fed with non-modified standard chow. The modified diet contained gliadin (20%) as single protein source and is high in proline compounds. Starting at an age of six weeks, animals were weighed at least once a week routinely or as experiments required.

Oral glucose tolerance test (OGTT) and determination of DP4-like enzymatic activity

Male DA.F344-*Dpp4*^{m}/SvH and DA/Ztm animals (21 ± 0.5 weeks of age) were used for these experiments being repeated at least three times. Additionally, some animals heterogeneous for the mutant *Dpp4* gene were included – coded DA.F344-*Dpp4*^{m}/SvH$^{(-/+)}$. Following an overnight fast (12 h) 1 h after the onset of the light phase, animals' basal blood glucose levels were determined (see below). If the glucose concentration was < 7.8 mmol/l (< 140 mg/dl), the animals were shortly anesthetized with ether and glucose (2.5 g glucose/kg animal) was orally given via a feeding tube. Blood samples (10 μl) were collected from the tail vein of conscious rats at 30, 60, 90, and 120 min following the oral glucose load and the glucose level was measured by a glucometer (Bayer, Leverkusen, Germany) using criteria as previously described (4). For determination of DP4 enzymatic activity, EDTA-plasma samples from experimentally naïve animals were kept at -80°C until being assayed using a microplate based chromogenic assay. The release of 4-nitroaniline (pNA) from the substrate glycyl-prolyl-4-nitroaniline (Gly-Pro-pNA) was monitored at 405 nm and 37°C using the PowerWave^{XS} Universal Microplate Spectrophotometer (BioTek Instruments, Bad Friedrichshall, Germany). The assay is selective for DP4-like activities, however, due to the alkaline pH it neglects the contribution by dipeptidyl peptidase 2. One unit is defined as the amount enzyme necessary to hydrolyze 1 μmol substrate (18).

Insulin, GLP-1, bound/free leptin

Insulin levels were determined using an Insulin-RIA-Kit (Biotrend, Chemikalien GmbH, Köln) according to the manufacturers instructions. Plasma samples were taken 15, 30, 45 min after oral glucose challenge under the condition described in the glucose tolerance test, respectively. For determination of active GLP-1(7-36) via ELISA kit (Linco; Glucagon Like Peptide-1 (Active)) EDTA-plasma samples were conditioned with 10^{-5} M of isoleucyl-thiazolidide and handled according to the manufacturer's instructions. Plasma samples were taken 30 min after oral glucose challenge. Bound leptin was measured using a specific radioimmunoassay (RIA) developed at the Department of Gastroenterology, Hepatology and Endocrinology, Hannover Medical School (19), while total leptin was measured using a RIA from Linco (Linco St. Charles, Missouri, USA).

Clinical chemistry

The auto-analyzer ADVIA 1650 (Siemens Medical Solutions Diagnostics GmbH, formerly: Bayer Vital GmbH), installed and validated at Clinical Pathology, Institute of Toxicology, Merck KGaA, Germany was used to determine various electrolytes and enzymes in 300-500 μl serum per animal derived from retro orbital blood samples. The data were generated according to standardized procedures and valid methods including internal controls (Duotrol Normal Lot No 5066, and Duotrol Abnormal Lot No 5077, BIOMED) resulting in scientifically reliable data.

Histology of pancreas, liver, and hypothalamus

Pancreas and liver samples were collected from either paraformaldehyde (PFA) fixed animals or HOPE fixation of samples was used. HOPE fixiation was conducted as recently described (20). Samples were transferred either into paraffin (HOPE) or kept frozen in cryoprotectant (PFA), sectioned (liver: 3-4 μm; pancreas: 6-7 μm; brain: 15 μm) and stained. Detection of free leptin (Ob-A20; Santa Cruz Biotechnology Inc., Santa Cruz, CA; 1:500 overnight at 4°C) in liver was conducted as previously described (21). Pancreatic beta cells were detected by insulin immunostaining with a polyclonal guinea-pig antibody (Insulin A 565, dil. 1:4000; DAKO, Hamburg, Germany). For double labeling of hypothalamus, animals were perfused using 4% PFA, brains removed, postfixed (12 h), cryoprotected (sucrose solution) and then cut coronally in a cryostat at 40 μm thickness. Free floating sections were processed for immunohistochemistry using a combination of the avidin-biotin-peroxidase complex (ABC) and the alkaline phosphatase-anti-alkaline phosphatase (APAAP) methods (7) by incubating

overnight at 4°C with rabbit anti-human/ratNPY polyclonal antibody (1:4000; affinity purified; Biotrend, Köln, Germany; # NA1233, Batch Z02052) and anti-rat DP4/CD26 monoclonal antibody (mAb) (clone 5E8, 1:500, Cell Science Inc., Canton, MA 02021). The dilutions of the antisera were made with Tris HCL containing 0.3% Triton X-100, and 5% normal goat serum. For ABC staining, the sections were first incubated for 1 h at RT with the biotinylated secondary anti-rabbit antibody (1:200 in 5% normal goat serum and 0.3% Triton X-100) and subsequently exposed to ABC complex (1:100; ABC Kit elite, Vector Labs, Burlingam, CA). After visualization of the APAAP reaction by Fast Blue reaction product (22), the reaction product of the ABC stain was visualized with 0.1% DAB. Control sections were included, in which one or both primary antibodies were omitted. Sections from the level of the hypothalamus, arcuate nucleus, and median eminence were evaluated using microscope.

General health and neurological examination

Gross abnormalities that would interfere with further behavioral testing such as general health, sensory abilities, and neurological reflexes were controlled and compared between the DA congenic and DA wild type rats as described previously for mice and rats (2; 3).

Determination of motor functions (Accelerod test) and circadian activity (Home cage activity)

An Ugo Basile accelerating rotarod (model 7750) for rats, supplied by Technical & Scientific Equipment GmbH (TSE GmbH), Bad Homburg, Germany, was used and training as well as experiments were conducted as previously described (11). For monitoring home cage activity, an infrared sensor controlled recording system (model No. E61-01/08; Coulbourn Instruments, Allentown, PA, USA) was used as previously described (23). This test is based on infrared detection of number and time of movements and it is useful to screen for differences in circadian rhythm. Activity peaks are defined as “small” and “large movements”, indicating when activity/movements took place within a duration being shorter or longer than 3 s, respectively. As a representative readout, the “time spent in large movements per time interval” was chosen as an indicator for circadian activity pattern.

Evaluation of stress-induced hyperthermia, anxiety and exploratory behavior

For determination of stress-induced hyperthermia, body temperature was repeatedly determined before and after a brief stressor (transport stress) according to Kask and colleagues (24). The elevated plus maze (EPM), social interaction (SI), and the holeboard assay were used to evaluate anxiety-like and exploratory-like behaviors, respectively. An EPM apparatus (TSE

GmbH) (23), a SI (24), and a self-made holeboard (23) were validated and used as previously described.

Two-way active avoidance shuttle box learning and test of prepulse inhibition (PPI)

Shuttle box conditioning was used as a test of associative learning and was conducted using a TSE Shuttle box system (TSE-Systems GmbH) following the protocol as previously described (11). PPI of a startle response is the phenomenon, in which a weak prepulse suppresses the response to a startling stimulus. Deficits in prepulse inhibition are common in schizophrenic patients. An automated startle system (TSE GmbH) was used as previously described (2).

Determination of corticosterone, ACTH, IL-6 and IL-10 levels

Corticosterone (AA-13F1, Lot 37170; IDS, Boldon, UK) levels were detected in EDTA-plasma samples via RIA (obtained via IBL, Hamburg, Germany). Detection was conducted in duplicates according to the manufacturer's guidelines. For corticosterone, the calculated sensitivity was 0.39 ng/ml and the intra- and interassay coefficients of variation were 5 and 9%, respectively. ACTH, IL-6, and IL-10 were measured by means of ELISA using previously described standard techniques for hormones and cytokines according to Straub et al. (25).

Quantification of NK cell cytotoxicity in spleens of DA substrains

NK cytotoxicity was measured in classical ^{51}Cr-release assays using splenocytes and YAC-1 target cells, which were derived from standard cell culture conditions, as previously described (22). The specific cytotoxicity was calculated by means of the following formula: [(experimental release) – (spontaneous release)] / [(maximal release) – (spontaneous release)] × 100. In addition, the percentage of $CD3^{-}CD161^{+}$ NK cells in each spleen was determined by FACS analysis as previously described (7; 26) and lytic units (LU) were calculated according to the method of Bryant et al. (27). Since mononuclear cells were used as effector cells, LU were further mathematically adjusted to NK cell numbers present in the respective assays by forming the quotient LU/NK cells (%).

Haematology

Two validated Bayer Diagnostics automated hematology systems (ADVIA 120) including species-specific software settings were used. EDTA blood samples (200 μl) were collected by retrobulbar venipuncture and analyzed using standard methods and controls.

FACS analysis

Leukocytes were counted using a Coulter counter and then further processed for flow cytometry using three colour stainings as previously described (26) with the following details being modified. Briefly, about 1×10^6 cells per well were incubated with mouse anti-rat mAb for 20 min at 4°C using the following marker for granulocytes (FSC/SSC/mAb HIS48), monocytes (mAb ED9/mAb W3/25), B lymphocytes (mAb OX12), $CD4^+$ T cells and $CD8^+$ T cells (mAb R73/mAb W3/25/mAb OX8), and NK^{bright} cells (mAb 10/78). Dendritic cells were defined using the mAbs OX62 and OX6. All antibodies were purchased from serotec (Germany).

T cell proliferation assay

Rat peripheral blood mononuclear cells (PBMC) were isolated from fresh, arterial EDTA blood via centrifugation on Ficoll gradient (Ficoll Paque ^{TM}Plus, Amersham, Sweden). PBMC number and viability was determined by cell counting using trypan blue staining. Cells were washed and the proliferation assays were conducted in 96 well flat-bottom plates. Therefore, 2×10^5 cells per well were cultured in the presence of 0.2 μg αβ TCR antibody (plate bound). Cells stimulated with 1 μg Concanavalin A served as positive controls. After 5 days of incubation the proliferation rate was quantified by BrdU incorporation and detected with a specific colorimetric BrdU Cell proliferation ELISA (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturers instructions. BrdU incorporation was measured using an ELISA reader (PowerWave^{XS} Universal Microplate Spectrophotometer; BioTek Instruments, Inc., Winooski, U.S.A.) at 370 nm and 492 nm as reference wavelengths. Results were expressed as absorbance rates (A_{370nm}-A_{492nm}).

Statistical analysis

Analysis of the various behavioral and physiological data was assessed either by applying repeated measures analysis of variance (ANOVA) on successive measurements or by one-way ANOVA. In repeated measures ANOVA the nominal independent variable “substrain” was used as the “between factors” and different continuous response variables within successive measurements were used as the “within-factors” (e.g. body weight over time). In case of significant differences in regard to the “between factor” or significant interactions of “between” and “within-factors”, this was followed by one-way ANOVAs (factor: “substrain”); split by the dimension of the continuous response variable (e.g. split by the different time-points of body weight determination). One-way ANOVAs were followed by the Fisher-PLSD-

test for post hoc comparison to evaluate pair wise differences among levels of main effects. The "between-subject effects" from ANOVAs are presented within the text in the results section by providing the degrees of freedom (for the "between factor" and for the "within subject error"), *F*-values, and *p*-values, while in figures and tables the *p*-values of the results obtained by the corresponding post hoc tests are provided, if appropriate. Differences were regarded as statistically significant if *p* was below .05. The number of animals per substrain (*n*) was at least 10. Presenting the *degrees of freedom* indicates exceptions from this. Significant post hoc effects versus the control animals of the DA/Ztm substrain are indicated by asterisks (*$p < .05$; **$p < .01$; ***$p < .001$). All data are presented as means ± standard error of the mean (SEM).

3.3. Results

DP4 deficiency reduces body weight, protects from high-fat diet induced obesity, improves glucose tolerance, increases GLP-1 and leptin, and lowers aminotransferases as well as triglycerides. Reduction of DP4 enzymatic activity became a widely accepted target that has been proven beneficial in the treatment of type 2 diabetes. Consequently, we studied parameters related to glucose metabolisms and body weight homeostasis in the DA.F344-*Dpp4*^{m}/SvH rats. Figures 1 and 2 illustrate these findings and clearly demonstrate various levels of beneficial effects in a genetically induced status of DP4 deficiency. While the genetic background of male DA wild type rats is characterized by high levels of DP4-like activity, DP4 deficient DA.F344-*Dpp4*^{m}/SvH rats exhibited negligible low, and heterozygous DA.F344-*Dpp4*^{m}/SvH$^{(+/-)}$ rats intermediate DP4-like activity ($F(2, 86) = 876.5$, $p < 0.0001$; Fig. 1A). Similar findings were observed in female animals ((-/-) 1.88±0.1 [mU/ml]; (+/+): 36.1±1.2 [mU/ml]; (+/-) 15.3±0.9 [mU/ml]). In support of DP4 being the key regulator in glucose homeostasis, testing glucose tolerance in the OGTT revealed a significantly improved response towards glucose load in DP4 deficient rats ($F(2, 20) = 10.04$, $p = 0.001$; Fig. 1B), being comparable to pharmacologically induced DP4 deficiency in mice (28; 29). Interestingly, heterozygous animals exhibited an intermediate phenotype, clearly illustrating an association between “gene dosis”, DP4 activity, and glucose tolerance.

Furthermore, these findings were associated with significantly elevated levels of active GLP-1(7-36) ($F(1, 6) = 8.8$, $p = 0.02$; Fig. 1C) in DA.F344-*Dpp4*^{m}/SvH rats being detectable at 30 min after oral glucose challenge. In addition, a trend toward elevated insulin levels (mean over three measurements at 15, 30, and 45 min: 0.4 ng/ml in DA.F344-*Dpp4*^{m}/SvH vs. 0.3 ng/ml in wild type; Fig. 1D) was found. Similar to *Dpp4*^{tm1Nwa} mice, interestingly, ß-islet size was reduced in the DA.F344-*Dpp4*^{m}/SvH rats (Fig. 1E), potentially suggesting an increase of insulin storage.

Screening of body weight gain (Fig. 1F) revealed a significantly reduced increase in DP4 deficient animals ($F(1,19) = 12.1$, $p < 0.001$), which parallels gain in body weight of wild type animals until about one year of age, when these differences became more apparent. Also, high-calorie diet induced weight-gain was significantly blunted in animals being about one year of age (Fig. 1F; small insert), although calorie up-take did not differ. Apart from this, no other obvious differences in general health, reflexes and sensory abilities were observed at any time. Furthermore, no significant differences for food or water consumption were observed at the age of 3, 6, and 9 months (data not shown).

Beneficial effects of DP4 deficiency in glucose metabolism (30; 31) may also be reflected in lipid metabolism (1,13). Actually, our findings provide evidences that DP4 deficiency facilitates leptin signaling (32; 33). This notion is reflected by histological evaluation of free leptin in liver tissue that illustrates increased levels in wild type animals (Fig. 2A) and by high levels of bound leptin in DA.F344-*Dpp4*^{m}/SvH rats ($F(1, 6) = 5.6$, $p = 0.04$; Fig. 2B). Levels of total leptin were not significantly altered in plasma (data not shown). As leptin is expressed predominantly by adipocytes, which represents to some extend the total mass of fat in the body, we also measured triglyceride levels (TG) and found a reduction in DA.F344-*Dpp4*^{m}/SvH rats ($F(1, 17) = 20.09$, $p < 0.001$). This is probably also sufficient to explain the observed statistical significant but albeit minor decreases in amino transferases (alanine aminotransferases (ALAT), ($F(1, 17) = 15.3$, $p = 0.001$), aspartate aminotransferases (ASAT), ($F(1, 17) = 7.08$, $p = 0.01$)) and alkaline phosphatase (AP), ($F(1, 17) = 8$, $p = 0.01$) (Fig. 2C). Table 1 illustrates that no other differences were found in parameters such as electrolytes except for minor increases of urea and inorganic phosphate in DA.F344-*Dpp4*^{m}/SvH animals. Thus, the DP4 deficient congenic rats also exhibit improved liver metabolism, which further hints to beneficial metabolic situation in DP4 deficient animals.

Having these findings on glucose and lipid metabolism along with corresponding GLP-1/leptin signaling in mind, it remained open to investigate the potential interaction of DP4 with proteins (e.g. gliadin, and proline-rich proteins) and protein metabolism especially in the intestine, as DP4 is highly expressed in the ileum and jejunum. In particular, the role of DP4 in the small intestine and the kidney is dipeptide re-absorption after cleavage of proline containing peptides and oligopeptides (17; 34; 35). This gives rise of problems that might occur as a possible consequence of chronic treatment using long acting DP4 inhibitors in diabetes type 2. Notably, it has already been shown that gliadin-based diet being rich of proline (17; 36) causes malabsorption of such proteins. Consequently, wild type and DA.F344-*Dpp4*^{m}/SvH rats received a modified gliadin-rich and otherwise non-modified diet. As expected, results (Fig. 2D) of three factorial ANOVA for repeated measures revealed full interaction of the between-subject factors "genotype", "diet composition", and the within subject-factor "delta body weight" in DP4 deficient rats showing a significant body weight loss ($F(4, 80) = 7.1$, $p < 0.0001$), and illustrating an impaired ability of protein utilization. Overall, these findings already confirmed that this new rat model of DP4 deficiency exhibit many if not all of the key features being targeted by DP4 inhibitor treatment. Nonetheless, as many of the substrates of DP4 (NPY, SP, endomorphin, etc) play a significant role in the central nervous system (CNS)

and peripheral nervous system (PNS), we then characterized the behavioral phenotype and the stress-response of these animals in-depth.

Multi-tired behavioral analysis of DP4 deficient DA.F344-*Dpp4*^{m}/SvH rats reveals a stress protective and anxiolytic-like phenotype. Multi-tired behavioral phenotyping compromises repeated tests for various behavioral domains being additionally complemented by tests of neurological reflexes and sensory abilities. Every behavioral domain (e.g. motorfunction, anxiety, cognition, etc.) should be screened repeatedly and confirmed by at least two different tests challenging similar adaptational responses (2; 3). In the case of genetically and pharmacologically induced DP4 deficiency, we have already shown that DP4 deficient F344 rats exhibit a phenotype of reduced stress-responsiveness and anxiety (2; 5). In the current study, we characterized a new animal model of DP4 deficiency (congenic DA.F344-*Dpp4*^{m}/SvH rats) that - in contrast to the previously used DP4 deficient F344 rat substrains - reveals a homogeneous genetic background and that exhibits higher DP4-enzymatic activity levels in wild type DA/Ztm rats. A priori, this difference should also cause a more pronounced anxiolytic and stress-protective like phenotype.

Results show that under home-cage conditions there are no differences in diurnal activity (Fig. 3A; $p > 0.05$ n.s.). Furthermore, testing of motor functions on the rotarod and of startle-response along with prepulse inhibition (PPI) (tested e.g. in repeated accelerod and PPI tests, data not shown), cognitive performance in associative learning task of the two way active avoidance shuttle box paradigm (Fig. 3B; $p > 0.05$ n.s.), as well as pain perception under habituated conditions (data not shown) revealed no differences between wild type DA/Ztm and DA.F344-*Dpp4*^{m}/SvH rats. In contrast, DP4 deficient congenic animals responded to all tests related to stress and anxiety in a very different way. Namely, stress-induced rise of body temperature (stress-induced hyperthermia, Fig. 3C) was significantly blunted ($F(1, 18) = 8.5$; $p = 0.009$), illustrating a reduced response to stress, probably mediated by decreased SNS activity (24). In support of this, reduced levels of ACTH (Fig. 3D) and corticosterone (Fig. 3E) were detected in plasma from DP4 deficient rats, thus underlining that the endocrine stress response corresponded to the behavioral phenotype. Furthermore, analysis of the social interaction test of anxiety revealed an increased total social interaction time for DP4 deficient DA.F344-*Dpp4*^{m}/SvH rats ($F(1,23) = 25.5$, $p < 0.0001$; Fig. 3F) being indicative for anxiolysis. Similarly, using the EPM test, DA.F344-*Dpp4*^{m}/SvH rats show higher values for percent of open arm entries ($F(1,14) = 4.5$, $p = 0.04$; Fig. 3G) and for percent of time spent in open arms ($F(1,14) = 4.9$, $p = 0.04$; data not shown). Concerning motor activity in this test (number of closed and total arm entries), no significant differences were found (data not shown).

Investigation of explorative behavior and locomotor activity in the holeboard test revealed that DP4 deficient DA.F344-*Dpp4*^{m}/SvH rats made significantly more head dips than DA/Ztm animals ($F(1,16) = 6.9$, $p = 0.02$; Fig. 3H). Additionally, DA.F344-*Dpp4*^{m}/SvH rats spent significantly more time in the center of the board ($F(1,16) = 5.3$, $p = 0.03$; data not shown). Again, no differences in motor activity - measured by the distance covered by entering different squares – were found (data not shown). Double immunohistochemistry of DP4 (blue APAAP staining) and NPY (dark brown vesicular precipitate) in the hypothalamus (section at the level of bregma -3.12 mm according to Paxinos and Watson, 2007; Fig. 3I) illustrates that at least NPY in the arcuate hypothalamic nucleus (ARC) as a anxiolytic- and stress-protective-like acting substrate of DP4, is expressed in the close vicinity of the corresponding DP4 enzyme. The latter is expressed in blood vessels of the MEE (blue staining, arrow heads) of DA wild type (Fig. 3I) but not in DA.F344-*Dpp4*^{m}/SvH rats (Fig. 3I, insert in upper right corner; lack of blue staining, arrows).

Differential immune cell distribution, blunted immune cell function and altered cytokine levels as consequence of DP4 deficiency in DA.F344SvH*Dpp4* rats. To complete the comprehensive phenotyping, we screened for immunological parameters that might be affected by DP4 deficiency (at experimentally naïve conditions or after *in vitro* stimulation of T and NK cells) and that possibly might indicate where adverse reactions of chronic DP4 inhibitor treatment may occur.

As DP4 deficiency as well as inhibitor treatment have been shown to affect hematopoiesis and the behavior of bone marrow stem cells, e.g. via modulation of the chemokine, stromal cell derived factor-1 (SDF-1/CXCL12) (37), we first screened hematological parameters. The hemogram showed significantly reduced absolute cell numbers in DA.F344-*Dpp4*^{m}/SvH rats for lymphocytes ($F(1, 18) = 5.5$, $p = 0.03$) and eosinophils ($F(1, 18) = 5.02$, $p = 0.04$) (Fig. 4A), which may demonstrate specific changes at local chemokine action at e.g. the level of bone marrow and thymus. In the case of eosinophils for example, we have recently shown regulatory effects of DP4 for the recruitment of eosinophils *in vivo* via prolonged action of the CCR3 ligand CCL11/eotaxin (Forssmann et al., 2007, unpublished), which may also explain reduced levels of eosinophils at baseline in this study. In addition, determination of blood leukocyte subsets by FACS analysis (Fig. 4B) revealed a significant increase of NK cells ($F(1, 7) = 11.06$, $p = 0.007$), B cells ($F(1, 7) = 6.2$, $p = 0.03$) and CD5 positive B1-like cells ($F(1, 7) = 8.6$, $p = 0.03$) in DP4 deficient rats. In previous studies we have shown that especially these leukocyte subpopulations are mobilized by NPY infusions (38), suggesting that apart from altered local chemokine metabolism also DP4 mediated N-terminal truncation of the

noradrenergic co-transmitter NPY at the local level of sympathetic innervated immune organs such as the spleen might be involved in this phenomenon. A similar mechanism may also lead to a NPY potentiated noradrenalin-mediated increase of the splenic IL-6 outflow (25) (Fig. 4G). Table 2 demonstrates that granulocytes, monocytes, dendritic cells and $CD4^+$ and $CD8^+$ T cells were not significantly affected. Surprisingly, similar to the blood pool, also the percentage of NK cells in the spleen was significantly increased in DA.F344-*Dpp4*^{m}/SvH rats – a finding which also has been observed in *Dpp4*^{tm1Nwa} mice (39) – suggesting that an overall increase of NK cells in these animals is evident. The determination of lytic units ($LU_{20}/10^7$, Fig. 4C) revealed that in comparison to DA/Ztm rats, an increased number of effector cells is needed in DA.F344-*Dpp4*^{m}/SvH rats to lyse 20% of the target cells. Due to the above mentioned higher percentage of NK cells in the spleen of DA.F344-*Dpp4*^{m}/SvH rats ($F(1, 6) = 24.7$; $p = 0.003$; Fig. 3D) the ratio of LU to percent of NK cells reveals a significantly reduced cytotoxicity per NK cell of DP4 deficient DA rats ($F(1, 6) = 6.1$, $p < 0.05$; Fig. 4E). Thus, the absolute capacity of a single NK cell to lyse tumor targets is reduced in DA.F344-*Dpp4*^{m}/SvH rats.

Besides differences in NK cell numbers and cytotoxicity, also a significantly blunted T cell proliferative response was observed. In comparison to NK cells, a priori it was more likely to find differences in this lymphocyte subpopulation, as T cell functions might be affected at very different regulatory levels including but not limited to antigen presenting cell (APC)-T cell interaction, T cell costimulation, and memory function (40), TGF-ß signaling (41; 42), T memory cell to regulatory T cell (Treg) switch, or chemokine metabolisms (14; 15). Here we found in DP4 deficient animals a five-fold reduced proliferation rate upon stimulation with anti-αβ-TCR mab ($F(1, 5) = 32.9$, $p = 0.001$ Fig. 4F). Furthermore, IL-6 levels are significantly reduced in DA.F344-*Dpp4*^{m}/SvH rats ($F(1, 7) = 7.6$, $p = 0.02$), whereas no significant differences were found in the determination of IL-10 (Fig. 4G).

3.4. Discussion

The recent introduction of sitagliptin (Januvia(R)) to the market as well as the fact that vildagliptin (Galvus(R)) has put on hold by the FDA both challenge an in-depth analysis of potential effects of chronic DP4-inhibition, other than improved glucose homeostasis (16). A priori, all studies investigating a status of long lasting DP4 deficiency in experimental animals, either achieved pharmacologically or genetically, are complementary to each other and relevant at this point. Here, we decided to take the advantage of the genetically induced chronic DP4 deficiency in the rat, and generated a novel DP4 deficient congenic model in order to facilitate an in-depth characterization of potential effects aside from the enteroinsular axis and to make use of the advantages of the species *Rattus norvegicus* e.g. with regard to behavioral alterations. Consequently, a comprehensive phenotyping approach was conducted, which not only was focused on endocrine but also on neurobehavioral and immune alterations as all these might result in side effects during chronic treatment of diabetes.

In the current study, we report that a comprehensive phenotyping of neurobehavioral, endocrine, hematological, metabolic, and immune parameters in DA.F344-*Dpp4*^{m}/SvH rats reveals DP4 dependent changes on at least three levels: (a) beneficial effects on the enteroinsular axis, glucose homeostasis, and body weight regulation not only via GLP-1 but also via leptin and liver dependent processes, (b) potent anxiolytic-like and stress-protective like effects, and (c) considerable changes in immune cell distribution and NK cell and T cell functions. While the metabolic effects largely cover the therapeutic targets of current drug development, the behavioral changes may represent a novel field of application for DP4 inhibitors, whereas the immune changes probably point to an area, where most likely unwanted effects might appear. Thus, the current results highly suggest tight supervision of immunological parameters in patients currently receiving DP4 inhibitors for the treatment of diabetes to detect any potential side-effects.

More specifically, the improved metabolic status of this novel DA.F344-*Dpp4*^{m}/SvH rat model largely reflects findings in *Dpp4*^{tm1Nwa} mice (8; 31), DP4 deficient F344 rats (2; 4) and in rats and mice receiving chronic DP4 inhibitor treatment (28; 29; 43), strongly supporting this concept for lowering blood glucose levels. In addition, our study reveals that the DP4 deficient phenotype of DA rats is characterized by reduced body weight gain as well as resistance to high calorie diet induced obesity suggesting that inhibition of DP4 may also be used for treatment of obesity without manifest diabetes type 2. Lower body weight may be a result from increased levels of bound leptin, but this is in contrast to an unaltered food intake, which was

observed in our study as well as in DP4 deficient F344 rats (2) and KO mice (31), which together hints to effects mediated either via higher metabolic rates or malabsorption. In this study, no differences in home cage activity were observed, thus, excluding that differential physical activity levels are responsible for the lower body weight in free feeding DA.F344-*Dpp4*^{m}/SvH rats. Although no differences in baseline body temperature were found in DA rats, leptin is reported to increase energy expenditure (44; 45) which might have contributed to differences in body weight. Interestingly, malabsortion of gliadin leads to a reduction of body weight (17) (Fig. 2D). Although, the latter represents an artificial model being unlikely to be observed in patients receiving DP4 inhibitors, the consequences resulting from reduced DP4 activity are non-negligible. Thus, malabsorption of other proline-rich diets as well as breast-feeding of children by mothers receiving DP4 inhibitors in conjunction with malabsorption of β-casomorphin, may both lead to loss of body weight or failure to thrive. In the latter case, a connection between DP4 activity and β-casomorphin in atopic dermatitis and potentially other immune disorders was suggested (46).

While the changes in body weight regulation and glucose homeostasis mentioned above, strongly support current drug developmental strategies, importantly, the stress-protective and anxiolytic-like phenotype observed in the present study as well as in our previous reports in DP4 deficient F344 rats (2; 5) may represent a novel target for drug development. We demonstrate here that DA.F344-*Dpp4*^{m}/SvH rats additionally show increased exploratory behaviors and reduced stress-like as well as anxiolytic-like behavioral responses that were also reflected on the endocrine level. While more frequent visits on open arms of the EPM and of social encounters in novel environment represent classical behavioral indicators of reduced anxiety in rodents (3) reduced corticosterone, ACTH, and stress-induced hyperthermia indicate that also the hypothalamic-pituitary-adrenal axis as well as the sympathetic response to stress are blunted. Our leading hypothesis is that DP4 deficiency in mutant F344 rats (2; 5) as well as congenic DA.F344-*Dpp4*^{m}/SvH rats prolongs the half-life of endogenous $NPY_{(1-36)}$, which binds with high affinity the NPY Y_1 receptor. This receptor is mainly responsible for NPY mediated anxiolysis and stress-protection (47; 48). N-terminal cleavage of NPY by DP4, leading to $NPY_{(3-36)}$ results in a much lower Y_1 receptor affinity by this truncated peptide, thereby abrogating anxiolytic-like action of NPY. To this end, the interaction of NPY and DP4 takes place at the level of the hypothalamus, where high expression of NPY and DP4 in blood vessels is apparent (Fig. 3I) but other limbic areas and Y_1 receptor expression sites may be involved as well. Last but not least, this concept of DP4 mediated modulation of NPY Y_1 receptor mediated may also account for the peripheral action of NPY in the PNS and immune

system as for example suggested by the increased levels of IL-6 (25). Concerning the CNS effects, presently, it remains open whether current DP4 inhibitors on the market will cross the blood-brain-barrier at a significant level thereby allowing interaction of CNS neuropeptide-substrates and DP4. Furthermore, little distinctive anxiolytic-like and stress-protective effects may have not been discovered in phase 2 and phase 3 studies, even when considering that such effects only become apparent after stress.

Whereas the "beneficial" metabolic as well as the appreciable anxiolytic/stress-protective-like actions, induced by DP4 deficiency, together represent a fascinating perspective for the application of DP4 inhibitors, probably, problems will appear in the area of immune regulation. Thus, already the present rat model exhibits several immune alterations at baseline or non-challenged conditions, which consist in differential leukocyte subset composition (eosinophils, NK cells, B cells), blunted NK cell and T cell functions, and altered cytokine levels. The most likely mechanisms for this plethora of effects probably have to be searched in different regulatory loops affected by DP4 deficiency. These are changes in T cell co-stimulation (40) and in chemokine metabolism (15), obviously also affecting hematopoetic stem cells (49). With regard to an altered chemokine metabolism it should be noted that at least several CCR3 ligands (eotaxin, RANTES), as well as the CXCR4 ligand CXCL12 (SDF-1; stromal derived factor 1) and all CXCR3 ligands all represent substrates of DP4. While in healthy, non-challenged conditions, i.e. phase 1 studies or genetically deficient animal models, modulatory effects induced by a prolonged half-life of these mediators may only be weakly expressed (i.e. alterations in blood and spleen leukocyte pools, as observed for eosinophils, B cells, and NK cells in the present study), at infectious or allergic states these processes might become crucial in patients (6; 50). As such conditions are not tested during phase 3 studies, their relevance for human patients remains open and probably will become overt during the introduction of DP4 inhibitor into clinics. Similarly, a modulated CD26 dependent T cell co-stimulation via caveolin-1 mediated, APC-dependent upregulation of CD86, the principle ligand of CD28, or CD26-mediated co-stimulation via intracellular signaling via Carma-1 (40) may represent another problem, which becomes relevant after introduction of CD26-inhibitors into clinics. Namely, the response to recall-antigens on T cell as well as B cell levels may be blunted (40). Last but not least, since also tumor cell adhesion (7) and – as shown here – NK cell distribution/function are modulated by DP4, it cannot be excluded at this stage that also NK dependent responses such as defenses against viral infections and tumors are modulated under chronic DP4 inhibition.

In conclusion, the present comprehensive characterization of DA.F344-*Dpp4*^{m}/SvH rats reveals a phenotype being composed of at least three major dimensions: (1) improvements in glucose and lipid metabolism being associated with a caveat of malabsorption of proline-rich diets, (2) surprising and very promising anxiolytic/stress-protective like effects, which need to be explored with regard to their clinical applicability, and (3) critical immune changes at baseline as well as after *in vitro* challenge, which hint to this area as the major impact regarding potential side-effects. Based on this plethora of findings in our novel model of DP4 deficiency, more specific DP4 inhibitors (i.e. organ specific) and more specific pharmacodynamics (i.e. short lasting compounds), appear to be necessary.

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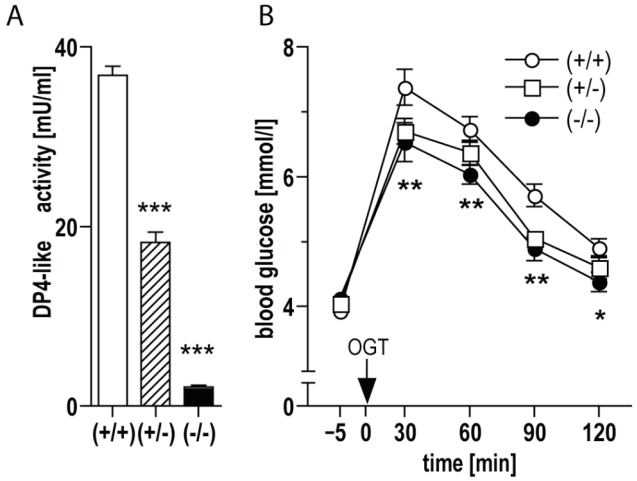
3.7. Tables and figures

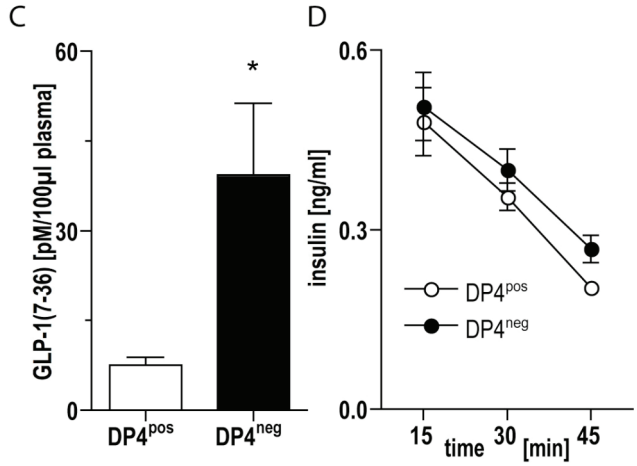
Table 1: Clinical chemistry in DP4 deficient DA rats

Parameter in serum	**DA/Ztm**	**DA.F344-*Dpp4^{m}*/SvH**
Inorganic posphate [mmol/l]	1.2 ± 0.0	1.4 ± 0.1*
Urea [mmol/l]	4.9 ± 0.1	5.7 ± 0.2**
Natrium [mmol/l]	142.1 ± 0.3	141.5 ± 1.3
Potassium [mmol/l]	3.5 ± 0.1	3.6 ± 0.2
Calcium [mmol/l]	2.5 ± 0.0	2.5 ± 0.0
Chloride [mmol/l]	100.8 ± 0.6	100.4 ± 1.0
Glucose [mmol/l]	9.2 ± 0.3	9.04 ± 0.4
Creatine [μmol/l]	37.2 ± 1.3	35.7 ± 1.4
Total bilirubin [μmol/l]	1.8 ± 0.1	1.8 ± 0.1
Cholesterol [mmol/l]	2.6 ± 0.1	2.5 ± 0.1
Total protein [g/l]	66.0 ± 0.5	65.03 ± 0.9
Albumin [g/l]	40.3 ± 0.4	39.8 ± 0.5

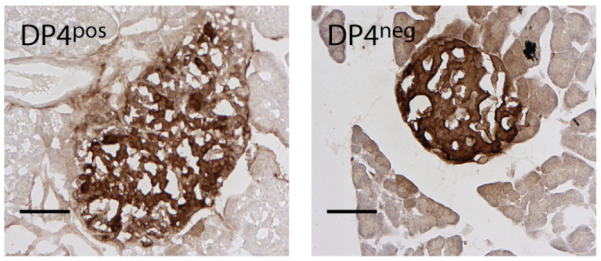
Table 2: Leukocyte subsets in DP4 deficient DA rats

	DA/Ztm	**DA.F344*Dpp4*^{m}/SvH**
Leukocytes [n x 10^3]	7170 ± 411	7284 ± 179
% of abs. leukocytes		
Mononuclear cells	86.22 ± 1.2	86.76 ± 1.3
Granulocytes	13.78 ± 1.3	13.24 ± 1.3
IgM^{pos} B cells	18.98 ± 1.1	21.96 ± 1.1
IgM^{pos} CD5^{pos} B cells	0.96 ± 0.2	1.20 ± 0.2
DC (MHC-II^{pos} αE integrine^{pos})	0.64 ± 0.1	0.62 ± 0.1
Monocytes (CD4^{pos} CD172a^{pos})	6.68 ± 0.5	7.20 ± 0.5
NK (CD161^{pos})	3.26 ± 0.2	4.28 ± 0.3
T cells (α/β TCR^{pos})	46.82 ± 3.1	43.86 ± 4.9
CD4^{pos} T cells	35.27 ± 1.9	33.25 ± 3.4
CD8^{pos} T cells	11.74 ± 0.6	11.04 ± 0.5





E Pancreatic islets: insulin stain



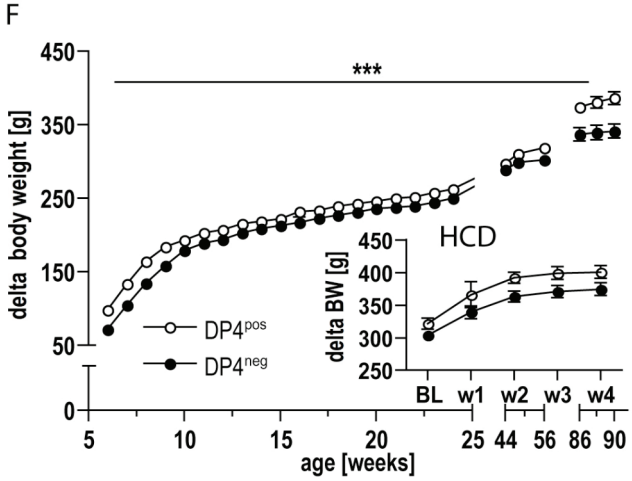


Figure 1: Glucose homeostasis and body weight related parameters in DP4 deficient (DP4^{neg}) DA.F344-*Dpp4*^{m}/SvH and wild type (DP4^{pos}) DA/Ztm rats: DP4-like activity in DA/Ztm (+/+), DA.F344-*Dpp4*^{m}/SvH (-/-) and additionally heterozygous DA animals (+/-) (A). Glucose tolerance 30-120 min following oral glucose challenge (OGC; indicated by arrow) in DA/Ztm (+/+), DA.F344-*Dpp4*^{m}/SvH (-/-) as well as DA congenics that are heterozygous for DP4 (+/-). Significant effects, indicated by asterisks, only refer to DA.F344-*Dpp4*^{m}/SvH vs DA/Ztm wildtype (B). GLP-1(7-36) levelsin DP4^{pos} and DP4^{neg} rats 30 min post oral glucose challenge (C). Insulin levels in DP4^{pos} and DP4^{neg} rats 15, 30, and 45 min post oral glucose challenge (D). Insulin staining of pancreatic islets in DP4^{pos} and DP4^{neg} rats. A representative of the maximal islets size shown in the beta cell area is illustrated, scale bar = 100 μm (E). Body weight of the different substrains between 6 and 90 weeks of age; bottom: body weight gain under high calorie diet (HCD), BL, baseline, w1-w4, week 1-4 of HCD (F). Data represent means ± SEM. Significant group difference derived from post-hoc analysis are indicated by asterisks (*p < .05; **p < .0 1; ***p < .0 01 vs DA/Ztm wild type).

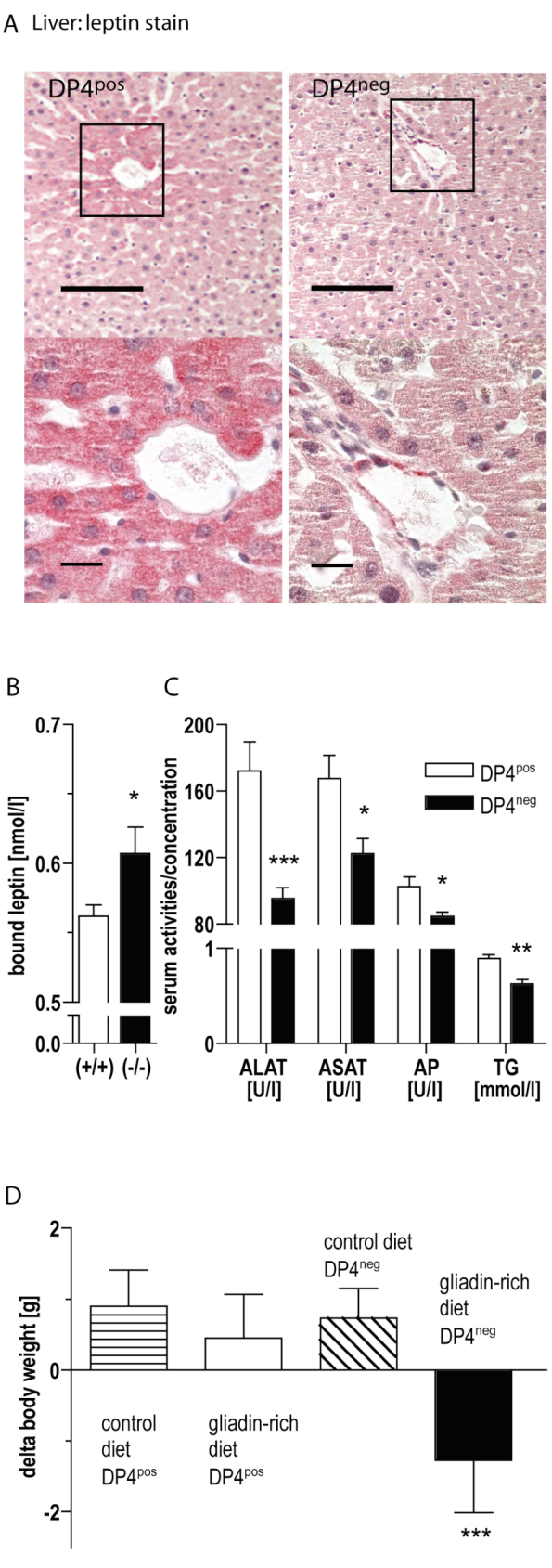

A Liver: leptin stain
DP4pos
DP4neg
B
bound leptin [nmol/l]
0.7
0.6
0.5
0.0
*
(+/+)
(-/-)
C
serum activities/concentration
200
160
120
80
1
0
DP4pos
DP4neg
***
*
*
**
ALAT [U/l]
ASAT [U/l]
AP [U/l]
TG [mmol/l]
D
delta body weight [g]
2
0
-2
control diet DP4neg
gliadin-rich diet DP4neg
control diet DP4pos
gliadin-rich diet DP4pos
***

Figure 2: Metabolic changes, and clinical chemistry in DP4 deficient (DP4^{neg}) DA.F344-*Dpp4^{m}*/SvH and wild type (DP4^{pos}) DA/Ztm rats: Immunohistological detection of free leptin in hepatocytes in DP4^{pos} and DP4^{neg} rats, scale bar = 100 μm. Strong immunoreactivity is represented by intensity of cytosolic red colour (A). Bound leptin levelsDP4^{pos} and DP4^{neg} rats (B). Clinical chemistry findings on alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT) and alkaline phosphatase (AP) [U/l], and triglycerides (TG) [mmol/l] in serum samples (C). Impact of gliadin-rich, modified food on weight gain. Animals were fed with modified and non-modified food over a three-week period (D). Data represent means ± SEM. Significant group difference derived from post-hoc analysis are indicated by asterisks (*p < .05; **p < .0 1; ***p < .0 01 vs DA/Ztm wild type).

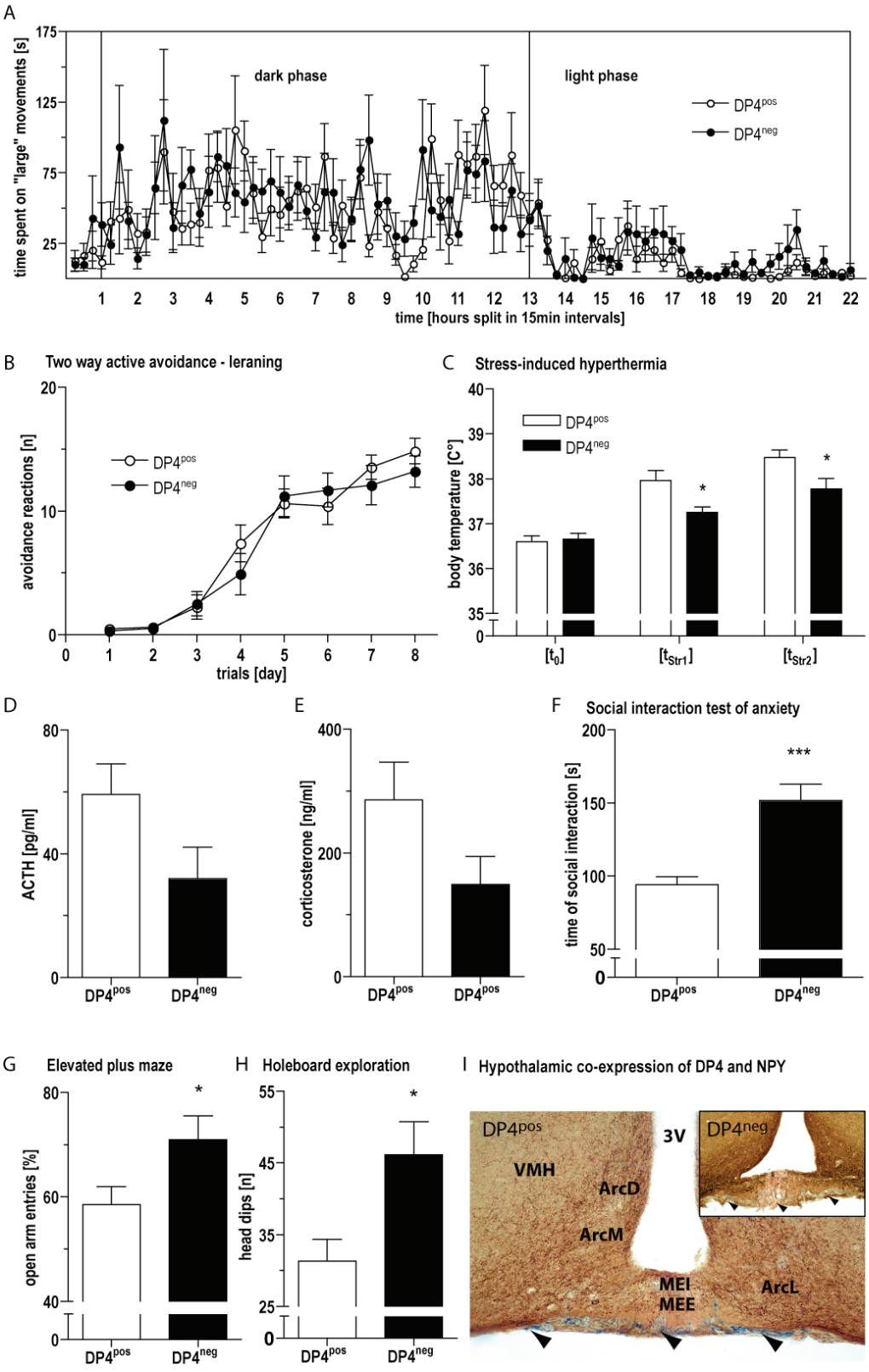


Fig. 3: Stress-protective and anxiolytic-like phenotype in DP4 deficient congenic DA rats: Diurnal home cage activity pattern. Activity of DA/Ztm (DP4^{pos}) and DA.F344-*Dpp4*^{m}/SvH (DP4^{neg}) rats was recorded over a period of three days. The diagram represents activity during a 22 h recording interval and displays the time spent in large movements (movements longer

than 3 s) being collapsed into sums 15 min total activity (A). Associative learning across 8 days in the two-way active avoidance shuttle box paradigm (B). Stress-induced hyperthermia. The rectal temperature was determined in non-stressed, experimentally naïve rats (t_0) as well as at two time points after a brief stressor (t_{Str1} and t_{Str2}) (C). in $DP4^{pos}$ and $DP4^{neg}$ rats (D). in $DP4^{pos}$ and $DP4^{neg}$ rats (E). Time spent in active social interaction as parameter for anxiety-like behaviors in the social interaction test; pairs of non-familiar either $DP4^{pos}$ or $DP4^{neg}$ rats were exposed to an open field 1h after onset of dark phase for 10 min and sum of time of their active "sociopositive" behaviors was recorded (F). Anxiety-like behaviors in the EPM test are reflected by the percentage of open arm entries. Animals were tested in the elevated plus maze 1 h after onset of dark phase (G). Exploratory behavior in the holeboard test. The number of head dips was recorded during a 10 min session (H). Hypothalamic co-expression of DP4 (blue) and NPY (brown vesicular staining) expression sites (I). Arrow heads indicate blue staining for DP4 in blood vessels the eminentia mediana, external layer, in $DP4^{pos}$ rats, while no such immunoreactivity was seen in $DP4^{neg}$ rats (arrows in the small insert, upper right of I); VMH = ventromedial hypothalamic nucleus, ArcD = arcuate hypothalamic nucleus, dorsal part, ArcM = arcuate hypothalamic nucleus, medial part, ArcL = arcuate hypothalamic nucleus, lateral part, V3 = 3rd ventricle, MEE = medial eminence, external layer, MEI = medial eminence, internal layer (I). Data represent means ± SEM. Significant effects are indicated by asterisks (*$p < .05$; ***$p < .0\ 01$ vs DA/Ztm wild type).

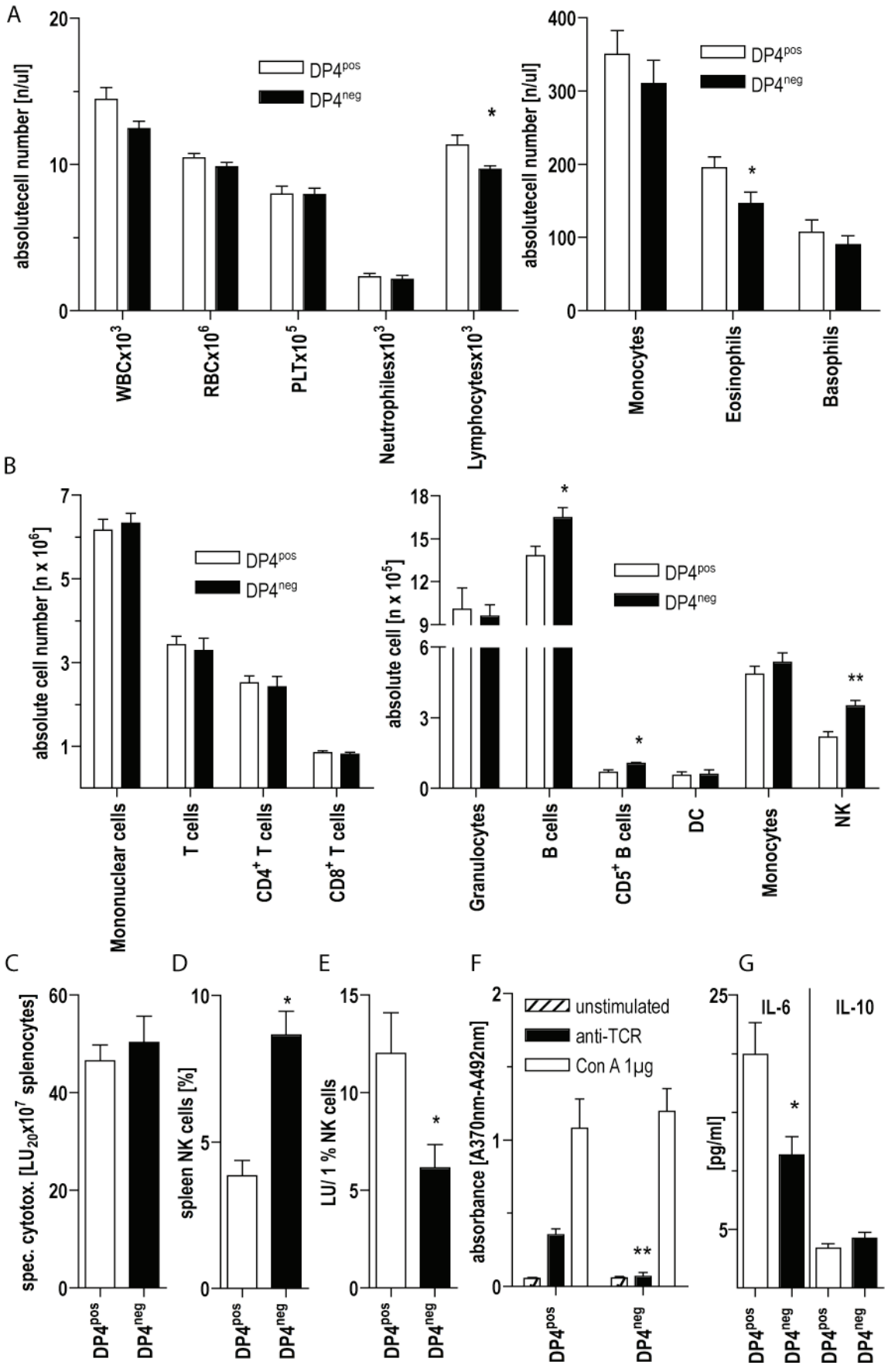

A
absolutecell number [n/ul]
DP4pos
DP4neg
WBCx10^3
RBCx10^6
PLTx10^5
Neutrophilesx10^3
Lymphocytesx10^3
Monocytes
Eosinophils
Basophils
B
absolute cell number [n x 10^6]
Mononuclear cells
T cells
CD4+ T cells
CD8+ T cells
absolute cell [n x 10^5]
Granulocytes
B cells
CD5+ B cells
DC
Monocytes
NK
C
spec. cytotox. [LU20x10^7 splenocytes]
D
spleen NK cells [%]
E
LU/ 1 % NK cells
F
absorbance [A370nm-A492nm]
unstimulated
anti-TCR
Con A 1µg
G
IL-6
IL-10
[pg/ml]

Figure 4: Immunological differences in DA congenics lacking DP4 activity (DA.F344-*Dpp4*^{m}/SvH) and in DA wild type (DA/Ztm) rats: Differential blood cell distribution of DP4^{pos} and DP4^{neg} rats; white blood cells (WBC), red blood cells (RBC), platelets (PLT) (A, left and right). Differential leukocyte subsets determined via FACS (B, left and right). Specific cytotoxity (spec. cytotox.) against and YAC-1 target cells (C). Splenic NK cells in % (D). LU per 1 % NK cells (E). T cell proliferation was assayed in PBMCs from DP4^{pos} and DP4^{neg} rats (F). Cytokine levels of IL-6 and IL-10 (G). Data represent means ± SEM. Significant effects made are indicated by asterisks (*p < .05; **p < .0 1 vs DA/Ztm wild type).

Loss of stress-induced analgesia in rat models of dipeptidyl peptidase 4 deficiency: Evidence for mediation via stress-protective effects of NPY

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Abstract

Dipeptidyl-peptidase IV (DP4) is involved in several physiological functions, some of which are dependent on DP4's ability to N-terminally truncate dipeptides of the neurotransmitter neuropeptide Y (NPY_{1-36}). The remaining C-terminal fragment NPY_{3-36} has a relatively lower affinity for the NPY receptor subtype Y_1. The Y_1 receptor is involved in stress-protective, anxiolytic-like and analgetic-like effects of NPY. In a genetic study, we tested stress-induced analgesia SIA in two spontaneously mutated DP4 deficient F344 rat substrains and in a DP4 deficient congenic DA rat strain. We also investigated whether intracerebroventricular (i.c.v.) administration of NPY (vehicle, 0.2, or 1.0 nmol) or the DP4 inhibitor isoleucyl-thiazolidide (*Ile-Thia*) (vehicle, 0.5, or 5.0 nmol) modulates the pain threshold in DP4 deficient F344 rat substrains compared to wild type-like F344 control animals. All three animal models for DP4 deficiency exhibited a blunted stress-dependent increase of the nociceptive threshold in the non-habituated hot plate paradigm illustrating a process of reduced SIA. This difference was lost after habituation to the hot plate paradigm. Centrally administered NPY decreased pain sensitivity of DP4 deficient F344 substrains in the non-habituated and habituated hot plate, whereas pharmacological inhibition of DP4 resulted in an increased pain threshold in wild type-like animals, which was similar to DP4 deficient animals. Immunohistology of the hypothalamus revealed expression of DP4 in the close vicinity of NPY positive neurons of the paraventricular nucleus. The study demonstrates that stress-induced analgesia depends on DP4 expression and suggests a mechanism via N-terminal truncation of NPY, subsequently resulting in reduced Y_1 receptor-like tone and associate loss of stress-protective/anxiolytic-like effects.

4.1. Introduction

The enzyme and binding protein dipeptidyl peptidase 4 (DP4; CD26) belongs to the class of membrane-associated peptidases (De Meester et al., 1999). Due to its unique ability to liberate *Xaa*-Pro and *Xaa*-Ala dipeptides from the N-terminus of regulatory peptides, substrates for DP4 include neuropeptides such as neuropeptide Y (NPY), endomorphin, and substance P (De Meester et al., 2000; Hildebrandt et al., 2000; Mentlein, 1999). These have been shown to exert analgetic-like effects, with NPY undergoing the highest relative cleavage rate and thus being a highly affine substrate (Bjelke et al., 2006; Mentlein, 1999).

We have previously investigated the role of loss of DP4 enzymatic activity on behavioural and physiological processes in two mutant F344 rat models for DP4 deficiency (i.e. F344/DuCrjSvH-*Dpp4*^{m} and F344/Crl(Wiga)SvH-*Dpp4*^{m}) – for details see Karl et al. (Karl et al., 2003a; Karl et al., 2003b; Karl et al., 2003c). In a systematical behavioural and physiological characterization, we observed reduced anxiety-like behaviours and an associated reduction in stress-induced analgesia in both DP4 deficient F344 rat substrains compared to wild type-like F344 rats (Karl et al., 2003c). Namely, we observed that under non-habituated conditions of the hot-plate assays, both DP4 deficient substrains exhibited a reduced threshold in showing aversive responses. This phenomenon was lost after habituation to the potentially stressful context of the hot plate assay (Karl et al., 2003c), thus being strongly suggestive for a status of blunted stress-induced analgesia under a condition of genetic DP4 deficiency and for a stress-protective effect of this deficiency.

NPY affects anxiety and nociception (Kask et al., 2002; Naveilhan et al., 2001; Wettstein et al., 1995) and an involvement of the Y_1 receptor subtype has been documented. Importantly, the described anxiolytic-like phenotype of the mutant substrains – giving rise to their reduced stress-induced analgesia (Wolf et al., 2007) – is very likely associated with differential degradation and utilization of NPY, strongly suggesting that a more potent NPY Y_1 receptor-like tone in these rats causes this behavioural effect (Karl et al., 2003b). However, the direct effect of NPY on nociceptive responses remains controversial: some studies describe an analgetic effect of exogenous NPY (Hua et al., 1991; Merlo Pich et al., 1990) and an involvement of the Y_1 receptor (Gibbs et al., 2004; Gibbs et al., 2006), others discuss a nociception-increasing effect (Broqua et al., 1996; von Horsten et al., 1998) or did not find any effect of NPY on pain threshold (Heilig et al., 1993; Jolicoeur et al., 1991).

As NPY represents one of the best substrates for DP4 (Bjelke et al., 2006), we considered here increased levels of this endogenous mediator first as being responsible for the stress-protective-

like phenotype of DP4 deficient F344 rats during their response to novelty (i.e. the non-habituated hot plate assay) and for the associated modulation of central pain processing (i.e. the blunted stress-induced analgesia).

In a first step we wanted to exclude any impact of reported variations in the genetic background of the F344 substrains (Karl et al., 2003a) on the nociceptive phenotype. Thus, we generated a DP4 deficient congenic strain on the Dark Agouti (DA) rat genetic background. For this, we transferred the mutant *Dpp4* allele of the F344/Crl(Wiga)SvH-*Dpp4*^{m} substrain onto a DA/Ztm background and tested these animals for SIA in the hot plate paradigm. In a second step, we characterized the phenomenon of SIA in mutant F344 substrains (F344/DuCrjSvH-*Dpp4*^{m}, F344/Crl(Wiga)SvH-*Dpp4*^{m}). We also investigated, whether identical doses of intracerebroventricular (i.c.v.) administered NPY in control and mutant F344 rats would lead to a differential nociceptive response in the hot plate design. Finally, we analysed whether pharmacological inhibition of DP4 (i.c.v. treatment with isoleucyl-thiazolidide) has a similar effect on pain sensitivity of wild type-like control F344 rats as caused by the genetic depletion of DP4 in the two mutant F344 substrains. This series of experiments was completed by immunohistological studies seeking for expression of NPY and DP4 in brain areas involved in the modulation of stress.

4.2. Materials and methods

Animals

F344 substrains obtained in 1998 from breeding colonies of Charles River Laboratories (Crl) in Sulzfeld, Germany (Thompson et al., 1991), and Atsugi, Japan (Tiruppathi et al., 1993; Watanabe et al., 1987), almost completely lack DP4-like activity and protein expression. This DP4 deficiency cannot be detected in wild type-like F344 rats from Crl breeding colonies in Portage, USA or Hannover (Ztm), Germany (Karl et al., 2003a). In our previous reports (Karl et al., 2003a; Karl et al., 2003b; Karl et al., 2003c) F344 rat substrains derived from breeding colonies of CR in Atsugi, Japan were named F344/DuCrj(DPPIV-), animals from breeding colonies in Sulzfeld, Germany, F344/Crl(Ger/DPPIV-), and wild type-like rats obtained from colonies in Portage, USA, F344/Crl(Por). Since we have further inbred these mutant lines for more than 10 generations, and since some of them are not commercially available from CR anymore, we code them nowadays F344/DuCrjSvH-*Dpp4*^{m} for the Japanese and F344/Crl(Wiga)SvH-*Dpp4*^{m} for the German DP4 deficient line as well as F344/Crl(USA) for the wild type-like control rats, respectively.

All adult, male test animals (F344/DuCrjSvH-*Dpp4*^{m}, F344/Crl(Wiga)SvH-*Dpp4*^{m}, F344/Crl(USA), DA.F344-*Dpp4*^{m}/SvH and DA/Ztm) were housed and bred at the Central Animal Facility of the Medical School Hannover. Animals were maintained in a separated minimal barrier sustained facility and kept in macrolon type III cages with standard bedding (Altromin GmbH, Lage, Germany). Food (Altromin Standard diet 1320: Altromin GmbH, Lage, Germany) and water were available *ad libitum*. Environmental temperature was automatically regulated at 21 ± 1°C and relative humidity was 60% with an air change rate of 15 times per hour. The animal rooms were operated with a positive pressure of 0.6 Pa. Rats were maintained under a 12:12 h light regime. They underwent routine cage maintenance once a week. Routine microbiologic monitoring according to FELASA recommendations (Rehbinder et al., 2000) did not reveal any evidence of infection with common murine pathogens except for *Pasteurella pneumotropica* and *Staphylococcus aureus*. All research and animal care procedures were approved by the Review Board for the Care of Animal Subjects of the district government, Hannover, Germany, and performed according to international guidelines for the use of laboratory animals.

Generation of congenic DP4 deficient DA strain

Development of the congenic strain was started with an initial cross between F344/Crl(Wiga)SvH-*Dpp4*^{m} females, bearing the loss-of-function-mutation in the *Dpp4* gene and male DA/Ztm rats. The DA rat strain was selected as a recipient strain due to its high endogenous DP4-like enzymatic activity, ensuring that congenics on DA background exhibit pronounced differences compared to the wild type-like strain (Karl et al., 2003c). Male F1 rats were then backcrossed to DA females. Heterozygosity of the *Dpp4* locus of N2 males was tested by analysing DP4 expression on T-cells using FACS analysis of T cell receptor (mAb R73) and DP4 (mAb OX61) immunopositive events and by genotyping the *Dpp4* using two informative microsatellite markers (D3cd26-7 and D3cd26-7).

The genetic background of heterozygous N2 DA.F344/Crl(Wiga)SvH-*Dpp4*^{m} males were genotyped using informative microsatellite markers with an intermarker distance of about 20 cM spanning the whole genome/chromosomes. The N2 male with the highest proportion of DA background was selected for the next cross. This scheme was used at each generation until N5. A N5 male and a N5 female, homozygous for the DA background, were then mated to produce DA.F344-*Dpp4*^{m}/SvH founders. The DP4 deficient congenic DA strain is maintained through brother x sister mating. We used DA.F344-*Dpp4*^{m}/SvH N5F2 rats for the experiments (DA/Ztm rats used as controls).

Determination of DP4-like enzymatic activity

All test animals were characterized for their DP4-like enzymatic activity as described previously (Karl et al., 2003a). For determination of plasma activity of F344 and congenic rats a microplate based chromogenic assay was used. EDTA-plasma samples were kept at –80°C until use. DP4 enzyme activity of the different rat substrains was determined by monitoring the release of 7-amino-4-methylcoumarin (AMC) from the substrate Gly-Pro-AMC at 360/480 nm (Ex/Em) and 30°C using the Novostar fluorescence microplate reader (BMG, Offenburg, Germany). The assay consists of 20 μl plasma sample, 100 μl H_2O and 100 μl HEPES buffer pH 7.6 and 50 μl Gly-Pro-AMC. Activity was calculated from the linear slope using a factor of 3.116×10^{-4} μmol/l calculated from an AMC standard curve and the sample dilution. One unit is defined as the enzyme activity, which cleaves 1 μmol Gly-Pro-AMC per minute. The assay is selective for DP4-like activities, however, due to the alkaline pH it neglects the contribution by dipeptidyl peptidase 2. Importantly, the chromophores are not released by other proline-specific peptidases, such as prolidase, prolyl endopeptidase or aminopeptidase P.

Surgery (I.c.v. cannulation)

For surgery F344 rats were anaesthetized with an intramuscular (i.m.) mix of ketamine hydrochloride (0.1 ml/100 g body weight; Albrecht, Aulendorf, Germany) and dormitor (0.01 ml/100 g body weight; Pfizer GmbH, Karlsruhe, Germany). The i.c.v. cannulation technique was identical to a previous report (von Horsten et al., 1998). After placement of the rat in a Kopf stereotactic apparatus (Model 900: David Kopf Instruments, Tujunga, USA), the incisor bar was adjusted on position zero and the ear bars were adjusted to equal positions so that the rat's head was fixed in the apparatus. The eyes were protected against drying with eye-salve (Bepanthen Augen- und Nasensalbe: Hoffmann-La Roche AG, Grenzach-Wyhlen, Germany). The skull was exposed by a midline incision, the periost was removed, the bone surface was dried, and the position of the bregma was identified. Three stainless steel anchor screws (Breitfeld & Schliekert, Karben, Germany) were secured to the skull and a stainless steel guide cannula (Plastics one, Roanoke, USA) was implanted in the right lateral ventricle and cemented in place with dental cement (Durelon Maxicap: Espe Dental AG, Seefeld, Germany). The coordinates for the lateral ventricle were 0.7 mm caudal and 1.6 mm lateral to the bregma, with the guide cannula (Plastics one) extending 3.4 mm ventral to the skull surface.

Flow of small amounts of 0.9% saline (Braun Melsungen AG, Melsungen, Germany) through the protracted injection (internal) cannula (Plastics one, Roanoke, Germany) was used to verify that the guide cannula was positioned just above the ventricular system. The guide cannula was fitted with a dummy cannula (Plastics one, Roanoke, USA) of the same length to prevent leakage of cerebrospinal fluid. Animals were housed individually after surgery. The anatomical position of the cannulation was verified by post mortem i.c.v. dye application (Berlin blue) and inspection of third ventricular staining in randomly chosen rats. The animals of the three F344 substrains F344/DuCrjSvH-*Dpp4*^{m}, F344/Crl(Wiga)SvH-*Dpp4*^{m} and F344/Crl(USA) were operated at the age of 95 (± 5) days. After a recovery phase of 10 days we commenced observation of the animals' behaviour in the hot plate task.

Drug dosages and i.c.v. application procedure

A stock solution of human/rat NPY (2 mol; Polypeptide GmbH, Wolfenbüttel, Germany) was adjusted under sterile conditions to final concentrations (0.2 nmol/5 µl and 1.0 nmol/5 µl) using 0.9% saline. The final concentrations were made 24 h before testing. Animals were habituated to experimental i.c.v. injections daily for seven days prior to the start of the first experiment. For i.c.v. administration, animals were taken out of the home cage and the dummy

cannula was replaced by the internal cannula. Peptide or 0.9% saline were injected i.c.v. at a volume of 5 µl over 20 s through the internal cannula extending 4.4 mm ventral to the skull surface. The internal cannula was attached to a microsyringe (Hamilton Bonaduz AG, Bonaduz, Switzerland) with approximately 30 cm of polyethylene tubing (Plastics one, Roanoke, USA), which allowed the animal to move freely during the i.c.v. injection. Before the rat was placed back into the home cage the dummy cannula was re-attached. Experiments started 15 min after administration. During the habituation phase the handling procedure was exactly the same but no compound was administered. Two different doses of NPY were used for this study and a 3 (substrain) × 3 (treatment) experimental design was set up. Thus, F344 rats of each substrain were subdivided into three treatment groups each, which were treated with either 0.9% saline (vehicle: 0.0 nmol/5 µl), 0.2 nmol/5 µl or 1.0 nmol/5 µl NPY. Seven days later animals were treated either with 0.9% saline (vehicle: 0.0 nmol/5 µl), 0.5 nmol/5 µl or 5.0 nmol/5 µl of the DP4 inhibitor isoleucyl-thiazolidide (*Ile-Thia*: Probiodrug AG, Halle, Germany).

Nociception (Hot plate)

A 30 x 30 cm hot plate analgesia meter (Columbus Instruments, Columbus, USA) was used for this experiment, which was carried out during the light phase of the light cycle. The experiment was performed as previously described (Karl et al., 2003c). The rat was placed on the surface of the apparatus, which was maintained at 52.5°C. The latency to respond (*lick* or *raise a hindpaw*) was recorded. To prevent any damage to the animals, rats were removed from the hot plate if they did not respond within 20 s (Naveilhan et al., 2001; von Horsten et al., 1998). F344/DuCrjSvH-*Dpp4*^{m}, F344/Crl(Wiga)SvH-*Dpp4*^{m} and F344/Crl(USA) rats' nociceptive response was tested 15 min after treatment with different dosages of either NPY (or saline) or *Ile-Thia* (or saline - seven days after NPY treatment). The recently generated DA.F344-*Dpp4*^{m}/SvH rats and their DA/Ztm controls were tested twice for nociception: 1) non-habituated (day 1) and 2) seven days later after being habituated to the test apparatus (habituation on the inactivated hot plate for 1 min per day on days two, four, and six).

Double immunoflourescence of hypothalamic immunoreactivity for NPY and DP4

Animals were perfused as previously described (Kask et al., 2001). 30 mm coronal cryosections of the rat hypothalamus that were defined by the anatomical atlas were cut on a cryostat (Paxinos and Watson, 1989). All sections were collected in 0.1 M PBS and processed after the free-floating method. After initial washing steps, sections were placed in a pre-incubation solution containing 10 % NDS, 0.3% Triton X-100 in 0.1 M PBS (1 h, RT).

Sections were incubated with the anti-rat DPPIV/CD26 monoclonal antibody (clone 5E8, 1:500, Cell Science Inc., Canton, MA 02021) for 48 h at 4^0C on a horizontal shaker. Subsequently, sections were washed and incubated with an anti-mouse-Cyanin 2 conjugated antibody (1:1500, diluted in 0.1M PBS) for 45 min. Finally, sections were washed extensively in 0.1 M PBS and further processing were performed in the dark. For double immunoflourescence staining, sections were incubated at 4°C with rabbit anti-human/ratNPY polyclonal antibody (1:200; affinity purified; Biotrend, Köln, Germany; # NA1233, Batch Z02052). Following subsequent washes, sections were labeled using Cyanin-3-coupled anti-rabbit antibody (1:1500, 2 hr, RT), diluted in 0.1 N PBS. In some cases, DAPI nuclear staining was applied according to standard procedures. Control sections were included, in which one or both primary antibodies were omitted. All sections were analyzed using a Nikon light microscope (Eclipse 80i; Nikon, Tokyo, Japan), Nikon objectives (Plan Apo, VC 2×, NA = 1.0; Plan Apo VC 40×, NA = 1.1), motorized specimen stage for automatic sampling (Märzhäuser, Wetzlar, Germany), electronic microcator (Heidenhain, Traunreut, Germany), a dedicated Nikon HiSN fluorescence system, a Nikon cooled DS-5Mc camera, and imaging software (Stereo Investigator, MicroBrightField, Williston, VT).

Statistical analysis

The behavioural data were analysed using a two-way analysis of variance (ANOVA; factor: "strain" × "treatment") and/or one-way ANOVA (factor: "strain" or "treatment" - split by the corresponding factor) followed by the Fisher-PLSD-test for posthoc comparison, if appropriate. Differences were regarded as statistically significant if $p < .05$. Results present the *degrees of freedom*, F-values, and *p*-values of the ANOVAs, while in figures and tables the *p*-values of the corresponding posthoc tests (Fisher-PLSD-test) are provided. The number of animals per strain/treatment group was $n = 8$, with exceptions indicated by the *degrees of freedom*. Significant posthoc effects for the factor "strain" versus the control animals of the F344/Crl(USA) substrain or the DA/Ztm strain are indicated by asterisks (*$p < .05$; **$p < .01$; ***$p < .001$). All data are presented as means ± standard error of the mean (SEM).

4.3. Results

DP4-like enzymatic activity

One-way ANOVA of the DP4-like activity of the congenic DA.F344-*Dpp4*^{m}/SvH rats revealed a significantly decreased level of activity for the congenic rats compared to the wild type-like DA/Ztm control strain [$F(1;7) = 416.4$; $p < .0001$; Fig. 1A]. Screening of the different F344 substrains confirmed a near complete lack of enzymatic DP4-like activity for F344/DuCrjSvH-*Dpp4*^{m} and F344/Crl(Wiga)SvH-*Dpp4*^{m} animals and a wild type-like pattern of DP4-like activity for the F344/Crl(USA) control substrain [$F(2;61) = 400.4$; $p < .0001$; Fig. 1B].

Nociception (Hot plate)

Non-habituated, vehicle-treated DP4 deficient F344 rats of both substrains exhibited a significantly increased nociception (two-way ANOVA for latency to *lick/raise a hindpaw* – factor "strain": $F(2;55) = 10.1$; $p < .0001$ – one-way ANOVA for "vehicle": $F(2;19) = 6.9$; $p < .01$; Fig. 2A) compared to control animals (F344/Crl(USA)). Importantly, i.c.v. NPY increased dose-dependently the pain threshold of mutant but not wild type-like F344 rats so that initially observed nociceptive differences disappeared (one-way ANOVA for "0.2 nmol": $F(2;189) = 4.2$; $p < .05$; for "1.0 nmol": non significant; Fig. 2A). Due to the reported lack of response to NPY treatment in F344 control rats, two-way ANOVA revealed only a trend (two-way ANOVA: $F(2;55) = 2.3$; $p = .08$) for i.c.v. NPY to increase the pain threshold.

Analysing the effects of DP4 inhibition on nociception in these F344 substrains confirmed once more the decreased pain threshold for vehicle-treated mutant F344 substrains "at baseline" (two-way ANOVA; factor "strain": $F(2;44) = 4.9$; $p = .01$; one-way ANOVA for "vehicle": $F(2;18) = 3.6$; $p < .05$; Fig. 2B). I.c.v. injections of 0.5 nmol or 5.0 nmol *Ile-Thia* attenuated the differences in nociception between the different substrains by increasing the pain sensitivity of the control F344/Crl(USA) substrain but not DP4 deficient rats (Fig. 2B).
Transferring the genetics for DP4 deficiency onto a DA background (DA.F344-*Dpp4*^{m}/SvH rats) resulted in a phenotype similar to mutant F344 substrains. The newly generated congenic DP4 deficient rats exhibited increased pain sensitivity in the non-habituated hot plate task (one-way ANOVA: F (1; 15) = 22.7; $p < .001$; Fig. 3). Similarly, the significant decrease in the latency to *raise* or *lick a hindpaw* disappeared after habituation (one-way ANOVA: F (1; 15) = 1.6; non significant; Fig. 3).

Histological findings

Figure 4 illustrates representative photomicrographs at the level of the third ventricle (3V) and the paraventricular hypothalamic nucleus (PVN) in lower (upper row) and higher magnification (lower row) of DP4 (A, D; green flourescence) and NPY (B, E; red flourescence) in wild type-like F344 rat brain (F344/Crl(USA)). Panels C and F represent overlays of A and B as well as D and E, respectively.

In general, strong DP4-like immunoreactivity (ir) was found at meninges, blood vessels, and the circumventricular organs such as the median eminence (data not shown). No evidence for neuronal expression of DP4-like protein was found in wild type-like and mutant brains. NPYir was present at multiple areas of the CNS including substantia gelatinosa of the dorsal horn of the spinal cord, several hypothalamic nuclei such as the paraventricular nucleus and the arcuate nucleus. Consistently, no obvious difference in the distribution pattern of NPYir fibres and cell bodies were found between the various F344 substrains. Although there was an overlap in the distribution pattern of DP4ir and NPYir within the before-mentioned brain areas, under a higher magnification, no clear co-localization of DP4ir and NPYir was observed at a cellular level (Fig. 4C and F). However, NPY positive neurons were found in close proximity to DP4-positive structures such as blood vessels or in the circumventricular organs (i.e. the median eminence on the level of the arcuate nucleus). Furthermore, DP4-like ir was found in the subependymal layer of the third ventricle close to the PVN of the hypothalamus (arrows). In addition, processes of NPYir neurons were closely associated with blood vessels.

4. 4. Discussion

We report here that mutant F344 as well as congenic DA substrains – all lacking endogenous DP4 enzymatic activity – show increased pain perception, interpreted as reduced stress-induced analgesia, in a non-habituated hot plate paradigm. I.c.v. administration of NPY reverses this increase in pain sensitivity in the DP4 deficient F344 substrains dose-dependently. In the same line, central inhibition of DP4 by the DP4 inhibitor *Ile-Thia* resulted in reduced stress-induced analgesia in wild type-like control F344 rats.

These effects show parallels to DP4 deficient congenic DA rats and to the previously reported mutant F344 substrains in the non-habituated hot plate test. Consequently, we conclude that central DP4-like enzymatic activity exerts an important modulatory effect on central pain processing. Furthermore, the data suggest that the DP4 substrate NPY is implicated in this process. In line with this, we hypothesize the involvement of NPY in nociception via two different levels/pathways: a) NPY's influence on stress-induced analgesia by mediating the amplitude of stress via the antinociceptive and stress-reducing action of its Y_1 receptor - with the endogenous NPY tonus being under control of DP4, and b) the antinociceptive effect of i.c.v. NPY, which produces analgesia/antinociception by activation of its Y_1 receptors.

So far, neither the interaction of NPY and DP4 nor the potential of NPY in reducing stress and thus controlling the dimension of stress-induced analgesia have been considered in pain perception. In two recent publications we described an anxiolytic-like and nociceptive phenotype for DP4 deficient F344/DuCrjSvH-*Dpp4*^{m} and F344/Crl(Wiga)SvH-*Dpp4*^{m} rats (Karl et al., 2003b; Karl et al., 2003c). The reduced behavioural stress response of mutant F344 rats is very likely based on differential NPY catabolism leading to an increased Y_1 receptor-like tone. The Y_1 receptor is the main mediator of NPY's well-described anxiolytic-like effects (Heilig, 2004; Kask et al., 2001). We also showed that the nociceptive phenotype of DP4 deficient F344 rats was associated with a reduced stress protection in these animals. This implies an impact of stress-induced analgesia (Kelley, 1986; Kelly, 1982) and endogenous NPY release (Heilig, 2004) on this nociceptive phenotype (for details see: Karl et al., 2003b).

Importantly, a similar increase in nociception was found for the newly generated congenic rats (DA.F344-*Dpp4*^{m}/SvH) that are also characterized by reduced stress and anxiolysis. Thus, we have excluded influences of the genetic background of the F344 substrains on their phenotype and confirmed that in fact differential expression and function of DP4 modulates pain perception in the non-habituated hot plate task.

In early experiments of acute stress (Akil et al., 1976; Madden et al., 1977), stress-induced analgesia was reversed by habituation to an experimental setting. In addition, stress-induced analgesia was accompanied by an increase in brain endogenous opioids (Madden et al., 1977). This phenomenon could be antagonized by non-specific centrally acting opioid antagonists and was found to be partially reversible by the opioid antagonist naloxone (Akil et al., 1976). Moreover, pain or any kind of stress – psychological, infectious or traumatic – activates corticotropin-releasing hormone (CRH) neurons (Chrousos and Gold, 1992; Crofford et al., 1994). Furthermore, stress-induced activation of the hypothalamic-pituitary axis has been shown to produce analgesia (Amit and Galina, 1986).

As NPY is a key mediator of stress-protective effects (Heilig, 2004; Kask et al., 2001), which could also be observed in the DP4 deficient rat substrains, the neuropeptide may very likely modulate stress-induced analgesia. In DP4 deficient rats, NPY may reduce stress levels via an increased activation of the Y_1 receptor. This increased Y_1 receptor-like tone would alter baseline arousal of these substrains in the non-habituated hot plate test. Attenuated stress levels would lead to a reduced release of opioids. In addition, it has to be mentioned that pronounced reduction of endogenous DP4 activity also affects DP4 substrate metabolism other than NPY and some of its substrates are powerful modulators of pain perception as well.

DP4 inhibition via i.c.v. *Ile-Thia* had no effect on hot plate latencies in mutant F344 substrains. However, control rats exhibited increased pain sensitivity after pharmacological DP4 inhibition, which was identical to the nociceptive phenotype of our genetic animal model for DP4 deficiency. Similar effects were found in another study for wild type-like but not DP4 deficient knockout mice (Guieu et al., 2006).

Histological findings indicate clear expression of DP4 immunoreactivity at sites of the blood-brain-barrier [i.e. blood vessels, circumventricular organs (CVOs) and meninges] but little or no evidence for DP4 expression/activity at distinct brain areas or even neurons. A close association of both proteins was found at the CVOs and ventricle walls at the level of the hypothalamus. The expression of DP4 at CVOs offers interesting possibilities for DP4-mediated alteration of endogenous stress modulating peptides such as NPY, the neuropeptide pituitary adenylate cyclase-activating polypeptide, and SP. As all of these peptides represent not only substrates of DP4 but also key players in adaptive/stress responses. Their local N-terminal truncation at the level of the hypothalamus (i.e. median eminence) might offer a novel and interesting avenue for the regulation of stress responsiveness.

Whereas NPY exerts a rather stress-modulatory role in the non-habituated hot plate task – resulting in a nociceptive response of DP4 deficient rats – i.c.v. NPY shows an antinociceptive effect, thus potentiating the analgesic action of NPY. NPY's function within pain perception is controversially discussed but its presence in areas involved in pain modulation such as the periaqueductal gray, locus coeruleus, thalamus or in the dorsal horn of the spinal cord suggests an important role for NPY as a putative regulator of pain transmission and perception (Broqua et al., 1996). Although some studies describe pronociceptive (Lin et al., 2004; Ossipov et al., 2002; Son et al., 2007; Tracey et al., 1995; White, 1997) or bilateral effects of NPY (Gibbs et al., 2007; Xu et al., 1999), the majority describes antinociceptive role of NPY (Hua et al., 1991; Naveilhan et al., 2001; Taiwo and Taylor, 2002). For example, intranasal NPY application in humans significantly reduced capsaicin-evoked pain by 34% (Lacroix et al., 1996). Using different animal models, NPY induces powerful antinociceptive effects in the acetic acid-induced writhing test and after thermal stimulation, which are probably mediated via the Y_1 receptor (Broqua et al., 1996; Gibbs et al., 2007; Taiwo and Taylor, 2002; Wang et al., 2001; Xu et al., 1999). However, the analgetic-like effects of exogenously applied NPY seem to be dependent on the route of administration, the application dosage, and the intensity and modality of the painful stimulus (Broqua et al., 1996; Mellado et al., 1996). So, apart from species-specific effects, variations of these parameters have very likely led to divergent results for the role of NPY in pain

In the current study, i.c.v. administration of NPY was found to have a more potent analgetic-like effect on mutant F344 substrains than on control rats. Differential degradation of NPY, caused by DP4 deficiency, could lead to an increased Y_1 receptor-like tone in mutant rats potentiating the dose-dependent analgesic/antinociceptive actions of NPY. The exclusive importance of the Y_1 receptor for the antinociceptive-like potential of centrally applied NPY is described in genetic and pharmacological studies using Y_1 receptor knockout mice (Naveilhan et al., 2001; Taiwo and Taylor, 2002). I.c.v. NPY had no further analgetic-like impact on control F344 rats as it is likely that these animals – caused by being tested in a non-habituated hot plate paradigm, which activates the rats' stress response and therefore NPY release and stress-induced analgesia – exhibit a pain threshold at its maximum.

Overall, these data provide further substantial evidence for the involvement of NPY-DP4-axis in stress-induced analgesia.

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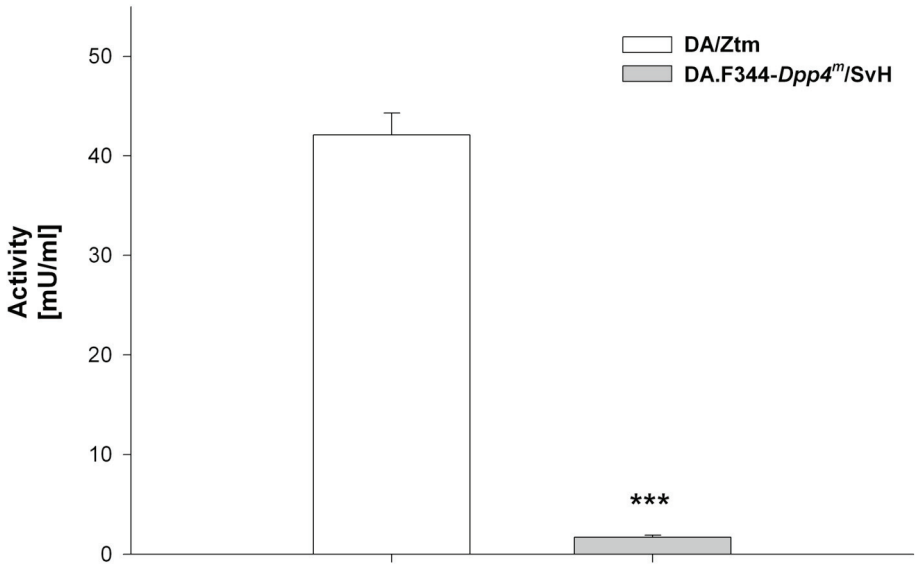
4.7. Figures



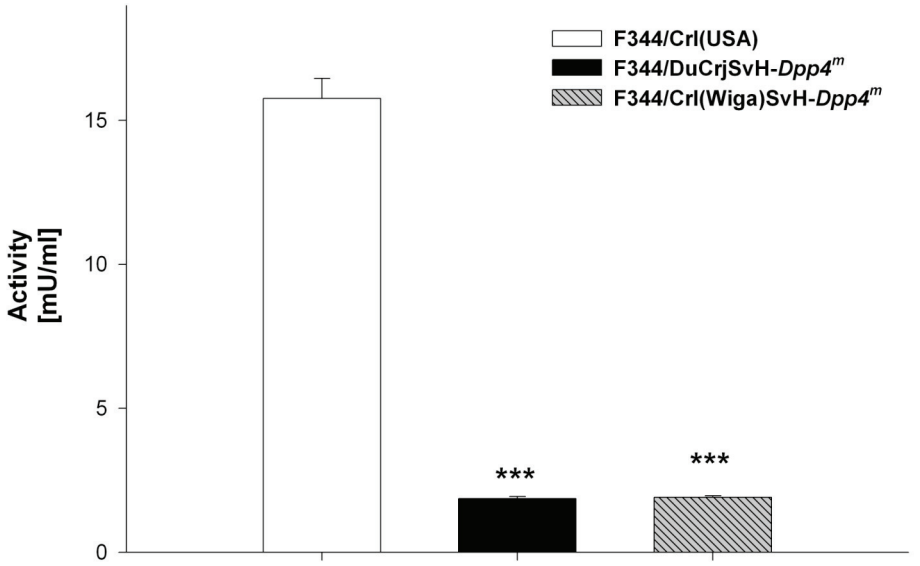


Fig. 1A (above) + B (below): DP4-like activity: DP4-like activity [mU/ml] of A) the newly generated congenic rat strain DA.F344-*Dpp4*^{m}/SvH and of B) three different F344 rat substrains (F344/DuCrlSvH-*Dpp4*^{m}, F344/Crl(Wiga)SvH-*Dpp4*^{m}, F344/Crl(USA)) was analysed using blood taken from the tail vein. Data represent means ± SEM. Asterisks (***$p <$ .001) reflect significant posthoc differences versus wild type-like rats of the A) DA/Ztm strain or B) F344/Crl(USA) substrain.

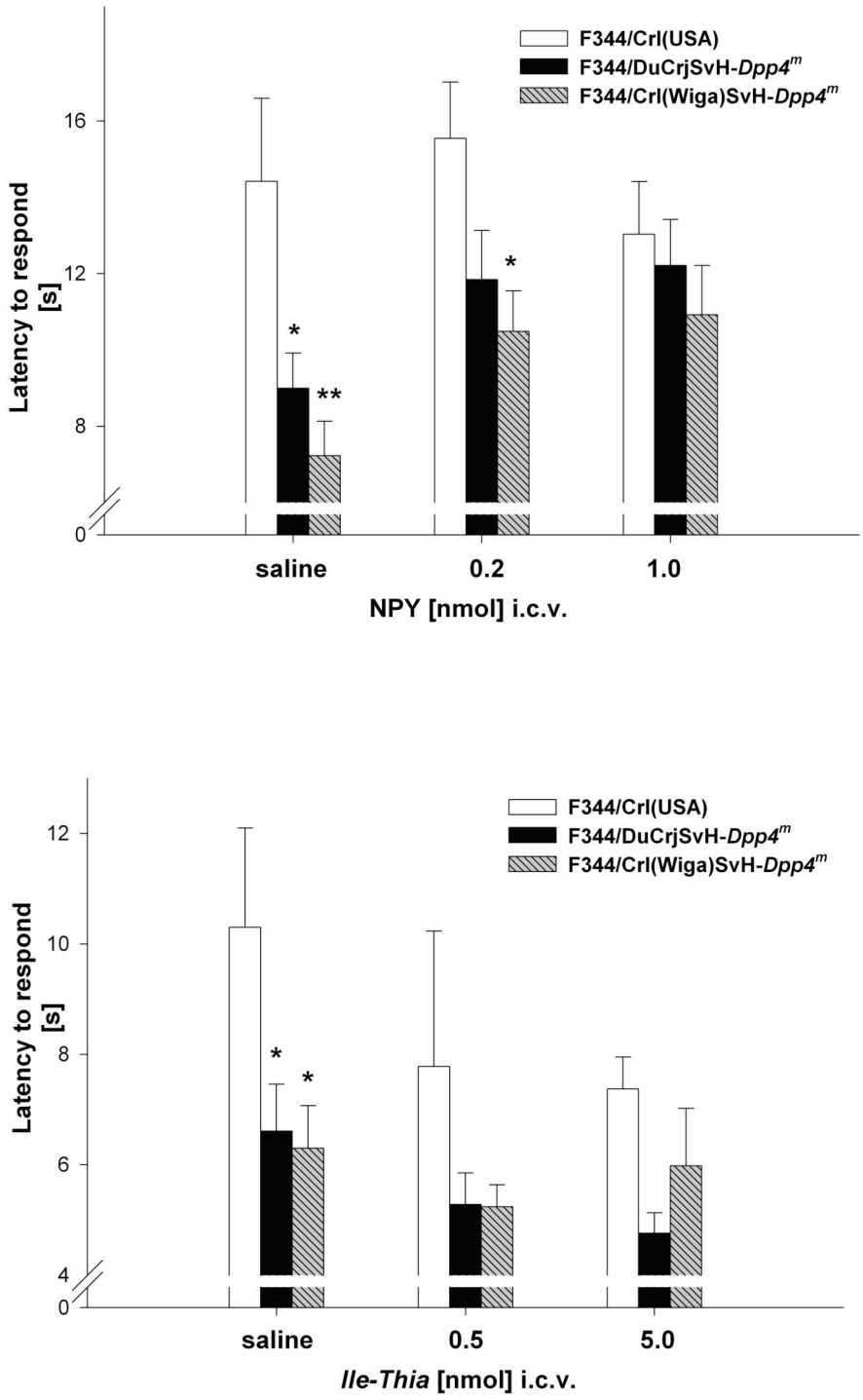


Fig. 2A (above) + B (below): Nociception: pain sensitivity was analysed in the non-habituated hot plate task - rats of the three different F344 substrains were tested after receiving an i.c.v. injection of vehicle or A) NPY (0.2 nmol/1 nmol) or B) *Ile-Thia* (0.5 nmol/5.0 nmol). The latency to respond (*lick* or *raise a hindpaw*) was recorded. Data represent means ± SEM.Asterisks (*p < .05; **p < .01) reflect significant posthoc differences versus F344/Crl(USA) rats.

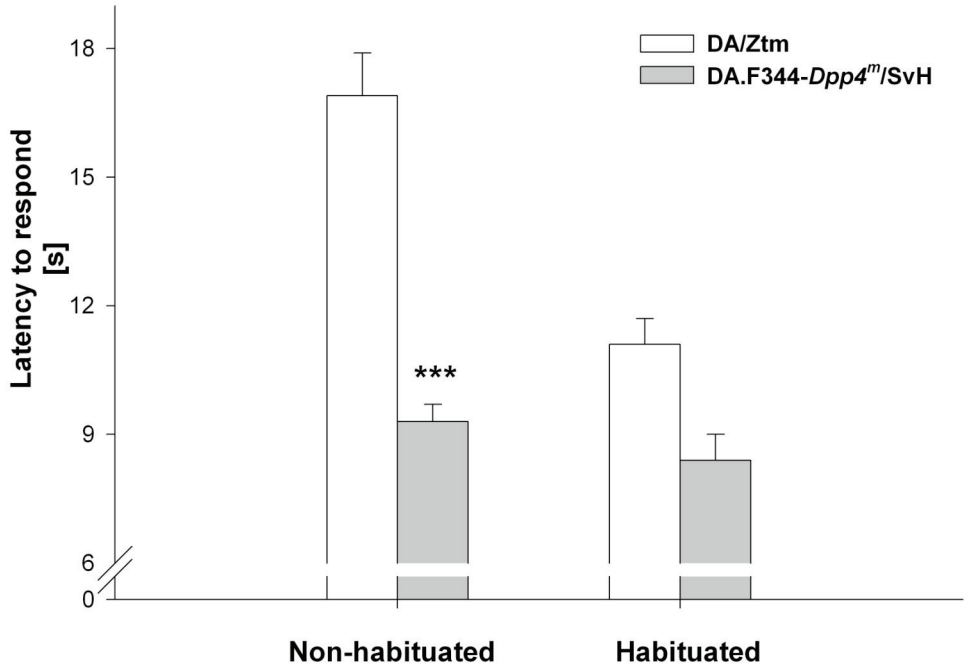


Fig. 3: Nociception: pain sensitivity was analysed in the non-habituated hot plate task and again seven days later in the habituated hot plate task - rats of a wild type-like DA/Ztm and of a DP4 deficient congenic DA.F344-*Dpp4*^{m}/SvH strain were tested. The latency to respond (*lick* or *raise hindpaws*) was recorded. Data represent means ± SEM. Asterisks (*$p < .05$; ***$p < .001$) reflect significant posthoc differences versus DA/Ztm rats.

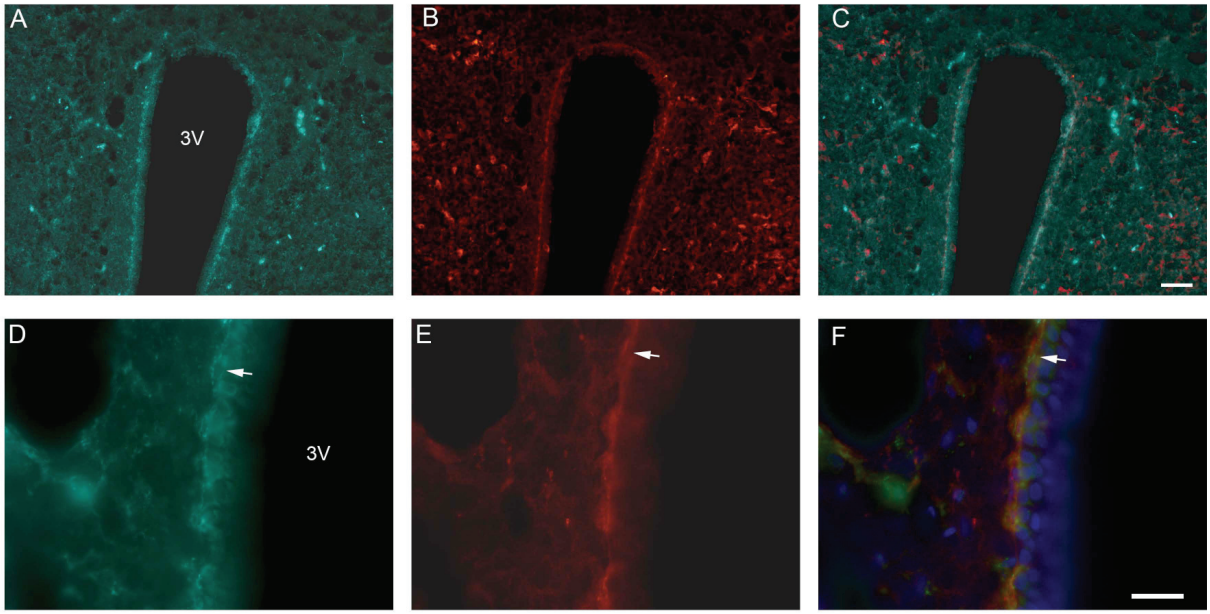


Fig. 4A-F: Immunofluorescence photomicrographs of double labelled sections of rat hypothalamus immunostained for DP4-like immunoreactivity (ir) (artificial green, A, D) and NPYir (artificial red, B, E) as well as overlays (green, red, and blue DAPI stain; C, F). Representative cryosections derived from a DP4 positive F344 rat brain. Under a low magnification (x10), DP4ir material bordering the third ventricle and blood vessels could be detected. On the same bregma level, NPYir cell bodies could be detected within the PVN and the periventricular nucleus of the hypothalamus (B). Overlay of the photomicrographs (C) show a co-distribution of both proteins within the region of interest, which lack clear co-expression. Under a higher magnification (x63), cells of the subependymal layer harbouring the third ventricle that show DP4ir cells are in direct contact with NPY neurophil. Abbreviations: 3V, third ventricle. Scale bar G, 100 mm; H, 10 mm.

5. Discussion

The neuropeptide NPY is involved in various physiological processes. Due to its size of 36 aa and the N-terminal structure, NPY is one of the best *in vivo* substrates for DP4 and *vice versa*, DP4 is the best peptidase for N-terminal dipeptide truncation of NPY – demonstrated by catalytic turnover rates [97, 156, 157]. Since this interaction is of exceptional significance, but not fully understood, we studied the NPY-DP4-axis at three levels of potential regulatory importance: (1) cleavage of NPY by DP4/DP4-like peptidases, (2) in a congenic animal model of DP4 deficiency, and (3) within central processing of pain perception.

Initially, we had to question which other peptidases are involved in the modulation of NPY receptor specificity. Another important aspect is the search for sources of N-terminally truncated NPY and related peptides (e.g. PYY). Thus, our first approach was the examination of DP4-NPY interactions on the molecular and cellular level combined with the potential cleavage of other DP4-like peptidases. Therefore, DP4 – isolated from wild type F344/Crl(USA) rats – and NPY were each cloned into fluorescence transport vectors. A simple transfection system was chosen to look for leadoff signs of possible shared transport processes in which intracellular cleavage of NPY by DP4 could occur. Internalization of DP4 is a reported mechanism in recycling of cell surface glycoproteins [158] or in association with T cell activation [159] that might offer the possibility of a co-localization with NPY and thus could potentiate intracellular truncation. Furthermore, co-localization of DP4 and NPY is suggested by Zukowska et al. who reported on a non-neuronal depot of NPY in endothelial cells [160]. So far, analysis of our model of co-transfected Cos-1 cells revealed that NPY and DP4 are not co-localized in transport vesicles. The process of co-localization could enable N-terminal cleavage of NPY and thus precise and quick intracellular degradation of NPY_{1-36} to NPY_{3-36}. This possibility could provide a tool for controlling activation of different NPY receptor subtypes and thus presents a fine-tuning mechanism in body functions. This idea is in contrast to the so far observed extracellular degradation of DP4 substrates which is based on circulating NPY and other substrates that get into contact with DP4. From this point of view, intracellular degradation seems to be a more efficient and economic way.

Several peptidases named “structural and functional homologues of DP4” (DASH) represent potential candidates for cleavage of NPY, however with different levels of specificity and activity. Little was known about the degradation of natural substrates by these DASH and their physiological role at the time when phenotyping and characterization of the previously used animal model of F344 rats started [146]. While former research distinguished between DP4

and DP2 activity and thus, mainly focused on DP4, the use of DASH specific inhibitors just became a standard in preclinical research [161-163]. So for example, Lankas et al. reported on studies with broad-specificity DASH inhibitors and highly selective inhibitors of DP4, DP2, and DP8/9 for the assessment of preclinical safety and tolerability in a study in 2005 [164].

To examine the potential involvement of different peptidases in NPY cleavage, selective inhibitors were applied against DP4, DP8/9 and DP2, and enzymatic distribution and activity studies associated with MALDI-TOF analyses were conducted. Using the chromogenic substrate Ala-Pro-AMC and selective inhibitors, examination of peptidase activity of brain extracts from wild type and DP4 deficient F344 rat substrains revealed highest distribution for DP2 followed by DP8/DP9 and finally DP4. However, DP2 was unable to hydrolyze human recombinant NPY and thus could be excluded. Although DP8/DP9 are presented in higher levels in the brain than DP4, MALDI-TOF-mass spectrometry showed similar cleavage of NPY by DP4 and DP8/DP9, confirming the higher catalytic efficiency of DP4 [156]. In contrast, analysis of human plasma showed highest degradation of NPY by DP4 and partial degradation by DP8/DP9. In addition, we report on the potency of APP from brain extracts on N-terminal cleavage of NPY resulting in NPY_{2-36} (detected via MALDI-TOF).

Preliminary results obtained by histochemistry on DASH specific enzymatic reactions suggest a dominant presence of DP4 at the meninges, CVOs and vessels and a dominant ubiquitously distribution of DP8/DP9 in the brain. Taken together, these findings support the idea that peripheral NPY is preliminarily truncated by soluble serum DP4, endothelial DP4 (blood vessels), or when crossing the blood-brain barrier by membrane-bound DP4 at the meninges, whereas NPY released from neurons in the brain also seems to be degraded to a large extent by DP8/DP9. Neither the histology nor the cytology of NPY cleavage are sufficiently understood at this time and demand further in-depth studies. Interestingly, there is no compensation of the loss of DP4 activity in DP4 deficient F344 rats by DP8 or DP9, which is in correspondence with the below mentioned differential NPY levels, which might influence behavioural responses.

In a second step we generated a novel DP4 deficient congenic animal model on a defined DA rat genetic background with pronounced differences in DP4 activity between congenic and wild type strains and studied the effects of this genetically induced DP4 deficiency in a comprehensive phenotyping approach. For generation of congenic DA.F344-*Dpp4*^{m}/SvH rats, the previously characterized spontaneous point mutation in the *Dpp4* gene of the F344 rat substrains F344/DuCrjSvH-*Dpp4*^{m} and F344/Crl(Wiga)SvH-*Dpp4*^{m} was transferred onto the

genetic background of DA/Ztm rats. These DP4 deficient animals are comparable to gene knockouts and provide a basis for learning more about the consequences of chronic DP4 deficiency, which is presently introduced into clinics as a treatment of diabetes type 2 via chronic pharmacological inhibition of DP4 [162, 165]. Whereas the previous model of DP4 deficient F344 rats mainly focused on behavioural tests to elucidate the role of DP4, our comprehensive phenotyping also comprised – next to neurobehavioural parameters – endocrine, hematological, metabolic, and immune parameters for better evaluation of effects that might be affected by DP4 deficiency.

First we studied parameters related to glucose metabolisms and body weight homeostasis in the DA.F344-*Dpp4*^{m}/SvH rats. Thus, we found that glucose tolerance seems to be inversely related to DP4-like activity levels because heterogenous animals – according to the characteristic semi-dominant mode of inheritance, expressing half of the DP4-like activity – show a rather intermediate development of glucose lowering in blood. The improved glucose tolerance in DA.F344-*Dpp4*^{m}/SvH rats is associated with increased levels of GLP-1 and higher amounts of circulating insulin which were additionally detected. GLP-1 is a substrate of DP4 that stimulates insulin gene expression, increases glucose-stimulated insulin secretion [166, 167], and inhibits glucagon secretion – all of which contribute to normalize elevated blood glucose levels [168]. DP4 deficiency leads due to missing protease activity to a prolonged half-life of GLP-1. Similar effects are achieved by use of DP4 inhibitors [166, 169, 170]. For example, the DP4 inhibitor *Ile-Thia* (isoleucine thiazolidide; P32/98) is reported to decrease circulating DP4 activity and to improve glucose tolerance in Zucker fatty rats [170], and the DP4 inhibitor NVP-Dpp728 is said to increase levels of intact GLP-1, to improve glucose tolerance, and to increase glucose-stimulated insulin secretion [171].

Although glucagon-like peptide-1(7-36)amide (GLP-1) and also the DP4 substrate glucose-dependent insulinotropic polypeptide (GIP) are involved in the regulation of postprandial nutrient homeostasis [172], no significant effects of these peptides on food intake are assumed in our congenic animal model. Interestingly, reduced body weight gain on standard but also on high fat diet was observed in DP4 deficient rats while food-intake was not significantly altered. These findings of reduced body weight gain combined with non-altered food intake are supported by data from Zucker fatty rats, where long-term treatment with *Ile-Thia* (P32/98) decreased body weight gain without affecting food intake [173]. Conarello et al. observed a significantly increased weight gain in wild type (C57BL/6) mice with restricted high calorie diet food intake in comparison to *Dpp4*^{tm1Nwa} mice, also suggesting that other factors than food intake contributed to reduced weight gain and to resistance in obesity [174]. Even though NPY

is known to stimulate the feeding behaviour [175-180] and to act as appetite transducer [108], food-intake was obviously not affected by endogenous NPY levels. In agreement with this, mice deficient for NPY were reported to have normal food intake and body weight, and become hyperphagic following food deprivation [154].

Interestingly, Hildebrandt et al. [181] suggest that there exists an interaction between DP4 and leptin, i.e. leptin deficiency. Leptin is reported to affect energy expenditure in rodens and humans [182, 183]. Activation of central leptin receptors (ObRbs) stimulates energy expenditure in adipose tissue [184]. Furthermore, the arcuate nucleus in the hypothalamus [113], a high expression site of ObRbs [185, 186], is a major site of leptin sensing [187-190]. Peripheral leptin is transported across the blood-brain barrier to reach areas distal to CVOs [191]. In this context it is of interest that the ARH contains at least two key populations of leptin-responsive neurons – one of them expresses the orexigenic peptide NPY and agouti-related peptide (AgRP) [192]. However, it has been shown that NPY deficient mice decrease their food intake and lose weight, initially to a greater extent than controls, when treated with recombinant leptin [154]. This leads to the suggestion that more complex systems are involved in body weight regulation than simple degradation of DP4 substrates that enhance or reduce stimulation of appetite. Moreover, differential effects resulting from various protein and peptides could possibly abrogate each other. So, the above mentioned findings suggest an interaction between DP4 and leptin and thus, it is very likely that increased levels of bound leptin and reduced levels of free leptin – that were observed in DA.F344-*Dpp4*^{m}/SvH rats – are cohesive with a reduction in body weight and are associated with DP4 activity or deficiency, respectively.

Additionally, reduced levels of triglycerides – also reported from studies of Ahren et al. [193] – and alanine aminotransferases as well as aspartate aminotransferases were observed, supporting the idea of an improved lipid metabolism. However, these coherences have to be studied in more detail. So far, the phenotype of DP4 deficient DA rats goes along with various levels of “beneficial” effects amongst others improved glucose tolerance and reduced body weight and obviously protects from high-fat diet induced obesity. The latter could also be observed in *Dpp4*^{tm1Nwa} mice, which are proposed to resist hepatic lipid accumulation when fed a high-fat diet [174]. Additionally, these mice are reported to show increased energy expenditure, which fits well with the abovementioned influence of leptin on energy expenditure.

Importantly, the influence of DP4 activity on digestion of gliadin-rich food could be demonstrated in our DA rats and is reflected by weight loss. Gliadin is a proline-rich

compound of gluten and causes, as a well-known allergen, the histamin reaction in celiac disease. DP4 is highly expressed in the ileum and jejunum. Since the role of DP4 in the small intestine is dipeptide re-absorption after cleavage of proline containing peptides and oligopeptides [149, 194], DP4 deficiency leads to an impaired intestinal absorption of certain proline-rich peptides [149] such as gliadin, as observed in F344 rats by Tiruppathi [194]. The small intestine has effective carrier transport systems for amino acids, di- and tripeptides, while peptides with longer chain length, e.g. tetrapeptides, require hydrolysis prior to absorption. In this context it was shown, that prolyl tetrapeptides such as Leu-Pro-Gly-Gly and Gly-Pro-Gly-Gly require hydrolysis by DP4 [195]. In addition, it has to be noted that DP4 is highly expressed in kidney and there – similar to the intestine – is involved in dipeptide re-absorption [196-199]. Thus, studies in DP4 deficient F344 rats have shown that DP4 deficient rats were unable to hydrolize and absorb urinary prolyl peptides, which are excreted in high amounts by these animals [199].

Furthermore, we suggest a connoting relevance of DP4 in regard to the processing of the casein-derived β-casomorphin that can be affected by application of DP4 inhibitors. In comparison to human β-casomorphin-7 (BCM-7; NH_2-Tyr-Pro-Phe-Val-Glu-Pro-Ile-CO_2H), bovine BCM-7 (NH_2-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-CO_2H) reveals an additional cleavage site for DP4. While the function of human BCM is rather restricted to the lactation period, bovine BCM-7 displays high relevance in human nutrition when consuming milk products. Although human BMC-7 shows high immune reactive (allergy-inducing) potency, the bovine BMC-7 and its derivates are reported to be much more reactive. Thus for example, regardless of the comparative physiological function between human and bovine derived casomorphins, human beta-casomorphin-5 (Tyr-Pro-Phe-Val-Glu) is about ten times less potent than bovine beta-casomorphin-5 (Tyr-Pro-Phe-Pro-Gly) [200]. To demonstrate the power of casomorphin, it can be noted that in that study approximately ten times more naloxone was required in rats to antagonize the beta-casomorphin-5 effect than that of morphine. Importantly, a potential connexion of bovine BMC-7 with schizophrenia is discussed. Since exorphins like casomorphin may be implicated in disorders such as autism and schizophrenia, attention should be paid to this aspect when targeting DP4 in *vivo*. Additionally, an association between casein-rich and casein-free nurishment and regulation of opiate receptor expression was observed in postnatal rats [201].

In this context, websites can be found in the Internet, selling or giving information on DP4-containing pharmaceuticals as alternative for a gluten-free, casein-free (GFCF) diet. These approaches are in contrast to the idea of a DP4 inhibitor and thus on the one hand, question the

effects of inhibitors on digestion but on the other hand, the so far observed effects – resulting from DP4 deficiency and inhibition – question these DP4-containing pharmaceuticals. This strongly requires to consider all consequences and functions of DP4 activity and inhibition and not to observe single aspects in a closed regulatory loop. The comprehensive phenotyping of novel animal models of DP4 deficiency represents an important approach to fulfill these rationals.

Thus, a comprehensive phenotyping is absolutely necessary for the appraisal of the influence of DP4 on neurobehavioural aspects in DP4 deficient DA.F344-*Dpp4*^{m}/SvH rats. Our analyses focused on behavioural domains related to anxiety, stress responsiveness, exploration, motorfunction, and cognition.

Testing of cognition or of schizophrenic-like responses (PPI) revealed no significant differences in our DP4 deficient model. Thus, the two-way active avoidance test reflected that both DA rat strains – DP4 deficient as well as wild type DA/Ztm rats – were able to learn and that none of the substrains differed significantly. This is also supported by findings in one-year-old transgenic NPY-overexpressing rats that showed no significant differences in spatial and non-spatial memory (Morris water maze and object recognition test) [150]. The PPI test showed that all animals clearly responded to the startle signaling in a similar manner – indicating healthy and equal hearing abilities. From this point of view and in addition to basic health control, we were able to state that no rat strain was advantaged or disadvantaged in the conduction of behavioural tests.

No significant differences in motorfunctions and behavioural activity were observed between wild type and DP4 deficient DA rats in the accelerod test, the holeboard test, and in the homecage activity test. Non-altered levels of activity and motorfunctions are important for the evaluation of further behavioural assays and have also been reported from NPY-overexpressing transgenic rats in another locomotor test [151]. Thus, the observed behavioural differences are not affected by altered levels of activity and motorfunctions. Furthermore, it can be excluded that differential activity levels have led to the reduced gain in body weight in DA.F344-*Dpp4*^{m}/SvH rats.

DP4 deficient DA.F344-*Dpp4*^{m}/SvH rats revealed significantly increased exploratory behaviour in the holeboard tests and significantly reduced anxiety-like behaviour in the classical and pharmacological validated EPM and SI test. In support of this, transgenic NPY-overexpressing rats were shown to be resistant to acute physical restraint stress – measured by the EPM – and showed anxiolytic-like activity in an open field test [150]. Stress-induced

hyperthermia revealed significantly reduced body temperature values in DP4 deficient rats, indicating that the appraisal of a novel environment is percepted less stressful in a status of DP4 deficiency. Moreover, reduced levels of ACTH and corticosterone were detected in blood plasma. This indicates that the hypothalamic-pituitary-axis as well as the sympathetic response to stress are blunted in a status of DP4 deficiency.

This finding of a reduced stress response in DP4 deficient rats probably also relates to the central processing of pain perception in our third approach concerning the NPY-DP4-axis. In this study, the nociceptive response in the DP4 deficient rat substrains F344/DuCrjSvH-*Dpp4*^{m}, F344/Crl(Wiga)SvH-*Dpp4*^{m}, and DA.F344-*Dpp4*^{m}/SvH was determined using the hot plate test for different approaches.

In comparison to wild type DA/Ztm rats DP4 deficient DA rats showed a significant earlier response towards the painful stimulus in the non-habituated hot plate test. Thus, previous findings from DP4 deficient F344 substrains [147] could be replicated – even with an earlier reaction in DA rats in comparison to F344 rats that might be influenced by two-fold higher endogenous DP4 activity levels. Significant response differences of the non-habituated hot plate tests were reversed after habituation – obviously resulting from a pronounced reduction in stress in wild type controls. These findings suggest a mechanism of reduced stress-induced analgesia in DP4 deficient rats, which might be caused by reduced release of endogenous opioids or other mediators due to the lowered stress-response at baseline conditions.

This phenomenon of stress-induced analgesia appears to present an adaptational response of the organism to stress, which also could be demonstrated in early experiments of acute stress preceeding the test of hot plate paradigm [202-204]. Here, stress-induced analgesia was reversed by habituation. Furthermore, stress-induced analgesia was accompanied by an increase in brain endogenous opioids [203]. This phenomenon can be antagonized by non-specific centrally acting opiod antagonists such as naloxone for example [202]. A further aspect is that pain, and any kind of stress – whether psychological, infectious or traumatic – activates corticotropin-releasing hormone (CRH) neurons [205, 206]. Stress-induced activation of the hypothalamic-pituitary axis has been shown to produce analgesia [207] and it was demonstrated that CRH can act at all levels of the neuronal axis to produce analgesia independent of the release of β-endorphin [208].

Furthermore, i.c.v. application of either NPY or the DP4 inhibitor *Ile-Thia* (P32/89), previously conducted in DP4 deficient F344 substrains, revealed an antinociceptive effect of NPY on the one hand, and a reduced treshold in pain perception caused by the DP4 inhibitor on the other

hand. While the latter approach supported the abovementioned observations of the genetic model (reduction in stress-induced analgesia), the role of NPY in pain perception and processing is controversially discussed. A survey of the literature on NPY in pain [209] has revealed that 52% of these studies describe an antinociceptive role [210-213], around 29% a pronociceptive action [214-219] and 19% reported on bilateral effects [220, 221]. A reason for divergent results might be derived from the experimental setting (e.g. study objects, way of application/local effect, pain stimulus), which again influences the activation of different receptors.

NPY is assumed to exert antinociceptive actions amongst others by inhibiting the release of SP and other "pain neurotransmitters" in the spinal cord dorsal horn. Studies in Y_1 receptor KO mice suggest the involvement of the Y_1 receptor in central physiological and pharmacological NPY-induced analgesia [212]. In agreement with this, Gibbs et al. suggest that the antinociceptive effects of NPY are likely due to activation of the Y_1 receptor, and could be mediated – at least in part – by inhibition of exocytosis of neuropeptides from the spinal cord [210, 222]. In addition, pain transmission through nociceptive sensory neurons expressing several types of NPY receptors might be modulated in different ways [209]. For example, seven distinct Y_1 receptor neuron populations have been identified in the rat lumbar spinal cord – all being located at different lamina layers [223]. Next to the Y_1 receptor, the Y_2 receptor is also taken into account in the role of pain transmission [209] dependent on local site of activation.

As nociceptive information is modulated on every level of the CNS via different receptor systems and mediators, this study is a first step in understanding the complex network of pain perception and processing and its endogenous modulation in response to challenge. However, this phenomenon clearly illustrates the stress-protective effects of DP4 deficiency.

Our novel animal model impressively exhibits a stress protective and anxiolytic-like phenotype that – following our hypothesis – is substantially caused by the prolonged half-life of endogenous NPY_{1-36} which binds with high affinity the NPY Y_1 receptor. This receptor is mainly responsible for NPY mediated anxiolysis and stress-protection [112, 151]. In wild type DA/Ztm rats N-terminal cleavage of NPY by DP4 leads to NPY_{3-36}, which again has a much lower Y_1 receptor affinity, thereby abrogating anxiolytic-like action of NPY and thus explaining the observed behavioural differences between DA/Ztm and DA.F344-*Dpp4*^{m}/SvH rats. We assume that these stress-reduced and anxiolytic-like responses are mediated via the interaction between DP4 and NPY at the level of the hypothalamus, where high expression of NPY and DP4 in blood vessels is apparent (Frerker et al., 2007 submitted).

Examination of the immunological status of DP4 deficient DA rats revealed a differential leukocyte subset composition, blunted immune cell functions and altered Il-6 levels. Accordingly, this first immunological approach questions – next to the abovementioned considerable impaired absorption and degradation processes – the so far declared "healthy phenotype" of our DP4 deficient animal models of F344 and congenic DA rats as well as of DP4 knockout in general.

The differential distribution of eosinophils and B cells might result from indirect effects of DP4 via cleavage of chemokines that might either affect hematopoetic stem cells or cell and progenitor cell mobilization. Concerning blunted T cell proliferation, this is very likely due to the direct role of DP4 as surface marker in T cell activation and co-stimulation as it could be shown that DP4 interacts with caveolin-1 and thus mediates the co-stimulatory and proliferation cascade [77, 78]. Thus, T cell functions might be affected at very different regulatory levels including but not limited to antigen presenting cell/T cell interaction, T cell co-stimulation, and memory function [77]. Furthermore, the switch of T memory cells to regulatory T cells [224] might be affected and, importantly, also the chemokine metabolism [225, 226]. With respect to the latter, affected chemokines themselves act again on the immune cells. Thus, chemokines such as RANTES have been shown to act as regulators of T cell differentiation [227] and have been associated with a T helper cell 1 response [228], which strongly underlines the importance of DP4 in chemokine cleavage and stresses the potential resulting effects of DP4 deficiency/inhibition on the immune system.

In this context, it could be shown that inhibition of enzymatic DP4 activity leads to increased release of the immuno-suppressive cytokine TGF-ß1 from T cells, which in turn leads to a suppression of the proliferation of these cells and to an inhibition of the production of immune-stimmulating cytokines (IL-2, IL-12, IFN-γ) [229, 230].

As previously reported from F344 rats [146], we confirmed blunted NK cell mediated cytotoxicity against tumor cell targets. However, in addition we also supply percentage and numbers, respectively, from splenic and peripheral blood NK cells and thus were able to specify this observation. Due to the higher number of NK cells in DP4 deficient DA.F344-*Dpp4*^{m}/SvH rats the over-all cytotoxicity (per rat) was not significantly altered in comparison to wild type DA/Ztm rats but taken the single NK cell, cytotoxicity is significantly reduced. Previous works showed that specific DP4 inhibitors had no effect on NK cell function/cytotoxicity but instead suppressed DNA synthesis and cell cycle progression of NK cells [42, 231] cells. They interpreted these findings with DP4 being involved in the regulation of NK cell proliferation whereas they suggested an independent regulation for natural

cytotoxicity. This is in contrast to our findings of increased NK cell levels in DP4 deficient rats, thus, disproving a suppression of DNA synthesis, and in addition, cytotoxic abilities per NK cell were affected – namely reduced. Probably these differential findings are derived from different experimental approaches or even might result as artefact from the DP4 inhibitors used. Nonetheless, our data suggest a role of DP4 in NK cell mediated tumor lysis and in NK cell distribution.

In summary, the key findings of this DP4 deficient congenic model are improved glucose tolerance, reduced body weight, improved leptin and liver metabolism, and an anxiolytic and stress-reduced phenotype, all of them presenting actual or potential fields of pharmaceutical application (e.g. diabetes, obesity, psychiatric disorders). However, further key aspects are mal-utilization of proline-rich proteins (intestine, kidney) and importantly, immune alterations.

By virtue of the restriction in a rats' or mice's life-span our observations are limited but might have severe effects in aging humans. In addition, effects of DP4 deficiency/inhibition might be clearly pronounced at infectious states. Due to the pleiotropic role of DP4, our animal model is pointing out that on the long run chronic DP4 inhibitors have the potential to interfere with CNS, immune, and physiological functions *in vivo*. Thus, we advise against side effects of the newly introduced anti-diabetic pharmaceuticals that might occur, if not tested appropriately. Furthermore, more specific inhibitors should be taken into account, for example DP4-resistant agonists on GLP-1 basis. A number of different GLP-1 analogues – so called "incretin mimetics" – with more favorable pharmacokinetic profiles have been generated and are currently studied in clinical trials.

Last but not least it has to be mentioned that specificity of DP4 inhibitors must not only be restricted to the target action but also to the inhibitor itself. This means that applied DP4 inhibitors have to be highly specific for DP4 and any kind of interference with the abovementioned functional homologues of DP4 or further peptidases has to be avoided. To give an example, a number of DP4 and DP4-like inhibitors have recently been tested for selectivity to DP4, DP8, DP9, and DP2 and their potential toxicity and tolerability was evaluated. The DP8/9-selective inhibitor produced thrombocytopenia, reticulocytopenia, alopecia, multiorgan histopathological changes, enlarged spleen, and mortality in rats. In dogs, this inhibitor produced gastrointestinal toxicity. Furthermore, the DP2-selective inhibitor produced reticulocytopenia in rats. Toxic effects of isomeric forms of the DP4 inhibitor *Ile-Thia* were observed at very high doses of administration and include thrombocytopenia, ataxia, seizures, convulsions, tremor, diarrhoea, and adverse effects on the lungs after four weeks'

treatment [164]. These findings were explained by off-target inhibition, which all in all again underlines the importance of highly selective inhibitors against DP4 itself.

Overall, these studies unequivocally demonstrate that DP4 represents indeed a multifunctional enzyme and remains the main dipeptidyl peptidase in modulation of the neuropeptide's receptor specificity. DP4 deficiency was shown to affect several adaptational responses many of them being under control of NPY.

As the "beneficial phenotype" of DP4 deficient rats is accompanied by considerable changes in immune functions, these findings are of particular interest for the further design and use of DP4-specific inhibitors that are presently available for treatment of diabetes type II and which – in the future – may also target immune and CNS functions.

6. References

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7. Curriculum Vitae

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Education and work experience

2004 - 2007	PhD thesis • Title: ”Functional and genetic analyses of dipeptidyl peptidase 4 (*Dpp4/Cd26*) deficiency in a rat model” • Department of Functional and Applied Anatomy, Hannover Medical School, Germany Supervisor: Prof. Dr. Stephan von Hörsten • PhD Student of Research Training Group (GRK 705) • Stipendiary of the German Research Foundation (DFG)
2005 - 2007	Co-operation with Probiodrug AG, Halle/Saale
03/2004	Scientific employee • Department of Physiological Chemistry, University of Veterinary Medicine Hannover Prof. Dr. Hassan Y. Naim
1998-2004	Studies of Biology University of Hannover
01/2004	• Graduation with Diploma • Diploma thesis: ”Investigation of the association of specific proteins components with apical transport vesicles“ • Department of Physiological Chemistry University of Veterinary Medicine Hannover Prof. Dr. Ralf Jacob
1997 - 1998	Stay abroad, Ramsgate, England Churchill House, School of English Language, • Participant in English courses and office work • Qualification: Cambridge Advanced Certificate
1990 - 1997	Upper-track Secondary School, Gymnasium Walsrode Graduation with “Abitur“

Conferences/Workshops

March 4 -5/2007	The 6th Meeting of the German-Endocrine-Brain-Immune-Network Educational Short Course
April 22 – 26/2006	8th NPY Meeting, St. Petersburg, Forida, U.S.A. • Poster
April 13 – 15/2005	2nd International Conference on Dipeptidyl Aminopeptidases, Magdeburg, Germany • Poster
March 16 – 19/2005	Annual meeting of the German Society for Cell Biology, Heidelberg, Germany • Talk • Poster

8. Publication list

Original publications

Jacob R, Heine M, Eikemeyer J, Frerker N, Zimmer KP, Rescher U, Gerke V, Naim HY: Annexin II is required for apical transport in polarized epithelial cells. *J Biol Chem* 279:3680-3684, 2004

Frerker N, Wagner L, Wolf R, Heiser U, Hoffmann T, Rahfeld JU, Schade J, Karl T, Naim HY, Alfalah M, Demuth HU, von Hörsten S: Neuropeptide Y (NPY) cleaving enzymes: structural and functional homologues of dipeptidyl peptidase 4. *Peptides* 28:257-268, 2007

Frerker N, Bode F, Nave H, Pabst R, Stephan M, Schade J, Brabant G, Wedekind D, Jacobs R, Jorns A, Forssmann U, von Hörsten S: Protection against obesity in speed congenic DA rats lacking dipeptidyl peptidase 4 is associated with behavioral and immune alterations. *Diabetes*, 2007, submitted

Karl T, Frerker N, Hoffmann T, Wedekind D, Appl T, von Hörsten S: Loss of stress-induced analgesia in rat models of dipeptidyl peptidase 4 deficiency: Evidence for mediation via stress-protective effects of NPY. *Pharmacol Biochem Behav*, 2007, submitted

Contributions to books

von Hörsten S, Krahn M, Frerker N, Gemeinhardt A, Schwab D, Slesiona S, Naim HY, Alfalah M: Intestinal Apical Protein Transport in Health and Disease. In *Proteases in Gastrointestinal Tissues* Hooper ULaNM, Ed., Springer Netherlands, 2006, p. 315-338

Abstract publication

Frerker N, von Hörsten S, Raber KA, Krahn M, Naim HY, Alfalah M.: A single mutation at amino acid 359 of dipeptidyl peptidase IV (CD26) causes a transport block in the endoplasmatic reticulum and cis-golgi compartment. In: *Deutsche Gesellschaft für Zellbiologie; European journal of cell biology* 84 (Suppl. 55); Jahrestagung der Deutschen Gesellschaft für Zellbiologie, Heidelberg, 16.-19.03.2005; Jena: Elsevier, 2005, S. 123; ISSN 0171-9335

9. Erklärung

Hiermit erkläre ich, Nadine Frerker, dass die hier vorliegende Dissertation von mir selbstständig verfasst wurde, alle benutzten Hilfsmittel und Quellen sowie die zur Hilfeleistung herangezogenen Institutionen vollständig angeben worden sind, und dass die Dissertation nicht schon als Diplomarbeit oder ähnliche Prüfungsarbeit verwendet worden ist.

Hannover, den 20.06.2007

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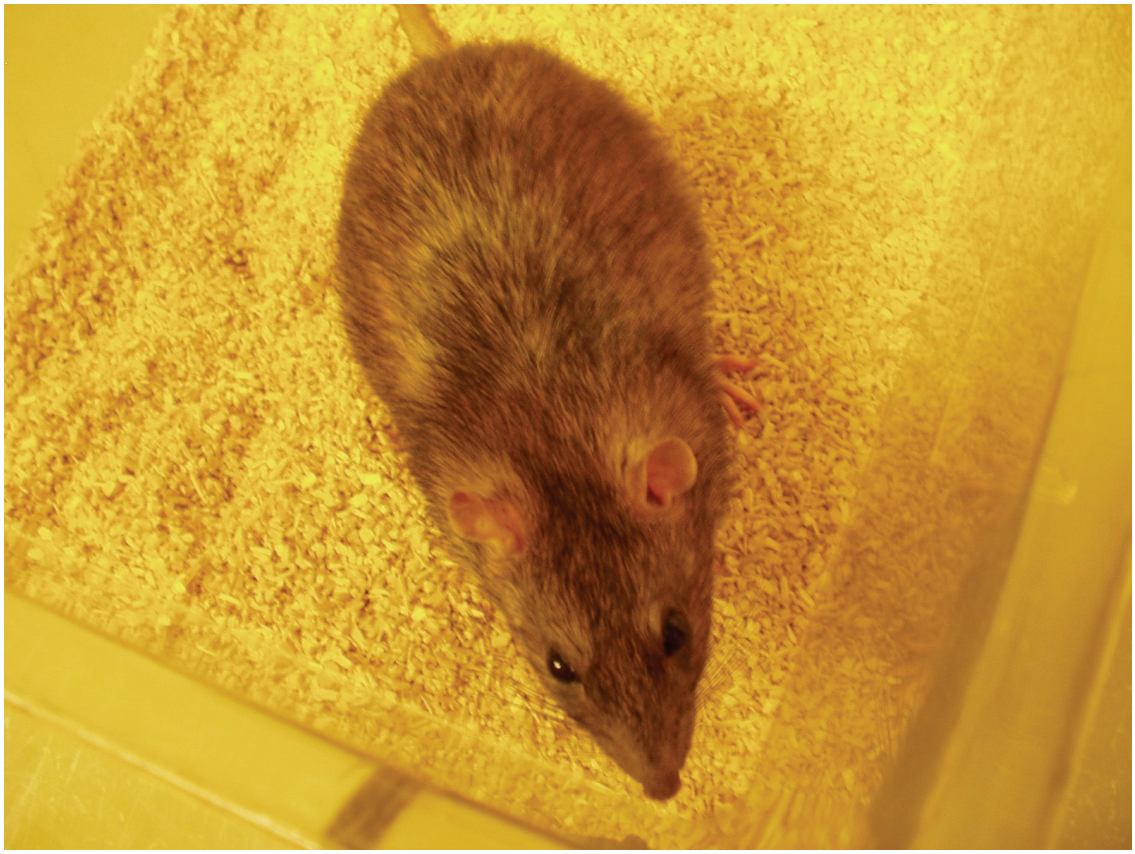

“Thanks, DA rats!“