Phenotyping of congenic dipeptidyl peptidase 4 (DP4) deficient Dark Agouti (DA) rats suggests involvement of DP4 in neuro-, endocrine, and immune functions¹⁾

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Abstract

Background: 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 have been described.

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.

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 glucagon-like peptide-1 (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. However, several immune alterations, such as differential leukocyte subset composition at baseline, blunted natural killer cell and T-cell functions, and altered cytokine levels were observed.

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 central nervous system and immune functions in vivo.

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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, also having modulating effects on a broad range of other substrates, unwanted effects cannot be excluded at this stage. 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,

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following the general rules and modifications for rats as described previously (2, 3). For the 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*] (2, 4–7) was used. Here, a transition from G to A at nucleotide 1897 in the mutant *DPP4* gene leads to a substitution from Gly to Arg in the catalytic center of the enzyme at amino acid position 633. This mutation causes a conformational change that leads to a rapid degradation in the endoplasmic reticulum and thus to a total loss of DP4 presence and activity (8). Marker-assisted breeding of congenic DA.F344-*Dpp4^m*/SvH rats was controlled via fluorescence activated cell sorting (FACS) and DP4 activity measurement. Furthermore, the mutation was confirmed by sequencing (data not shown). This strategy resulted in DP4 deficient animals, being comparable to CD26 knock-out mice (9), but providing the advantages of the species *rattus norvegicus*, being in metabolism, toxicology, and neurobiology more comparable to humans (10–13). 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 (14). Relevant substrates in immune reactions are, for example, chemokines (eotaxin, RANTES) (15, 16). 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 characterize this new congenic DP4 deficient 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 [adrenocorticotrophic hormone (ACTH)] of the hypothalamic-pituitary-adrenal (HPA) axis associated with anxiolytic-like responses in several behavioral assays were observed, which can also be considered beneficial. Probably the 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 monitor patients more closely, who are presently receiving gliptins (17) as a novel therapy inducing chronic DP4 inhibition.

Materials 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.

Generation of congenic animals

Generation 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 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: GGAAGCTTTGAATTAGCTCTCTGC; reverse: CTCTGGACTGCCATCTCC-TACTTC) 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 approximately 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. An N5 male and an 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. The nucleus colony of the congenic-inbred strain DA.F344-*Dpp4^m*/SvH is maintained as modified parallel line system to satisfy the demand for genetically uniform animals. When the colony has been established, DA.F344-*Dpp4^m*/SvH rats were checked for homozygosity of the *Dpp4* mutation by sequencing of exon 22. The genetic background of the nucleus colony was genetically monitored regularly using serological and molecular markers as well as skin grafting techniques. In addition, deficiency of DP4 expression is controlled by through flow analysis using mAb R73 and mAb Ox61. 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 described previously (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 groups 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 uptake was determined. For analysis of body weight gain under gliadin-rich diet – providing insight into the metabolism with regard to DP4 activity (18) – DP4 deficient and wild type DA rats were fed a modified rat diet (modified Altromin Standard Diet 1320) for a 3-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 6 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 heterozygous 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 given orally 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 described previously (4). For determination of DP4 enzymatic activity, EDTA-plasma samples from experimentally naive 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 of enzyme necessary to hydrolyze 1 µmol substrate (19).

Insulin, GLP-1, bound/free leptin Insulin levels were determined using an Insulin-RIA-Kit (Biotrend, Chemikalien GmbH, Cologne, Germany) according to the manufacturer's instructions. Plasma samples were taken 15, 30, and 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 [Glucagon Like Peptide-1 (Active), Linco, St. Charles, MO, USA] 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 (20), while total leptin was measured using a RIA from Linco.

Clinical chemistry The auto-analyzer ADVIA 1650 [Siemens Medical Solutions Diagnostics GmbH (formerly Bayer Vital GmbH), Fernwald, Germany], 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, Oberschleißheim, Germany) resulting in scientifically reliable data.

Histology of pancreas and liver Pancreas and liver samples were collected from either paraformaldehyde (PFA) fixed animals or HOPE fixation of samples was used. HOPE fixation was conducted as described recently (21). 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, USA; 1:500 overnight at 4°C) in liver was conducted as described previously (22). Pancreatic β-cells were detected by insulin immunostaining with a polyclonal guinea pig antibody (Insulin A 565, diluted 1:4000; DAKO, Hamburg, Germany).

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 described previously (12). For monitoring home cage activity, an infrared sensor controlled recording system (model No. E61-01/08; Coulbourn Instruments, Allentown, PA, USA) was used as described previously (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 hole-board 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 hole-board (23) were validated and used as described previously.

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 described previously (12). 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 described previously (2).

Determination of corticosterone, ACTH, IL-6, and IL-10 levels Corticosterone (AA-13F1, Lot 37170; IDS, Bolder, 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 var-

iation 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 described previously (26). The specific cytotoxicity was calculated by means of the following formula: $[(\text{experimental release}) - (\text{spontaneous release})] / [(\text{maximal release}) - (\text{spontaneous release})] \times 100$. In addition, the percentage of $\text{CD3}^+\text{CD161}^+$ NK cells in each spleen was determined by FACS analysis as described previously (7, 27) and lytic units (LUs) were calculated according to the method of Bryant et al. (28). Because mononuclear cells were used as effector cells, LUs were further mathematically adjusted to NK cell numbers present in the respective assays by forming the quotient LU/NK cells (%).

Hematology Two validated, automated hematology systems (ADVIA 120, Siemens Medical Solutions Diagnostics GmbH, Fernwald, Germany) 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 (Beckman Coulter Inc., Fullerton, CA, USA) and then further processed for flow cytometry using three color stainings as described previously (26) with the following details being modified. Briefly, approximately 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 (Düsseldorf, Germany).

T-cell proliferation assay Rat peripheral blood mononuclear cells (PBMCs) were isolated from fresh, arterial EDTA blood via centrifugation on Ficoll gradient (Ficoll Paque™ Plus, Amersham, Uppsala, 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 $\alpha\beta$ 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 manufacturer's instructions. BrdU incorporation was measured using an ELISA reader (PowerWave^{XS} Universal Microplate Spectrophotometer; BioTek Instruments, Inc., Winooski, VT, USA) at 370 nm and 492 nm as reference wavelengths. Results were expressed as absorbance rates ($A_{370\text{nm}} - A_{492\text{nm}}$).

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 the case of significant differences with 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 pairwise 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 a p-value was <0.05 . The number of animals per substrain (n) were at least 10. Presenting the degrees of freedom indicates exceptions from this. Significant post hoc effects vs. the control animals of the DA/Ztm substrain are indicated by asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). All data are presented as means $\pm$ standard error of the mean (SEM).

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 metabolism 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 [$F(2,86) = 876.5$, $p < 0.0001$; Figure 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 [$F(2,20) = 10.04$, $p = 0.001$; Figure 1B], being comparable to pharmacologically induced DP4 deficiency in mice (29, 30). 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) [$F(1,6) = 8.8$, $p = 0.02$; Figure 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; Figure 1D) was found. Similar to CD26 knockout mice, interestingly, β -islet size was reduced in the DA.F344-*Dpp4*^m/SvH rats (Figure 1E), potentially suggesting an increase of insulin storage.

Screening of body weight gain (Figure 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 approximately 1 year of age, when these differences became more

Figure 1 Glucose homeostasis and body weight related parameters in DP4 deficient (DP4^{neg}) DA.F344SvHDpp4 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 wild type (B). GLP-1(7–36) levels in DP4^{pos} and DP4^{neg} rats 30 min post-OGC (C). Insulin levels in DP4^{pos} and DP4^{neg} rats 15, 30, and 45 min post-OGC (D). Insulin staining of pancreatic islets in DP4^{pos} and DP4^{neg} rats. A representative of the maximal islets size shown in the β -cell area is illustrated, scale bar = 100 μ m (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 $\pm$ SEM. Significant group difference derived from post hoc analysis are indicated by asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. DA/Ztm wild type).

apparent. Also, high-calorie diet induced weight gain was significantly reduced in animals approximately 1 year of age (Figure 1F, small insert), although calorie uptake 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 (31, 32) may also be reflected in lipid metabolism (1, 13). Actually, our findings provide evidence that DP4 deficiency facilitates leptin signaling (33, 34). This notion is reflected by histological evaluation of free leptin in liver tissue that illustrates increased levels in wild type animals (Figure 2A) and by high levels of bound leptin in DA.F344-*Dpp4*^m/SvH rats [$F(1,6) = 5.6$, $p = 0.04$; Figure 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 extent the total mass of fat in the body, we also measured triglyceride levels 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 significant, but albeit minor, decreases in aminotransferases (alanine aminotransferases) [$F(1,17) = 15.3$, $p = 0.001$], aspartate aminotransferases [$F(1,17) = 7.08$, $p = 0.01$] and alkaline phosphatase [$F(1,17) = 8$, $p = 0.01$] (Figure 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 congenic DP4 deficient rats also exhibit improved liver metabolism, which further suggests a beneficial metabolic situation in DP4 deficient animals.

With these findings on glucose and lipid metabolism along with corresponding GLP-1/leptin signaling in mind, it remains 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 ile-

Figure 2 Metabolic changes, and clinical chemistry in DP4 deficient (DP4^{neg}) DA.F344SvH*Dpp4* 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 μ m. Strong immunoreactivity is represented by intensity of cytosolic red color (A). Bound leptin levels of DP4^{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 3-week period (D). Data represent means $\pm$ SEM. Significant group differences derived from post hoc analysis are indicated by asterisks (* p <0.05, ** p <0.01, *** p <0.001 vs. DA/Ztm wild type).

um 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

Table 1 Clinical chemistry in DP4 deficient DA rats.

Parameters in serum	DA/Ztm	DA.F344- <i>Dpp4</i> tm /SvH
Inorganic phosphate, mmol/L	1.2 $\pm$ 0.0	1.4 $\pm$ 0.1*
Urea, mmol/L	4.9 $\pm$ 0.1	5.7 $\pm$ 0.2**
Sodium, mmol/L	142.1 $\pm$ 0.3	141.5 $\pm$ 1.3
Potassium, mmol/L	3.5 $\pm$ 0.1	3.6 $\pm$ 0.2
Calcium, mmol/L	2.5 $\pm$ 0.0	2.5 $\pm$ 0.0
Chloride, mmol/L	100.8 $\pm$ 0.6	100.4 $\pm$ 1.0
Glucose, mmol/L	9.2 $\pm$ 0.3	9.04 $\pm$ 0.4
Creatine, μ mol/L	37.2 $\pm$ 1.3	35.7 $\pm$ 1.4
Total bilirubin, μ mol/L	1.8 $\pm$ 0.1	1.8 $\pm$ 0.1
Cholesterol, mmol/L	2.6 $\pm$ 0.1	2.5 $\pm$ 0.1
Total protein, g/L	66.0 $\pm$ 0.5	65.03 $\pm$ 0.9
Albumin, g/L	40.3 $\pm$ 0.4	39.8 $\pm$ 0.5

* p <0.05, ** p <0.01.

oligopeptides (18, 35, 36). This gives rise to 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 a gliadin-based diet being rich in proline (18, 37) causes malabsorption of such proteins. Consequently, wild type and DA.F344-*Dpp4*tm/SvH rats received a modified gliadin-rich and otherwise non-modified diet. As expected, results (Figure 2D) of three factorial ANOVAs 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 confirmed that this new rat model of DP4 deficiency exhibits many 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-tiered behavioral analysis of DP4 deficient DA.F344-*Dpp4*tm/SvH rats reveals a stress protective and anxiolytic-like phenotype

Multi-tiered behavioral phenotyping comprises repeated tests for various behavioral domains being additionally complemented by tests of neurological reflexes and sensory abilities. Every behavioral domain (e.g., motor function, 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*tm/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 (Figure 3A; $p > 0.05$, n.s.). Furthermore, testing of motor functions on the rotarod and of startle response along with PPI (tested, e.g., in repeated accelerated and PPI tests, data not shown), cognitive performance in an associative learning task of the two-way active avoidance shuttle box paradigm (Figure 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*tm/SvH rats. In contrast, congenic DP4 deficient 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, Figure 3C) was significantly reduced [$F(1,18) = 8.5$, $p = 0.009$], illustrating a reduced response to stress, probably mediated by decreased sympathetic nervous system (SNS) activity (24). In support of this, reduced levels of ACTH (Figure 3D) and corticosterone (Figure 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 SI test of anxiety revealed an increased total SI time for DP4 deficient DA.F344-*Dpp4*tm/SvH rats [$F(1,23) = 25.5$, $p < 0.0001$; Figure 3F] being indicative for anxiolysis. Similarly, using the EPM test, DA.F344-*Dpp4*tm/SvH rats showed higher values for percent of open arm entries [$F(1,14) = 4.5$, $p = 0.04$; Figure 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 hole-board test revealed that DP4 deficient DA.F344-*Dpp4*tm/SvH rats made significantly more head dips than DA/Ztm animals [$F(1,16) = 6.9$, $p = 0.02$; Figure 3H]. Additionally, DA.F344-*Dpp4*tm/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).

Differential immune cell distribution, diminished immune cell function, and altered cytokine levels as a consequence of DP4 deficiency in DA.F344SvHDpp4 rats

To complete the comprehensive phenotyping, we screened for immunological parameters that might be affected by DP4 deficiency (at experimentally naive 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) (38), we first screened hematological parameters. The hemogram showed significantly reduced absolute cell numbers in DA.F344-*Dpp4*tm/SvH rats for lymphocytes [$F(1,18) = 5.5$, $p = 0.03$] and eosinophils [$F(1,18) = 5.02$, $p = 0.04$] (Figure 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, e.g., we have recently shown regulatory effects of DP4 for the recruitment of eosinophils in vivo via prolonged action of the CCR3 ligand CCL11/eotaxin (unpublished data), which may also explain reduced levels of eosinophils at baseline in this study. In addition, determination of blood leukocyte subsets by FACS analysis (Figure 4B; presented in percentage in Table 2) 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 (39), 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 an NPY potentiated noradrenalin-mediated increase of the splenic IL-6 outflow (25) (Figure 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*tm/SvH rats – a finding which has also been observed in DP4 knockout mice (40) – suggesting that an overall increase of NK cells in these animals is evident. The determination of LUs (LU_{20/10⁷}, Figure 4C) revealed that in comparison to DA/Ztm rats, an increased number of effector cells are needed in DA.F344-*Dpp4*tm/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*tm/SvH rats [$F(1,6) = 24.7$, $p = 0.003$; Figure 3D], the ratio of LU to percent of NK cells reveals a significantly reduced cytotoxicity per NK cell of DP4 deficient DA rats [$F(1,6) = 6.1$, $p < 0.05$; Figure 4E]. Thus, the absolute capacity of a single NK cell to lyse tumor targets is reduced in DA.F344-*Dpp4*tm/SvH rats.

Besides differences in NK cell numbers and cytotoxicity, also a significantly diminished 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 co-stimulation, and memory function (41), tumor growth factor- β signaling (42, 43), T-memory cell to regulatory T-cell (Treg) switch, or chemokine metabolisms (15, 16). Here, we found in DP4 deficient animals a five-fold reduced proliferation rate upon stimulation with anti- $\alpha\beta$ -TCR

Figure 3 Stress protective- and anxiolytic-like phenotype in congenic DP4 deficient 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 3 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 of 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 naive rats (t₀) as well as at two time points after a brief stressor (t_{Str1} and t_{Str2}) (C). ACTH levels in DP4^{pos} and DP4^{neg} rats (D). Corticosterone levels 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 1 h 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 hole-board test. The number of head dips was recorded during a 10-min session (H). Data represent means ± SEM. Significant effects are indicated by asterisks (*p < 0.05, ***p < 0.001 vs. DA/Ztm wild type).

Figure 4 Immunological differences in DA speed-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 by FACS (B, left and right). Specific cytotoxicity (spec. cytotox.) against 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 $\pm$ SEM. Significant effects made are indicated by asterisks (* $p < 0.05$, ** $p < 0.01$ vs. DA/Ztm wild type). DC, dendritic cells.

mab [F(1,5)=32.9, $p=0.001$; Figure 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 (Figure 4G).

Discussion

The introduction of sitagliptin (Januvia®) to the market as well as the fact that vildagliptin (Galvus®) has been put on hold for some time by the Food and Drug

Table 2 Leukocyte subsets in DP4 deficient DA rats.

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

DC, dendritic cells.

Administration (FDA) both challenge an in-depth analysis of potential effects of chronic DP4 inhibition, other than improved glucose homeostasis (17). A priori, all studies investigating a status of long lasting DP4 deficiency in experimental animals, achieved either 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 was not only 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*tm/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*tm/SvH rat model largely reflects findings in DP4 knockout mice (9, 32), DP4 deficient F344 rats (2, 4), and in rats and mice receiving chronic DP4 inhibitor treatment (29, 30, 44),

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 knockout mice (32), which together is indicative for effects mediated either via higher metabolic rates or malabsorption. In this study, no differences in home cage activity were observed, thus excluding differential physical activity levels being responsible for the lower body weight in free feeding DA.F344-*Dpp4*tm/SvH rats. Although no differences in baseline body temperature were found in DA rats, leptin is reported to increase energy expenditure (45, 46), which might have contributed to differences in body weight. Interestingly, malabsorption of gliadin leads to a reduction of body weight (18) (Figure 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 breastfeeding of children by mothers receiving DP4 inhibitors in conjunction with malabsorption of β -casomorphin may lead to both loss of body weight or failure to thrive. Presently, breastfeeding represents a contra-indication for the use of gliptins. In the latter case, a connection between DP4 activity and β -casomorphin in atopic dermatitis and potentially other immune disorders has been suggested (47).

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 on DP4 deficient F344 rats (2, 5) may represent a novel target for drug development. We demonstrate here that DA.F344-*Dpp4*tm/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 HPA axis as well as the sympathetic response to stress are diminished. Our leading hypothesis is that DP4 deficiency in mutant F344 rats (2, 5) as well as congenic DA.F344-*Dpp4*tm/SvH rats prolongs the half-life of endogenous NPY₍₁₋₃₆₎, which binds with high affinity the NPY Y1 receptor. This receptor is mainly responsible for NPY mediated anxiolysis and stress protection (48, 49). N-terminal cleavage of NPY by DP4, leading to NPY₍₃₋₃₆₎, results in a much lower Y1 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, but other limbic areas and Y1 receptor expression sites may be involved as well. Last but not least, this concept of DP4 mediated modulation of NPY Y1 receptor mediated responses may also account for the peripheral action of NPY in the PNS and immune system as, e.g., 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 II and phase III 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, most likely problems will appear in the area of immune regulation. Thus, the present rat model exhibits several immune alterations at baseline or non-challenged conditions, which consist of differential leukocyte subset composition (eosinophils, NK cells, B-cells), diminished 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 and the “multifunctionality” of CD26. These are changes in T-cell co-stimulation (41) – though in this case the involvement of CD26 enzymatic activity is still under debate – and in chemokine metabolism (16), obviously also affecting hematopoietic stem cells (50). 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 I 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, 51). As such conditions are not tested during phase III 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 (41) 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 diminished (41). Last but not least,

also as 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 comparison to the existing CD26 deficient F344 rat models, this new model of DA.F344-*Dpp4*tm/SvH rats confirms several neurobehavioral changes that have been observed in the F344 models of DP4 deficiency, but apparently they appear to be more pronounced in the congenic line. While reduced body weight, improved glucose homeostasis, and altered NK cell cytotoxicity were also described in F344 rats, further endocrine, hematological, metabolic, and immune parameters were demonstrated in the DA.F344-*Dpp4*tm/SvH rats for the first time.

In conclusion, the present comprehensive characterization of DA.F344-*Dpp4*tm/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 suggest this area as the major impact regarding potential side-effects.

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