Characterization of neuropeptide Y-mediated behavioral effects in F344 rat substrains with a differential dipeptidyl-peptidase IV (DPPIV; CD26) enzymatic activity

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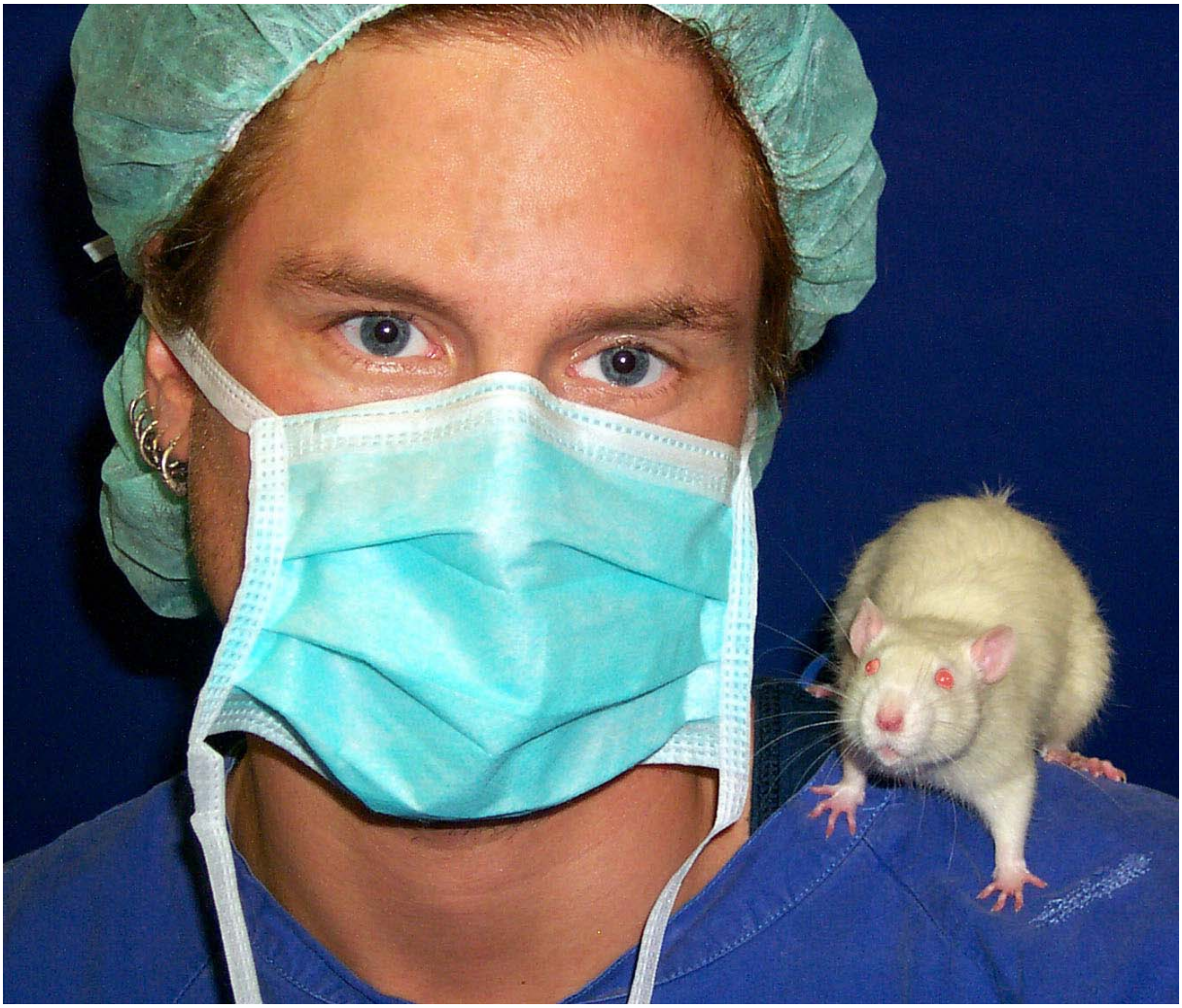


Abstract

The ectopeptidase dipeptidyl-peptidase IV (DPPIV; CD26) specifically truncates dipeptides from substrates with an alanine or proline at the second N-terminal position of the amino acid sequence. Due to this specificity, the neurotransmitter neuropeptide Y (NPY) represents one of the best substrates for DPPIV but implications of the "DPPIV-NPY" interactions on behavioral responses *in vivo* have not been investigated.

The aims of the present studies were (1) to characterize the phenotype of two mutant F344 rat substrains [F344/DuCrj(DPPIV-) and F344/Crl(Ger/DPPIV-)], which exhibit an extreme reduction of their endogenous DDPIV activity, and of a wildtype-like F344 substrain [F344/Crl(Por)], and (2) to test the hypothesis whether this lack of enzymatic activity is associated with differential *in vivo* effects of centrally applied NPY. In a first experimental step, we mapped the *Dpp4* gene, performed breeding experiments, and determined the glucose tolerance and the natural killer (NK) cell function in the three different substrains. In a second step, the influence of DPPIV deficiency on behavioral domains such as feeding behavior, anxiety, nociception, memory, and the sedative effect of ethanol was analyzed. In a third step, we investigated the effects of intracerebroventricularly (i.c.v.) applied NPY in various behavioral tasks for feeding behavior, anxiety, and nociception.

Results demonstrated the position of the *Dpp4* gene on rat chromosome 3 (RNO3) and a semi-dominant mode of inheritance. DPPIV deficiency improved the glucose tolerance and blunted the NK cell function. Furthermore, it was found to be associated with a reduced body weight and water intake, a hyperalgesic response under stress, and an anxiolytic-like phenotype. In addition, we observed a decreased susceptibility for the sedative effect of ethanol. After i.c.v. administration of NPY most behavioral effects of this peptide were more potent in the mutant substrains strongly suggesting that the anxiolytic- and hyperalgesic-like phenotype of the DPPIV-deficient F344 substrains is caused by a differential degradation of NPY.

In conclusion, a differential cleavage of NPY in our different substrains can be considered as at least one of the main reasons for the differences in the behavioral and physiological phenotype. Overall, these animals provide a useful model to study various behavioral and physiological effects associated with DPPIV-enzymatic activity and to study the "DPPIV-NPY-axis". Furthermore, these animals could serve as a physiological and molecular model for behavioral modulations.

Keywords: Dipeptidyl-peptidase IV – neuropeptide Y – behavioral effects

Kurzzusammenfassung

Die Ektopeptidase Dipeptidyl-Peptidase IV (DPPIV; CD26) spaltet spezifisch Dipeptide von Substraten ab, die ein Alanin oder Prolin an der zweiten N-terminalen Position der Aminosäuresequenz aufweisen. Aufgrund dieser spezifischen Eigenschaft ist der Neurotransmitter Neuropeptid Y (NPY) eins der besten Substrate für die DPPIV. Die Auswirkungen von „DPPIV-NPY“ Interaktionen auf Verhaltensantworten *in vivo* wurden allerdings bisher nicht untersucht.

Ziele der vorliegenden Studie lagen (1) in der Charakterisierung des Phänotyps zweier mutanter F344 Rattensubstämme [F344/DuCrj(DPPIV-) und F344/Crl(Ger/DPPIV-)], die eine extreme Reduzierung der endogenen DPPIV-Aktivität aufweisen, und eines wildtyp-ähnlichen F344 Substammes [F344/Crl(Por)], sowie (2) in der Überprüfung der Hypothese, ob das Fehlen der enzymatischen Aktivität mit unterschiedlichen *in vivo* Effekten von zentral appliziertem NPY verbunden ist.

In einem ersten experimentellen Schritt kartierten wir das *Dpp4* Gen, führten Vererbungsexperimente durch, bestimmten die Glucosetoleranz und die natürliche Killerzellfunktion in den drei verschiedenen Substämmen. In einem zweiten Schritt wurde der Einfluss der DPPIV-Defizienz auf Verhaltensdomänen wie Fressverhalten, Ängstlichkeit, Schmerzwahrnehmung, Erinnerungsleistung und sedative Effekte von Ethanol analysiert. In einem dritten Schritt wurden die Effekte von intrazerebroventrikulär (i.c.v.) injiziertem NPY in verschiedenen Verhaltenstests zum Fressverhalten, zur Ängstlichkeit und zur Schmerzwahrnehmung untersucht.

Die Ergebnisse bestätigten die Lokalisierung des *Dpp4* Gens auf Chromosom 3 (RNO3) der Ratte und eine semi-dominante Vererbung. DPPIV-Defizienz verbessert die Glucosetoleranz und schwächt die Killerzellfunktion. Ausserdem ist sie mit reduziertem Körpergewicht und reduzierter Wasseraufnahme, einer hyperalgetischen Reaktion unter Stress und einem angstlösenden Phänotyp assoziiert. Zusätzlich wurde eine verringerte Empfänglichkeit für sedativen Effekte von Ethanol beobachtet. Nach i.c.v.-Gabe von NPY waren die meisten Verhaltenseffekte von NPY deutlicher in den mutierten Substämmen nachweisbar. Dies deutet darauf hin, dass angstlösende und der hyperalgetische Phänotyp der DPPIV-defizienten Tiere auf einer differentiellen Spaltung von NPY basiert.

Zusammenfassend zeigt sich, dass die unterschiedliche Spaltung von NPY in den verschiedenen Substämmen zumindest einer der Hauptgründe für die Unterschiede im

verhaltensbiologischen und physiologischen Phänotyp ist. Insgesamt lässt sich feststellen, dass unsere Tiere ein nützliches Tiermodell zur Untersuchung von verschiedenen verhaltensbiologischen und physiologischen Effekten, die mit der DPPIV-Aktivität assoziiert sind, und zur Untersuchung der „DPPIV-NPY-Achse“ darstellen. Des Weiteren könnten diese Tiere als physiologisches und molekulares Modell für Verhaltensmodulationen dienen.

Schlagworte: Dipeptidyl-Peptidase IV - Neuropeptid Y - Verhalten

Inhaltsverzeichnis

1. Introduction

The aim of the present study was to characterize the behavioral and physiological phenotype of three F344 rat substrains, which differ in their endogenous dipeptidyl-peptidase IV activity (DPPIV; CD26). Two F344 rat substrains, which were commercially available from Japanese and German breeding colonies of Charles River Laboratories (F344/DuCrj, Atsugi, Japan and F344/Crl, Sulzfeld, Germany) in the year 1998, exhibit an extreme reduction in endogenous DPPIV activity, whereas F344 substrains from breeding colonies in the USA (Portage and Raleigh) exhibit a wildtype-like phenotype (same laboratory code as the German substrain: F344/Crl). In this study the impact of the substrains´ differential DPPIV activity on the behavior and physiology as a model for DPPIV deficiency was analyzed. We focused especially on behavioral and physiological effects, that are possibly mediated by a differential cleavage of the DPPIV substrate neuropeptide Y (NPY), which is one of the best substrates for DPPIV. Using a systematic and multi-tiered approach for the phenotyping of the three different substrains (Karl et al., 2003a), we first characterized the genetics and some general physiological effects of the mutation in the *Dpp4* gene (Karl et al., 2003b), applied a broad screening to identify key behavioral differences (Karl et al., 2003c), and tested finally by intracerebroventricular (i.c.v.) application of NPY if some of the observed differences could be due to a differential cleavage of NPY in the different substrains (Karl et al., 2003d; Karl et al., 2003e).

1.1 Discovery and distribution of DPPIV

The enzyme and binding protein dipeptidyl-peptidase IV was discovered in rat liver homogenates in 1966 (Hopsu-Havu and Glenner, 1966). The ectopeptidase DPPIV is a type II integral membrane peptidase being anchored to the plasma membrane by its signal sequence and is identical to the leukocyte differentiation marker CD26 (De Meester et al., 2000; De Meester et al., 1999). The enzyme is highly conserved among different species (Ludwig et al., 2003).

DPPIV is expressed at different levels in tissues like kidney, lung, liver, adrenal gland, spleen, testis, and brain. Due to its abundant expression on endothelial cells, the enzyme is in close contact to hormones, chemokines, and cytokines circulating in the blood. Moreover, the peptidase is found as a soluble enzyme in plasma and cerebrospinal fluid (CSF), in the latter at low levels. Furthermore, DPPIV/CD26 is expressed on cells of the immune system and here especially on activated T-helper lymphocytes and subsets of macrophages. In the adult

central nervous system (CNS) DPPIV has contact with neuropeptides mainly in the CSF, brain vessels, and leptomeningeal cells. Based on protein detection by immunohistology, DPPIV is particularly high expressed in the circumventricular organs and on leptomeningeal cells, whereas in the embryonic brain it is abundantly present on neuronal cells (Mentlein, 1999). In the peripheral nervous system DPPIV is located in the perineurium and on Schwann-cells. The peptidase is also present at sites of physiological barriers (i.e. blood-brain barrier). Its location allows DPPIV to act on bioactive peptides in body fluids or during communication processes between immune cells (Mentlein, 1999).

1.2 Functional role of DPPIV

The role of DPPIV in the inactivation of bioactive peptides was recognized 25 years ago (Heymann and Mentlein, 1978; Kato et al., 1978) due to its unique ability to cleave dipeptides from the N-terminus from polypeptides and proteins carrying a *Xaa*-proline or *Xaa*-alanine in the second (penultimate) position of the amino acid sequence (De Meester et al., 1999; Reinhold et al., 2002). Many neuropeptides, immunopeptides (i.e. cytokines), and peptide hormones have proline residues at specific positions in their amino acid sequence, which serve as a cleavage point for DPPIV (Table 1). Thus, due to this specificity, DPPIV is potentially involved in the regulation of nervous, endocrine, and immune functions at various functional levels (Hildebrandt et al., 2000).

Table 1:

Neuropeptides	Endomorphine-2, kentsin, enterostatin, and neuropeptide Y
Immunopeptides	RANTES, TNF-α, TNF-γ, IL-1, IL-2, IL-6 , IL-11, and IP-10
Peptide hormones	Growth-hormone-releasing factor, glucagon-like peptide 1, glucose-dependent insulinotropic peptide, peptide YY, and substance P

Substrates of DPPIV (data are displayed according to Hildebrandt et al., 2000).

1.3 Genetics of DPPIV and DPPIV deficiency

Several studies focused on the location and expression of the *Dpp4* gene in man and mice. In humans the *DPP4* gene is located on the long arm of chromosome 2 (HSA2, 2q24.3) and spans approximately 70 kb. It contains 26 exons, ranging in size from 45 b to 1.4 kb. The active site Ser^{630} of human *DPP4* is surrounded by Gly-Trp-Ser^{630}-Tyr-Gly, which corresponds to the motif Gly-*Xaa*-Ser-*Xaa*-Gly proposed for serine-type peptidases (De Meester et al., 1999). The cDNA codes for a polypeptide of 766 residues. The mouse *Dpp4*

gene is located on chromosome 2 (MMU2; partially homologous to HSA2/HSA11), encompasses more than 90 kb, and is also composed of 26 exons. Their length varies from 100 bp to more than 20 kb. The cDNA codes for a polypeptide of 760 residues (Bernard et al., 1994).

In rats the *Dpp4* gene maps to a region of chromosome 3 (RNO3; genome.ucsc.edu/cgi-bin/hgGateway?org=rat), which is homologous to the *Dpp4* gene containing MMU2 region. The mode of inheritance of the *Dpp4* gene still remains unclear. Tsuji and coworkers (1992) analyzed the genetics of DPPIV in wildtype-like F344/Clea rats (Nihon Clea, Osaka, Japan) and DPPIV-deficient F344/DuCrj rats from a breeding colony of Charles River Laboratories (Crl) in Atsugi, Japan. On the basis of the cDNA sequence for DPPIV the primary structure of the enzyme seems to consist of 767 amino acid residues. It is initially synthesized as a 103 kDa form. Afterwards, DPPIV is processed to the mature form of 109 kDa during the intracellular transport. Like in the human *DPP4* gene a serine-protease typical Gly^{629}-Trp-Ser-Tyr-Gly^{633} sequence surrounds the active-site Ser^{631} of DPPIV. Recently, the three-dimensional structure of rat DPPIV was analyzed. The protein exists as a dimer with structural analogies to the serine peptidase POP (Ludwig et al., 2003).

Cloning and sequencing of *Dpp4* cDNA in the DPPIV-deficient F344/DuCrj rats revealed a G to A transition at nucleotide 1897 in the cDNA sequence, which results in a substitution of Gly^{633} to Arg in the active-site sequence (catalytic center). This substitution leads to a conformational change of the ectopeptidase, which appears to be responsible for the loss of DPPIV activity and the absence of DPPIV in the plasma membrane of F344/DuCrj (Tsuji et al., 1992). Based on this point mutation, DPPIV activity is reduced or deficient in kidney, lung, liver, submaxillary glands, brain, and urine of these animals (Watanabe et al., 1987), although *Dpp4* mRNA levels are similar in mutant F344/DuCrj and wildtype-like F344/Clea rats (Erickson et al., 1992). In contrast to the wildtype-like enzyme, mutant DPPIV of the F344/DuCrj substrain, although also being synthesized as a 103 kDa form, is rapidly degraded without normal post-translational processing to the mature form. The single substitution of Gly^{633} to Arg is sufficient to cause this rapid intracellular degradation of DPPIV (Tsuji et al., 1992).

The mutation in the German F344/Crl substrain has not been characterized to that extend. But similar to the F344/DuCrj substrain, comparable mRNA levels are detected in mutant F344/Crl rats from German breeding colonies and in wildtype-like F344/Crl rats from breeding colonies in Portage, USA. Only wildtype-like DPPIV is expressed in an active form (Gossrau, 1990; Thompson et al., 1991).

Because of the involvement of DPPIV in cleavage processes of several neuro- and immunopeptides (Table 1), differences in DPPIV expression and/or activity between different rat strains or substrains can be of considerable importance for research regarding the direct and indirect role of DPPIV in the regulation of nervous, endocrine, and immune functions. Thus, in a first step, we mapped *Dpp4* using a gene linked SSLP marker, performed breeding experiments to identify the mode of inheritance of DPPIV-like activity (cooperation with W.T. Chwalisz and Dr. D. Wedekind: Institute for Laboratory Animal Science and Central Animal Facility, Hannover Medical School, Germany), and screened several laboratory rat strains including F344 rat substrains from different breeding colonies obtained at different time points from Charles River Laboratories (Crl) for their endogenous DPPIV-like activity (cooperation with Dr. T. Hoffmann: Probiodrug AG, Halle, Germany). Furthermore, we determined the glucose tolerance and the natural killer cell function (the latter one in cooperation with Dr. R. Jacobs: Department of Clinical Immunology, Hannover Medical School, Germany) in mutant (F344/DuCrj, Atsugi, Japan and F344/Crl, Sulzfeld, Germany) and wildtype-like (F344/Crl, Portage, USA) F344 rat substrains in order to exemplify the functional importance of spontaneous mutations in the *Dpp4* gene (Karl et al., 2003b).
For clarity, the different F344 substrains were coded as followed: DPPIV-deficient F344 rats derived from breeding colonies in Atsugi, Japan were designated as F344/DuCrj(DPPIV-), DPPIV-deficient animals from breeding colonies in Sulzfeld, Germany, as F344/Crl(Ger/DPPIV-), wildtype-like rats obtained from colonies in Portage, USA, as F344/Crl(Por), and wildtype-like rats from colonies in Raleigh, USA, as F344/Crl(Ral). Additionally, the time point, at which the animals were obtained from Charles River Laboratories (1998/2001) was indicated in our code (“98” for 1998 and “01” for 2001), whenever this appeared to be necessary.

1.4 Substrates of DPPIV and their physiological impact

Exogenously administered inhibitors of DPPIV prolong the biological half-life of DPPIV substrates, with several of them being highly important clinical and pharmaceutical targets for drug development. For example, DPPIV inhibition stabilizes and hence improves the intestinal absorption of enterostatin, which, having an anorectic effect (Erlanson-Albertsson and York, 1997), produces a dose-dependent reduction in fat intake and body weight (Bouras et al., 1995; Bouras et al., 1996; De Meester et al., 2000). Furthermore, the insulinotropic hormones glucagon-like peptide 1 (GLP-1) and the glucose-dependent insulinotropic peptide (GIP) are inactivated by DPPIV truncation (Kieffer et al., 1995). GLP-1 has multifaceted

actions including stimulation of insulin gene expression (Hildebrandt et al., 2000) and the inhibition of food intake (Turton et al., 1996). Inhibition of DPPIV increases the amount of the intact incretins GLP-1 and GIP, improves their insulinotropic effect, and has been proposed as a valid therapeutic approach for lowering glucose levels in type 2 diabetes or other disorders involving glucose intolerance (Mentlein, 1999). Interestingly, elevated urinary concentrations of DPPIV have been shown in non-insulin-dependent diabetic patients (Nagata and al, 1988).

DPPIV inhibition also elicits analgesia by potentiating the actions of the endogenous opioid peptide endomorphine-2 (and endomorphine-1), which has a high affinity at μ opioid receptors and produces potent analgesia (Shane et al., 1999). In addition, DPPIV enhances nociception by processing substance P to a more potent derivative, a process, which may involve additional circuits via other substrates for DPPIV like NPY and endomorphine-2 (Hildebrandt et al., 2000). These modulators of pain perception and processing may result in multiple interactions (and may modulate - as a net effect - the expression of pain). In addition, several studies demonstrate the involvement of DPPIV in the metabolism of NPY, which is involved in various other physiological and behavioral processes (see below and De Meester et al., 2000; Mentlein, 1999; Wettstein et al., 1995).

Furthermore, DPPIV seems to be involved in human diseases like rheumatoid arthritis (Kamori et al., 1991; Williams et al., 2003), AIDS (Vanham et al., 1993), major depression (Maes et al., 1991), schizophrenia (Maes et al., 1996), and anorexia nervosa (Hildebrandt et al., 1999). Patients with psychiatric disorders (anxiety-disorders or psychosis) show a decrease in serum DPPIV activity (Maes et al., 1996; Maes et al., 1991), whereas an increase in DPPIV serum activity was reported in patients with hyporectic eating disorders (van West et al., 2000).

Inhibition of DPPIV/CD26 plays also an important role in the process of activation and proliferation of human lymphocytes and can provoke many cellular effects, including suppression of DNA synthesis and reduced production of various cytokines (Kahne et al., 1999). Elevation of DPPIV/CD26 activity exerts an immunoprotective effect, mainly via expansion of T cell activation (Morimoto and Schlossman, 1998).

Because of the ability of DPPIV/CD26 to cleave several substrates and its involvement in various human diseases and immune functions, it is of great interest to investigate an animal model with a differential endogenous DPPIV activity. The DPPIV-deficient F344 substrains F344/DuCrj(DPPIV-) and F344/Crl(Ger/DPPIV-), which exert an extreme reduction in endogenous DPPIV activity but produce DPPIV mRNA (Erickson et al., 1992), could be a

useful tool for investigating the effects of "natural" DPPIV inhibition via "germline-knockout"-comparable mechanisms on the behavioral and physiological phenotype but with the advantage of using the species *Rattus norvegicus* instead of *Mus musculus laboratorius*. Theoretically, differences in the behavioral phenotype between the mutant F344 substrains [F344/DuCrj(DPPIV-) and F344/Crl(Ger/DPPIV-)] and wildtype-like animals from Portage, USA [(F344/Crl(Por)] could be attributed to differential degradation processes of various DPPIV substrates such as enterostatin, GLP-1, endogenous opioids, NPY, or substance P in the CNS. Therefore, in a second step of this study, the influence of DPPIV deficiency on behavioral and physiological topics was analyzed in the mutant and wildtype-like F344 substrains. For this the different substrains were observed in several schedules (Table 2), which are potentially affected by above-mentioned DPPIV substrates (Table 1) (Karl et al., 2003c) after a systematic behavioral phenotyping (Crawley, 1999; Crawley and Paylor, 1997; Karl et al., 2003a). A differential degradation of these substrates (caused by the differences in endogenous DPPIV activity) could have a high impact on the various behavioral and physiological domains.

Table 2:

Behavioral and physiological domains	**Related test paradigmes**
Feeding behavior	Voluntary food and fluid consumption
Glucose homeostasis	Glucose tolerance test
Nociception	Tail flick and hot plate
Anxiety	Open field, elevated plus maze, and social interaction
Memory and learning	Radial maze, object exploration, and passive avoidance
Ethanol	Voluntary consumption of ethanol Sedative effect of ethanol
Tests related to symptoms of human psychiatric disorders	Schizophrenia: Startle response and prepulse inhibition Depression: Porsolt swim test

Schedules for various behavioral and physiological domains.

Among the various substrates for DPPIV, NPY is the most abundant neuropeptide in the brain (Allen et al., 1983) and it is rapidly cleaved by DPPIV (Mentlein, 1999). Thus, we focused especially on the endogenous metabolism of the DPPIV substrate NPY and its involvement in behavioral and physiological processes in the three F344 substrains.

1.5 Neuropeptide Y as a substrate of DPPIV-like enzymatic activity

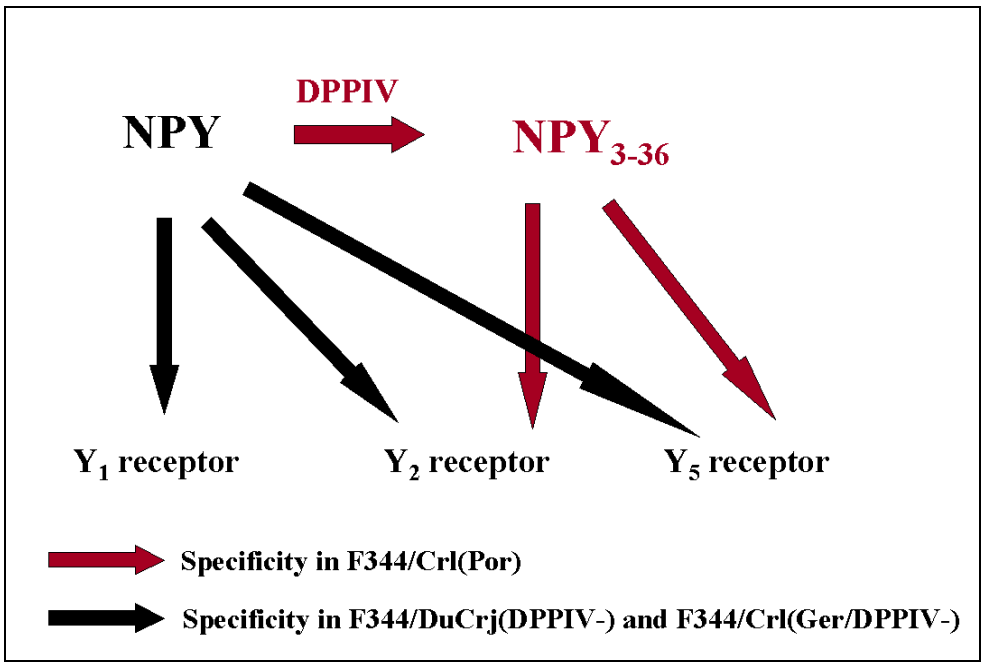
1.5.1 Discovery, distribution, and function of NPY

NPY, discovered in 1982 (Tatemoto, 1982; Tatemoto et al., 1982), is a member of the pancreatic polypeptide (PP-fold) family of peptides (Gehlert, 1998). It is composed of 36 amino acids and its sequence is one of the most conserved peptide sequences known (Larhammar et al., 2001). The neuropeptide has a C-terminal amide that is essential for its biological activity and contains a large number of tyrosine residues on both ends of the molecule (Colmers and Wahlestedt, 1993; Gehlert, 1998; Michel et al., 1998). Significant NPY levels are found in most brain regions including cerebral cortex, hippocampus, thalamus, hypothalamus, and brainstem. NPY-containing cell bodies have a broad distribution as well. NPY-containing fibres are found in the peripheral nervous system within a variety of organs including the pancreas, intestinal tract, heart, thyroid, lung, kidney, and gonads (Allen et al., 1983). The localization of nerve terminals and cell bodies suggest a wide-ranging role for NPY on behavior and physiology. In the periphery, NPY functions as a potent vasoconstrictor and has effects on reproduction, on the gut, and on the gastrointestinal and renal epithelial secretion. In the CNS, NPY seems to be implicated in feeding (food intake) and obesity, anxiety, nociception, memory retention, seizure susceptibility, neuronal excitability, circadian rhythm, inhibition of insulin release, metabolism, and human psychiatric disorders such as schizophrenia and depression (Gehlert, 1998; Kalra et al., 1999; Kask et al., 2002; Wettstein et al., 1995). Furthermore, NPY regulates the secretion of various hypothalamic neuropeptides, stimulates the corticotropic axis and has potent inhibitory effects on the gonadotropic and somatotropic axis. Interestingly, studies with NPY knockout animals negotiate some of the assumed effects of endogenous NPY (Bannon et al., 2000; Erickson et al., 1997; Erickson et al., 1996). NPY is stored in synaptic vesicles often with classical neurotransmitters such as norepinephrine. The release of NPY is prejunctionally regulated by endogenous noradrenaline acting on α_2-adrenoreceptors and is increased in response to stress (Colmers and Wahlestedt, 1993).

1.5.2 Receptors for NPY

All cloned NPY receptor subtypes [Y_1, Y_2, Y_4, Y_5, and y_6 - the latter is not present in rats and its function is obscure in mice and humans (pseudo-receptor)] belong to the large super-family of G-protein-coupled heptahelical receptors. Y_1 and Y_2 receptors are the major receptor subtypes expressed in the rat brain (Blomqvist and Herzog, 1997; Parker and Herzog, 1999). DPPIV rapidly cleaves the first two N-terminal amino acid residues (Tyr-Pro) with high turnover rates from native NPY. The resulting C-terminal fragment NPY_{3-36} has a markedly reduced affinity to the NPY Y_1 receptor subtype, while being as potent as the native peptide on the NPY Y_2 and Y_5 receptor subtype (although the receptor affinity is dependent on the endogenous level of NPY_{3-36}). In the present study it is hypothesized that the loss of DPPIV activity in the mutant F344 substrains leads to changes in the NPY catabolism. We expect that in the wildtype-like F344/Crl(Por) rats DPPIV converts NPY to NPY_{3-36} and therefore to an agonist of Y_2 and Y_5 receptors, but terminates NPY´s action at the Y_1 receptor (De Meester et al., 2000; Mentlein, 1999). In the mutant DPPIV-deficient substrains F344/DuCrj(DPPIV-) and F344/Crl(Ger/DPPIV-) NPY should not be cleaved and, therefore, still exhibits its high affinity to the Y_2, Y_5, and especially the Y_1 receptor and acts prolonged at the latter one (Fig. 1). But since these rats could be regarded as “germline-knockout” animals, developmental compensation of the enzyme deficiency on various levels (including up-regulation of related enzyme systems or down-regulation of NPY receptor expression) may have taken place.

Fig. 1: hypothesized differential cleavage of NPY in the different F344 substrains.



The Y_1 receptor is the best-characterized receptor for NPY and predominates in the cerebral cortex, the thalamus, the hypothalamus, and in certain nuclei of the amygdala (Dumont et al., 1998). The Y_2 receptor subtype is found in the hippocampal and hypothalamic formation, the septum, and nuclei of the brainstem (Bischoff and Michel, 1999). Y_5 receptor mRNA has been reported in several brain structures, including the limbic system (paraventricular hypothalamic nucleus, lateral hypothalamus, and arcuate nucleus) and the brainstem. Interestingly, Y_5 receptor mRNA expression always coincides with the presence of Y_1 receptor mRNA (although not *vice versa*), possibly due to the overlapping organization of both receptors (Bischoff and Michel, 1999).

One of the earliest discovered central effects of NPY was a profound increase in food intake along with an involvement of the peptide in body weight regulation (Kalra et al., 1999). Recent studies suggest that this response is likely to be mediated by the Y_1 receptor (Inui, 1999; Kushi et al., 1998; Pedrazzini et al., 1998), although considerable numbers of investigations also reported the Y_5 receptor as being an appetite receptor (Bischoff and Michel, 1999; Gerald et al., 1996; Inui, 1999; Marsh et al., 1998). The distribution of the Y_5 receptor in the CNS hints to its role in hypothalamic processes such as feeding. Very recently, also the Y_2 receptor has been discussed in regard to its feeding (food intake) regulation (Inui, 2000; Kaga et al., 2001; Naveilhan et al., 1999). Interestingly, patients with anorexia nervosa and bulimia show altered levels of NPY in the CSF (Gehlert, 1998; Kalra et al., 1999). In addition to its effects on feeding behavior, NPY seems to produce potent anxiolytic-like effects in animal models of anxiety (Broqua et al., 1995; Heilig et al., 1989; Kask et al., 2002). This effect appears to be mediated via specific Y_1 receptors localized primarily in the amygdala (Heilig, 1995; Heilig et al., 1993; Kask et al., 2001a; Sajdyk et al., 1999; Wahlestedt et al., 1993). Central administration of NPY also reduces locomotor activity and homecage activity in rodents interpreted as sedation, presumably mediated also through the Y_1 receptor activation (Heilig and Murison, 1987; Heilig et al., 1988; von Horsten et al., 1998a). The antinociceptive effect of NPY (mediated by Y_1 and perhaps Y_2 receptors) seems to be particularly dependent on either the intensity or the modality of the painful stimulus (Lecci, 2001; Naveilhan et al., 2001b; Wettstein et al., 1995). In addition, very early studies on NPY found that the neuropeptide probably improves memory retention via Y_2 receptors (Clearly et al., 1994; Flood and Morley, 1989; Morley and Flood, 1990). The same receptor seems to be involved in the regulation of the circadian rhythm of rodents (Biello, 1995; Biello et al., 1994; Biello and Mrosovsky, 1995; Calza et al., 1990; Golombek et al., 1996).

Furthermore, NPY influences the neuronal excitability, which is proved by its protective effect against seizure activity. This could be mediated through Y_2 and Y_5 receptors (Baraban et al., 1997; Erickson et al., 1996; Vezzani et al., 1994; Woldbye et al., 1997). NPY and its Y_1 receptor are also involved in the neurobiological response to the sedative effect of ethanol and the ethanol preference (Kelley et al., 2001; Thiele and Badia-Elder, 2003; Thiele et al., 2002; Thiele et al., 1998; Thiele et al., 2000).

In humans, levels of NPY in the temporal cortex (a region, whose dysfunction may be related to schizophrenia) of schizophrenic patients are reduced (Colmers and Wahlestedt, 1993). Furthermore, NPY levels in the CSF seem to be negatively correlated with anxiety scores in patients diagnosed with major depression (Colmers and Wahlestedt, 1993). Reduced concentrations of NPY have been measured in the brain and CSF of suicide patients and antidepressants have been reported to increase NPY levels in rodent brains (Redrobe et al., 2002).

To prove our hypothesis that differences in the behavioral and physiological phenotype between the wildtype-like and DPPIV-deficient F344 rats could be based especially on differences in the metabolism of NPY and in receptor specificities, in a third experimental step of this study, we analyzed the effect of i.c.v. administration of different doses of NPY (0.0/0.2/1.0 nmol) in the wildtype-like F344/Crl(Por) and in the DPPIV-deficient F344/DuCrj(DPPIV-) and F344/Crl(Ger/DPPIV-) rats on behavioral tasks like feeding (food intake), anxiety, and nociception (Karl et al., 2003d, Karl et al., 2003e).

1.6 Aims of the present study

Because of the influence of DPPIV activity on several substrates, especially NPY (and associated changes in NPY receptor specificity) and possible resulting effects on different behavioral and physiological processes, we hypothesize differences in the phenotype of wildtype-like and DPPIV-deficient F344 rats. Therefore, we first investigated the genetics and variations of DPPIV activity in different inbred rat strains, especially in F344 substrains. Furthermore, we validated the functional relevance of these differences in DPPIV-like activity in regard to the glucose homeostasis and the NK cell function (Karl et al., 2003b). In a second step, a systematic behavioral phenotyping was performed based on a multi-tiered strategy (Karl et al., 2003a), which was focused on behavioral and physiological domains, which are well-known to be influenced by DPPIV substrates such as GLP-1, enterostatin, endomorphin-2, substance P, and especially NPY (Table 1). For this purpose, the different substrains were screened in various experiments (Table 2) to get a largely complete overview about their

behavioral and physiological phenotype (Karl et al., 2003c). To investigate the hypothesis if differences in the phenotype between mutant and wildtype-like rats could be associated with differences in the NPY metabolism and receptor specificity (Fig. 1), in a third step, animals were tested in selected paradigms after i.c.v. treatment with different doses of NPY (Karl et al., 2003d) and additionally after i.c.v. treatment with the DPPIV inhibitor isoleucyl-thiazolidide *Ile-Thia* (Karl et al., 2003e). More potent effects of NPY in the DPPIV-deficient rat substrains would suggest that observed differences in behavior and physiology between mutant and wildtype-like F344 substrains would be at least particularly caused by a DPPIV-dependent differential degradation of NPY (or other DPPIV substrates).

Behavioral phenotyping of mice in pharmacological and toxicological research

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Abstract

The evaluation of behavioral effects is an important component for the *in vivo* screening of drugs or potentially toxic compounds in mice. Ideally, such screening should be composed of monitoring general health, sensory functions, and motor abilities, right before specific behavioral domains are tested. A rational strategy in the design and procedure of testing as well as an effective composition of different well-established and reproducible behavioral tests can minimize the risk of false positive and false negative results in drug screening. In the present review we describe such basic considerations in planning experiments, selecting strains of mice, and propose groups of behavioral tasks suitable for a reliable detection of differences in specific behavioral domains in mice. Screening of general health and neurophysiologic functions (reflexes, sensory abilities) and motor function (pole test, wirehang test, beam walking, rotarod, accelerod, and footprint) as well as specific hypothesis-guided testing in the behavioral domains of learning and memory (water maze, radial maze, conditioned fear, and avoidance tasks), emotionality (open field, hole board, elevated plus maze, and object exploration), nociception (tail flick and hot plate), psychiatric-like conditions (porsolt swim test, acoustic startle response, and prepulse inhibition), and aggression (isolation-induced aggression, spontaneous aggression, and territorial aggression) are described in further detail. This review is designed to describe a general approach, which increases reliability of behavioral screening. Furthermore, it provides an overview on a selection of specific procedures suitable for but not limited to behavioral screening in pharmacology and toxicology.

Keywords: Mouse - Behavioral phenotyping – Neurological reflexes – Motor functions – Learning and memory – Emotionality - Anxiety – Nociception – Psychiatric-like conditions - Aggression

2.1 Introduction

The detection of discrete behavioral difference in rodents is a time consuming, cost intensive and laborious task in current biomedical research. Furthermore, scientists are challenged by a large amount of different techniques and may experience problems in developing a meaningful and rational strategy in planning their experimental designs. Several experimental steps must actually precede before hypothesis-driven behavioral differences in a specific behavioral domain can reliably be evaluated. This presumption has re-gained considerable attention during the past ten years since many researchers were faced with an unknown phenotype in their transgenic or knockout mice (CRAWLEY and PAYLOR 1997). However, the development of a "multi-tiered" strategy in the field of behavioral phenotyping of transgenic and knockout mice (CRAWLEY 1999) has generated certain novel implications for the general approach of behavioral testing, which are also important in screening for epigenetic, pharmacological, and toxic effects of novel compounds on behavioral performances.

Uncoordinated experimental approaches in applying behavioral tests for specific behavioral domains bear a high risk of false negative and/or false positive results, if not a basic phenotyping does precede such tests. For example, if a compound induces blindness during development of mice, these mice perform badly in spatial learning tasks such as the Morris water maze or the radial maze. Both of these tests code for the behavioral domain "learning and memory" and essentially rely on the ability of rodents to use and to store visual information. The interpretation of differences in a spatial learning task as "learning impairments", thus, is only valid if the mouse can properly see its surroundings and thereby develop spatial memory. Another example from recent studies in Huntington transgenic mice (CARTER et al. 1999), is diabetes mellitus, which – if remains undetected - most likely interferes with motoric and cognitive functions of mice. The consequence of this consideration is that every firm conclusion based on certain difference in a specific behavioral performance must be substantiated by the proof that the experimental animals are equipped with the corresponding sensory and sensorimotoric abilities. Otherwise, the finding bears a high risk to be false positive or false negative.

Therefore, every experimental design in drug screening and toxicological research should include a proper evaluation of general health, sensory functions, and motor abilities of mice, before specific behavioral domains are tested. If this is done, the resulting "multi-tiered" rational strategy minimizes the danger of misleading findings.

In the present review we describe an approach based on these basic considerations in planning

experiments and furthermore propose groups of behavioral tasks suitable for a reliable detection of drug or toxicological effects on the behavior of mice (summarized in Table 1). Screening of general health and neurophysiologic functions (reflexes, sensory abilities) and motor function (pole test, wirehang test, beam walking, rotarod, accelerod, and footprint) as well as specific hypothesis testing in the behavioral domains of learning and memory (Morris water maze, radial maze, conditioned fear, and avoidance tasks), emotionality (open field, hole board, elevated plus maze, and object exploration), nociception (tail flick and hot plate), psychiatric-like conditions (porsolt swim test, acoustic startle response, and prepulse inhibition), and aggression (isolation-induced aggression, spontaneous aggression, and territorial aggression) are described in further detail.

2.2 Behavioral tests for a comprehensive behavioral screening

Animals

The mouse strain can greatly affect the behavioral phenotype and this fact actually must be taken into account before the behavioral screening starts. For example, 129/J and 129/SvJ mice exhibit a complete or incomplete lack of the corpus callosum and some C57BL/6J mice produce amyloid plaques, both which make these strains poor learners (HSIAO et al. 1996; WEHNER and SILVA 1996).

2.2.1 Systematic behavioral phenotyping

First steps and preliminary observations

The first step in a behavioral analysis is to obtain a sufficient number of animals for each treatment group. Breeding problems often limit the availability of sufficient numbers of individuals. It is also important that the test animals are in the same range of age (differences of up to 10 days in age can be tolerated). For most behavioral paradigms, animals have to be adult (at least 70 days of age) to obtain general and reproducible results, which are not influenced by endocrinological processes during ontogenetic development. In general, 10 to 12 mice of each treatment group or genotype should be used to minimize derivations within one group because of individual differences possibly occurring between animals (CRAWLEY and PAYLOR 1997). Furthermore, it is extremely important that each animal is screened for general health, home cage behavior, sensory abilities, and motor performance (see below). Usually this latter point is disregarded, if the animals are obtained from commercial breeders. Normally, researcher can rely on a constant genotype of mice provided by commercial

breeders. However, very recently we observed a segregation in the genotype of F344 rats obtained from a commercial breeder (KARL et al. 2003). Thus, at least in the case that a large research program will be based on a particular strain, a screening of all animals provides important information on several baseline performances of the particular genotype and might allow also to confirm this phenotype at later stages. Furthermore, if the ultimate hypothesis-driven specific behavioral domain is "emotionality", also observations of the social status and aggression of each mouse may provide important additional information.

Neurophysiology and health

It is extremely important to detect any gross abnormalities, sensory, or motor deficits in the animals that will interfere with further behavioral testing. Therefore a "physical exam" is required (CRAWLEY 1999). Illness (signs are, e.g., poor grooming, labored breathing, very low body weight or other easily observable symptoms) will compromise the performance of the animals in different tests. Indices of general health are obtained by measuring body weight and rectal temperature and recording observations of any abnormal physical features (like poorly groomed fur, bald patches in the coat or absence of whiskers, which can lead to unusual home cage behavior). Illness can lead to increased aggression, hypoactivity, or also hypersensitivity to handling (CRAWLEY 1999). Furthermore, a blind or deaf mouse will not perform tasks, which require visual or auditory cue perception and discrimination. Therefore it is important to control sensory abilities and neurological functions. The following battery of tests, which are useful for detecting severe neurological dysfunctions, can be used (CRAWLEY 1999; PICCIOTTO and WICKMAN 1998):

1. The animal is placed in an empty cage for 3 min to record abnormal spontaneous behavior (like *wild-running*, excessive *grooming*, *freezing*).
2. The response to an approaching unknown object is observed (normally, the mouse will sniff or approach the object, will then turn away and avoid this object – abnormally, the mouse will attack the object or ignore it).
3. modificated "visual cliff": the mouse is placed onto the center of a platform. The latency to reach the edge of the platform (normally less than 10 s) and the frequency of *dipping the head over the edge* (normally several times a minute) is measured.
4. A simple olfactory test can be used (e.g. latency to locate an odiferous piece of food, buried under the litter in a clean test cage - or time spent *sniffing* a novel odor painted onto the test cage wall, i.e. vanilla or lemon extract) to measure the olfactory abilities of the animal.

5. Measuring of several reflexes (CRAWLEY and PAYLOR 1997; MIYAKAWA et al. 2001):
    a) Balance: The mouse is placed in an empty cage, which is rapidly moved from side to side and then up and down. The normal postural reflex is to extend all four legs in order to maintain an upright, balanced position.
    b) Righting reflex: The postural reflex is measured. The animal is turned on its back and the time to right itself to an upright position is measured. Mice normally will right themselves up within seconds.
    c) Eye blink reflex and ear twitch reflex: These paradigms are measured by simply touching the eye with a cotton-tip swab and by slightly pinching the tip of the ear with a tweezers.
    d) Whisker-orienting reflex: The whiskers of a freely moving animal are touched lightly. Normally, the continual moving of the whiskers will stop and in many cases, the mouse will turn its head to the side of the stimulus.
    e) Constriction and dilatation reflex: This test determines the visual response of the pupil to light. In a dimly lit room, a penlight or a small flashlight beam is directed at the eye and the constriction and following dilatation (when the light is removed) of the pupils are observed.
6. It is also important to observe the behavior of the mouse in the home cage (without any influences): locomotion, grooming, nesting, sleeping, reproductive (lactation and reproductive success) and aggressive behavior as well as playing behavior of pups.

On the basis of these considerations it can be concluded that each behavioral investigation should start with a basic behavioral phenotyping. The resulting information about the animal's health, neurophysiology, and sensory abilities are extremely important for all further experiments. This characterization should not only be performed prior to studies regarding the behavioral phenotype of animals but also prior to physiological measurement, because of the influence of health and neurophysiologic functions on both, the behavior and the physiology of animals. A systematic behavioral phenotyping must be a first step in research and all the different tasks mentioned above should be screened comprehensively.

2.2.2 Motor functions

Locomotion is a complex behavior affected by many different brain systems, including the telencephalic dopaminergic system and the cerebellum, as well as by peripheral abnormalities

(i.e. muscle weakness). Because locomotor activity is required for many complex behavioral tasks, increases or decreases in locomotor activity nonspecifically affect performance in many behavioral tests and should be measured before any other behavioral characterization is performed. The following tests are not only a sensitive control for more complex behavioral tasks, but also a useful tool to study cerebellar or dopaminergic functions. Several aspects of locomotor activity can be measured in parallel with tests for the response to novelty, exploratory behavior, or the locomotor response to drug treatment. All measures of activity are also sensitive to the circadian clock. Therefore, the experiments should be performed and compared under consideration of the circadian rhythm of the animals. The following list provides a considerable number of tests, which – if performed in a group – provide a rather comprehensive picture on the various aspects of motor functions.

Pole test

The pole test was developed in 1985 (OGAWA et al. 1985) to measure bradykinesia (slowed down movement ability). The mouse is confronted with a situation, in which it has to turn round and to climb down a pole. For this a wooden stick (diameter: 1 cm; length: 50 cm) with a cork ball on its top (diameter: 1.5 cm) is installed vertical on a heavy platform (SEDELIS et al. 2000). The mouse is placed directly under the ball at the top - the head held upwards. The time to turn round and to reach the platform at the bottom is measured (cut-off time: 120 s). If the animal slides down the wooden stick without active climbing or turning round, both parameters are recorded as 120 s. The apparatus is cleaned after each trial with 70% ethanol. Healthy animals can climb down the pole in 10-20 s. After loss of dopamine in the striatum the behavior is slowed down or vanishes completely.

Beam walking test

Motor coordination and balance of mice are assessed by measuring the ability of the mice to traverse a graded series of narrow beams to reach an enclosed safety platform (PERRY et al. 1995). The beams consists of long strips of wood (1 m) with 28 mm, 12 mm, or 5 mm square cross sections and/or 28 mm, 17 mm, or 11 mm round diameters (CARTER et al. 1999). The beams are placed horizontally, 60 cm above the floor, with one end mounted on a narrow support and the other end attached to an enclosed box (20 cm square) into which the mouse can escape. Two lights (60 W) are positioned above the beam and to the narrow support (start of the beam). During training, the mouse is placed at the start of the 12 mm square beam and trained over 3 days (4 trials per day) to traverse the beam to the enclosed box. Once the mouse is trained (i.e. crossing of the 12 mm square beam in less than 20 s) it receives two consecutive trials on each of the square and round beams, in each case progressing from the

widest to the narrowest beam (cut-off time: 60 s). The latency to traverse each beam and the number of times the hind feet slip off each beam are recorded for each trial. Analysis of each measure is based on the mean scores of the two trials for each beam. To avoid influences of possible differences in the learning abilities of the mice the task can also be realized without any training session. Experimenters may experience with the motivation of animals to traverse the beams. In this case it may help to use the home cage of each animal as the escape box.

Wirehang test

Neuromuscular strength is tested by the wirehang test (CRAWLEY 1999; MIYAKAWA et al. 2001). The mouse is placed on a wire cage lid and the lid is gently waved in the air so that the mouse grips the wire. The lid is then turned upside down approximately 50 cm above the surface of soft bedding material. The latency to fall onto the bedding is recorded (cut-off time: 60 s).

Rotarod and accelerod

The rotarod/accelerod is the widely used test of neuromotor performance – a balance task performed on a motor-driven, rotating rod. It was developed in 1968 (JONES and ROBERTS 1968). Motor coordination, balance and ataxia can be tested with the rotarod test. The rotarod measures the ability of the mouse to maintain balance on a rotating rod (BARLOW et al. 1996). Thus, the fore- and hind limb motor coordination and balance can be analyzed. This task requires an intact cerebellar function and motor coordination (CARTER et al. 1999). Mice with severe motor coordination problems will have difficulties to remain on the rotating rod. The motivation to stay on the rod can be increased by raising the rod to greater heights above a soft landing surface. Two methods have been established:

1) Rotarod: each mouse is placed on the rotating rod and the time to fall off is measured (cut-off time: 60 s). Different constant rotation speeds are used in this paradigm.
2) Accelerod: an accelerating rotarod allows the rotation speed to be constantly increased from 4 to 40 revolutions per minute (rpm), over a 5 min period. In the accelerating rotarod test the latency and the rotation speed, at which the animal falls off the rod, are recorded.

The accelerod is more sensitive than the rotarod in detecting motor function deficits, i.e. induced by acrylamide or ethanol.

Training: In order to exclude difference in motivation and motor learning, it is very important to sufficiently train the animals before testing. During the training phase for 2-4 consecutive days each mouse is placed on the rotarod at a constant speed (12 rpm) for a maximum of 120

s and the latency and frequency to fall off the rotarod within this time period is recorded. During the 120 s of the training trials, the animals are instantly replaced on the rotarod.

Testing: a) Rotarod: Animals are subjected to two consecutive test sessions (trials) on each level (ranging from 4-40 rpm in 4 rpm steps) with an intertrial interval of 1 h for four consecutive days (SEDELIS et al. 2000). The average of the latency to fall off the rotating rod of the two trials per day per rotation speed level is recorded. b) Accelerod (can follow directly after the training or after the rotarod test session): Duration of each trial is in the maximum 5 min. During this time, the rotation speed is constantly increased, i.e. from 4 rpm to 40 rpm within four and a half minutes. The latency to fall off the rod and the actual rotating speed level are measured. The animals are tested in two trials per day for four consecutive days with an intertrial interval of 1 h. The average latency of falling off the rod and the average actual rotating speed per day are recorded.

Footprint test

The footprint test evaluates the walking pattern of mice and thereby allows gait abnormalities to be detected. Footprint patterns of mice are analyzed after the mouse was walking along a narrow corridor (BARLOW et al. 1996). To obtain footprints, the hind and fore paws are coated with blue and black non-toxic paints. The mouse is then placed at one end of a dark tunnel (10x10x50 cm). A light (60 W) is positioned above the starting point of the tunnel. All animals have (optional) three training runs before the test session, which is performed during the dark phase of the light cycle. The mouse has to walk along the narrow, dark corridor (the motivation of the animal to walk along this tunnel can be increased by stimulation with food rewards). The footprints are recorded on a sheet of white paper that is placed on the floor of the tunnel. The footprint patterns are analyzed for three step parameters (CARTER et al. 1999):

1) The stride length is analyzed by measuring the distance between each step on the same side of the body (distance between one right front or hind footprint and the next right front or hind footprint).
2) The hind-base and front-base widths are measured as the average distance between left and right hind footprints and left and right front footprints.
3) The distance from front footprint/hind footprint overlap is used to assess uniformity of step alternation. When the center of the hind footprint falls on top of the center of the preceding front footprint, a value of zero is recorded (when the footprints do not overlap, the distance between the centers of the footprints is recorded).

For each parameter, three values are measured of each run, excluding footprints made at the beginning and the end of the run (because of initiating/finishing movements). The mean value of each set of three values is used in subsequent analyses.

Open field

(see section "Emotionality/Anxiety and exploration")

Thus, several tests are available for the characterization of motor functions in rodents. As part of a systematic behavioral phenotyping, the pole test, the wirehang test, and the accelerod test should be realized to control the basic motor functions in mice. These tasks can be performed easily and briefly and detect gross abnormalities in natural climbing abilities, neuromuscular strength, motor coordination, and balance abilities. If a study concentrates on investigations regarding possible motoric dysfunctions, additionally, the beam walking test, the rotarod task, and the footprint paradigm should be included in order to obtain a more comprehensive analysis of the mice´s motor abilities. The open field is an additional and important tool for analyzing the locomotion of rodents. However, open field behavior of mice and rats is affected by two behavioral dimension, activity and emotionality/anxiety (DEFRIES et al. 1966; VON HORSTEN et al. 1998; WHIMBLEY and DENENBERG 1967). The interpretation of results is confounded by these two underlying constructs. Therefore, this test usually should not be used as a single measure of activity or anxiety.

2.2.3 Learning and memory

Learning is a complex phenomenon subserved by the activity of many brain regions. Some aspects of learning that can be measured in rodents include attention, working memory (the short-term memory used while a task is being performed), memory consolidation, and reference memory (the long-term memory, which lasts from 24 h to the lifetime of the animal). Various tests have been developed that evaluate preferentially one or another of these aspects of learning (HODGES 1996).

Morris water maze

Hidden platform version of the Morris water maze (MORRIS 1981; OWEN et al. 1997):

This is a task for measuring spatial memory. Each mouse learns to swim in a circular pool of water to locate a submerged hidden platform. On the first day the mouse is alternated between standing on this platform and swimming for 10 s in the pool to familiarize the animals with the test apparatus. The mouse is given three blocks consisting of four trials each for a total of three days. On each trial, the starting position is randomized between four possible positions. The platform's location remains constant throughout training for each mouse but is rotated

over three possible positions between treatment groups or genotypes. Each trial lasts 60 s or until the animal locates the platform. Mice that do not find the platform are guided to the platform and given a latency score of 60 s. All animals receive a 10-15 s rest period on the escape platform between trials. The time to reach the platform (latency to escape) is recorded for each trial (LIONE et al. 1999). Between blocks, the animals are placed individual in a heated enclosure (metal holding cages that have a single paper towel at the bottom under a heat light).

After training on the third day, animals are given a 60 s probe trial during which the platform is removed from the pool. The probe trial starts from a start position opposite to the quadrant that originally contained the platform. The number of times the mouse crosses each of the four possible platform positions (platform crossings) is calculated from a videotaped recording of the probe trial. Then the preference score is calculated. It is defined as the number of crossings over the trained position minus the mean number of platform crossings over the other three positions. Therefore, a higher preference score indicates better spatial selectivity for the trained platform location.

Cued (visible platform) version of the Morris water maze (OWEN et al. 1997):

A white plastic sail (5 cm^2) is attached to the platform and sits approximately 12 cm above the water surface. The sail is outlined with black electrical tape to provide visual contrast. Other than the following two exceptions, the training procedure is identical to the spatial (hidden platform) version of the task: 1) training takes place on only two consecutive days and 2) both the visible platform's position and the animal's starting position are randomized between each trial.

Radial maze

Radial mazes are frequently used to study hippocampal function, and different procedures permit to dissociate spatial and non-spatial memory capabilities in rodents (CRUSIO et al. 1987). In the radial arm maze paradigm, rodents are trained to visit a pattern of arms in an 8 arm maze to receive a food reward (for this food deprivation of the mice is necessary). The animal must keep the egozentric or allocentric/spatial information, which arms of the maze it has already visited during the course of the task, to perform well. Recent developments of mazes often try to assess choice accuracy for information presented within the day's test session, and learning that has accumulated across days of testing. These two different types of tests are often referred to as working memory (WM) and reference memory (RM) tasks respectively (OLTON and SAMUELSON 1976). Nowadays most tasks are particularly geared towards measuring working and reference memory.

The original radial arm maze consists of an octagonal central area from which 8 arms radiate outwards, like spokes around a hub (HOLSCHER and SCHMIDT 1994). Optional, all arms can be closed by doors so that the mouse can be forced to stay in an arm or the center platform for a defined time. The maze is elevated above the ground, and has no high walls at the sides so that the room view is unobstructed. The maze is normally placed in a well-lit room containing several other objects. During all experiments the maze is kept in a constant position in the room (BUBSER and SCHMIDT 1990). At the outer end of each arm a food cup is located, which can be baited before each test. The cups are not rebaited during the test session. A food-deprived mouse is placed into the maze, and quite rapidly learns to avoid choosing arms it already visited during the test session. It seems that mice can learn to visit each spatial location once, and remember the locations they visited (SUZUKI et al. 1980).

Up to 2 weeks before starting the maze experiment, the food-deprivation schedule should be introduced gradually. Body weight should be kept at 80-90% of the pre-test body weight (MIYAKAWA et al. 1996). The mice should also be accustomed to the reward. Furthermore, mice should be picked up and put down so that they are used to the handling procedure (RAWLINS and DEACON 1993).

There are some factors, which influence the spatial memory in the radial maze: a) motivation, b) stress and anxiety, c) age and gender (TROPP and MARKUS 2001; WINTER 1997). The operator must clean the apparatus after each trial, because mice use also olfactory cues to orientate in the maze (HODGES 1996).

Working memory:

In the training phase, food pellets are scattered along the arms. The mouse is placed in the center of the maze and can explore the apparatus for 10 min. On the consecutive days food is increasingly restricted to the further ends of the arms until on day 4 it is only available in the food cups.

In the following test phase (day 5-18), the mouse is placed into the central area of the maze. All arms are baited. The tests ends either if the mouse has collected all pellets or after a maximum of 5 min. The chosen arms are recorded (if it is a previously visited arm – WM error) and numbers of right choices in the first eight tries, number of errors, the latency for the first arm entry, the total time for collecting all pellets, entry angles, and ratio of time and errors are analyzed.

Reference and working memory:

Animals are trained and tested like in the working memory schedule. But in this schedule only four arms are baited at the beginning of the test session – the other four arms are never baited.

Learning to avoid selecting these never baited arms constitutes a RM task. A reentry in an already visited arm is recorded as a WM error. To avoid errors in the results because of possible simple learning patterns (LANKE et al. 1993), each treatment group or genotype should be split in two groups so that baited arms for the one group constitute unbaited arms for the other group and baited and unbaited arms should be chosen randomly and not alternating.

Conditioned-fear paradigm

Cued and contextual conditioning is a fear-conditioning task that measures memory of an aversive experience and the stimuli present during this aversive experience. A standard foot shock shuttle box is used to control foot shock delivery and to measure the duration of freezing behavior (OWEN et al. 1997). *Freezing*, a standard response to a sudden aversive stimulus, is defined as a complete behavioral immobility except for natural respiratory motions (STIEDL et al. 2000; STIEDL et al. 1999; STIEDL and SPIESS 1997).

Day 1: The first day of training consists of pre-exposure. The mouse is placed in the chamber and left there for 10 min to explore the environment.

Day 2: 24 h later the animal is returned to the same test chamber. The mouse is allowed to explore the context for 2 min. During this time, baseline-freezing behavior is observed at 10 s intervals. After 2 min, an 80 dB auditory clicker stimulus sounds for 30 s immediately followed by a 0.35 mA foot shock (2 s duration). This pairing is repeated after a 2 min intertrial interval. Baseline-freezing is only measured before the first shock is administered. The mouse is returned to its home cage 30 s after the second foot shock.

Day 3: 24 h later, the mouse is returned to the testing chamber. During the 5 min contextual phase, the animal's contextual-freezing behavior is examined every 10 s. No auditory stimulus is presented at this time. Approximately 1 h later the mouse is returned once more to the now altered testing chamber (Plexiglas floor instead of grid floor) so that associations to the testing context are reduced. Furthermore, a divider is placed in the cover restricting the animal's space to a small triangular area. Olfactory cues are changed by placing approximately four drops of liquid orange extract in the corner of the testing chamber, out of the animal's reach. Once in the altered context, the animal is observed for 6 min. During the first 3 min, no auditory stimulus is presented (pre-CS). During the last 3 min, the auditory stimulus of Day 2 is presented (CS). The freezing behavior is examined at 10 s intervals for the entire 6 min testing period. A total percent freezing score is calculated for each animal at each of the four time periods tested: baseline, contextual, pre-CS, and CS (OWEN et al. 1997; VAN GAALEN and STECKLER 2000).

Active and passive avoidance

Maze learning and avoidance tasks are among the oldest learning and memory tasks used in rodents. Passive and active avoidance tasks measure the memory of an aversive experience through the simple avoidance of a location, in which the aversive experience occurred. A commercially available automated apparatus consists of two connected chambers (for the passive avoidance task: one lighted, one dark). The animals will readily learn an avoidance response only if this response is closely related to the animals species-specific defensive behavior.

Passive avoidance:

Passive avoidance is a two-day task. This one-trial test of aversive memory pairs a mild foot shock with the entry into a dark chamber (BOVET et al. 1969). This task depends on fear of the foot shock and involves activation of the amygdala. Lesion studies and dopaminergic antagonists have also shown that both passive avoidance and active avoidance, in which the mouse is trained to avoid a shock, are dependent on an intact striatal function. Mice tend to prefer a dark, not brightly illuminated environment and will immediately enter the dark chamber when placed in the lighted chamber of the apparatus (CHAOULOFF et al. 1997; CRAWLEY and GOODWIN 1980). The animals should not be tested in the light phase because then they do not show an aversion to the lighted test box. The conclusion that a brightly lit, white environment is aversive to mice and inhibits their exploratory behavior is based on data, which are recorded in the dark phase of the animals (COSTALL et al. 1989).

Day 1: In the training session, the mouse is placed in the lighted chamber for 10 s. The door to the dark chamber is then opened, and the latency to enter the dark chamber is measured as a control for visual ability and motor activity. Immediately after the mouse entered the dark chamber, a 0.3 mA, 1 s foot shock is delivered. The animal remains in the dark compartment for further 10 s to allow the formation of an association between the dark compartment and the received foot shock. The mouse is then returned to the home cage.

Day 2: In the retention test session, the mouse is placed in the lighted compartment and the door is opened. The latency to enter the dark compartment is measured (cut-off time: 300 s). The mouse is then returned to its home cage.

Active avoidance:

Two-way active avoidance is a type of conditioning that results in associative learning. Essentially the animals learn to avoid a signaled noxious stimulus (electrical foot shock – signaled by light or sound) by initiating a specific locomotor response (moving to the other compartment of the apparatus). In the other compartment the signaled noxious stimulus

occurs again. This procedure is repeated several times. The task contains elements of conflict because the animal has to initiate a response towards a location where it previously experienced a noxious stimulus (WADENBERG and HICKS 1999). Hence speed of conflict resolution, anxiety, and fear are elements that influence avoidance probability. These seem to be especially important in the early phase of acquisition and have led to the use of this paradigm also as a model of anxiety. Anxiolytic drugs improve learning and performance in the avoidance task at low doses, probably due to the stress-reducing effect of anxiolytic agents.

For active avoidance, using the same chambers as in the passive avoidance task, the mouse must move into the opposite chamber after the presentation of a light or sound stimulus to avoid receiving the foot shock. This procedure is repeated twice a day for several days. The latency to enter the non-shocked chamber is the measure of learning.

Object exploration

(see section "Emotionality/Anxiety and exploration")

In conclusion, different learning paradigms measure different types of cognitive processes. The Morris water maze and the radial maze appear to be very suitable and well-validated tasks for spatial memory. However, the mouse is forced to swim in the Morris water maze test (which induces very high stress levels in mice) while - in contrast - the radial maze test uses food rewards to motivate cognitive processes. It is very likely that testing mice in the water maze represents a more potent stressor for the animals than radial maze testing. Furthermore, it should be kept in mind that stress represents an important determinate of cognitive performance, suggesting that a priori the selection of one of these tests should be based on the level of stress necessary for the experiment. In addition, stress levels must be considered when applying the conditioned-fear paradigm or the active/passive avoidance task. One advantage of the passive avoidance task, when evaluating drug effects, is the brief, two-day-spanning design. In this design the aversive treatment is minimized to only one single event. Finally, it should be kept in mind that in all tests, which are based on painful stimuli such as electrical foot shocks, nociception of the animals has to be analyzed right before the learning paradigm, in order to avoid confounding effects of differences in pain perception on the learning process.

2.2.4 Emotionality/Anxiety and exploration

Open field

Locomotor activity and emotionality can be evaluated by placing the mouse in a square or circular (BADISHTOV et al. 1995) open field arena (OF) under standard room lighting (CRAWLEY 1985). The OF is divided in a peripheral and center area by a grid cross on the floor (SATINDER 1982). This paradigm mimics the natural conflict in mice between the tendency to explore a novel environment and the tendency to avoid a brightly lit open area (DEFRIES et al. 1966). The behavior is also influenced by thigmotaxis (TREIT and FUNDYTUS 1988). Exploratory tendencies, emotional reactivity, or both can motivate a mouse exposed to a novel environment. Crawley (1985) mentioned that anxiolytic agents minimize the activity of the mice in the OF and so the exploratory behavior. The stimulating effect of emotionality on distance traveled in the OF was confirmed by another study (EIKELIS and VAN DEN BUUSE 2000), although Denenberg (1969) discussed an inverse relation between the ambulation and the emotionality of mice.

In the most schedules the mouse is tested only once in the arena for 10 min. In other studies the animals were tested four times and the summed activity and defecation scores were used as a measure of emotionality (WHIMBLEY and DENENBERG 1967). The level of illumination of the OF and also the background noise influences the locomotor activity and emotionality of the animals (WALSH and CUMMINS 1976). Environmental odors should be removed by cleaning the OF after each session to avoid influences of the behavior by odor trials. Other factors, which determine the behavior in the OF, are genetic, experiential and developmental background, biological rhythm, litter size and sex of the test animal – also pre- and post weaning treatment, and observation methods (WALSH and CUMMINS 1976).

The animal's horizontal activity and total distance (number of total square entries), vertical activity (number of *rearings*), and center distance are recorded. The ratio of center distance to total distance and the defecation rate can be taken as a measure of emotionality (DENENBERG 1969). Furthermore, the latency and frequency of *self-grooming* should be recorded. *Rearing* combined with ambulation is proved to reflect a stable individual trait called "nonspecific excitability level". Normally, it is taken as a measure for activity.

Activity can also be scored by its absence. Two major parameters are the latency: 1) to leave the start area (to reach the periphery) or 2) to show freezing behavior (defined as the absence of movement). This is a widely used parameter usually taken as indicative of a high stress

rate. The recording of the OF behavior should be split in 2 min sessions so that also the development of the behaviors during 10 min can be analyzed.

Hole board

The hole board test provides independent measures of locomotor activity and directed exploration (VAN GAALEN and STECKLER 2000). The mouse is placed in a Plexiglas box with four up to sixteen holes equally spaced in the floor. Infra-red photocells directly beneath each hole provide automated measures of the number of *head dipping* and time spent *head dipping* in a 3 min test session (LISTER 1987).

Head dipping or *hole-poking* is a spontaneous elicited behavior in the mouse. It is suggested that the frequency of *head dipping* represents inquisitive exploration, whereas the duration reflects inspective exploration. The hole board is validated pharmacologically: anxiolytic agents increase significantly the number of holes explored in a standard 3 min session (PELLOW et al. 1985).

Elevated plus maze

The elevated plus maze (EPM) is an ethologically-based approach-avoidance conflict test, which is sensitive to anxiolytic drug treatment (CRUZ et al. 1994; PELLOW and FILE 1986). Mice prefer a dark, enclosed, small place over a brightly lit, open, large space. However, mice are also highly exploratory. The EPM represents the natural conflict between the tendency of mice to explore a novel environment and the tendency to avoid a brightly lit open field like in the OF paradigm (MONTGOMERY 1958). The behavior is also influenced by thigmotaxis (TREIT and FUNDYTUS 1988) and the fear of heights. The elevated plus maze is in the shape of a "+". The four arms extend from a central platform. Two alternate arms are dark and enclosed, while two alternate arms are open, lit and optional available with or without ledges. These ledges influence the behavior of mice (FERNANDES and FILE 1996). Additionally, the surface of all arms is raised 1 m above the floor (BALDWIN and FILE 1986).

One of the first elevated plus maze tasks for mice was developed by Montgomery et al. (1955). The hypothesis that novel stimuli generate both an exploratory drive leading to approach behavior and a fear drive leading to avoidance behavior was employed to develop an elevated plus maze, in which the intensity of the fear drive (anxiety) could be measured by the ratio of open and enclosed arm entries (MONTGOMERY and MONKMAN 1955). The open and enclosed arms of the plus maze generate exploratory behavior and the avoidance of elevated open arms is an indication of the intensity of anxiety (HOGG 1996; LISTER 1987). This model was pharmacologically validated (HANDLEY and MITHANI 1984) – anxiolytic drugs were found to increase open arm entries (anxiogenic agents decrease number of

entries). These results were confirmed and extended (FILE 1987; PELLOW et al. 1985; RODGERS and COLE 1993).

The mouse is placed onto the center field of the "+" (faced to an open arm) and is allowed to explore the maze for 5 min. Anxiety can be measured by the time spent on open arms as well as the percentage of open arm entries (HOGG 1996; PELLOW et al. 1985; PELLOW and FILE 1986). These parameters are inversely related to anxiety. The number of total arm entries reflects also the general motor activity. Also the number of enclosed arm entries and *rearing* seem to reflect general motor activity (FILE 1986). Furthermore, *head dipping* over the edges of the open arms, *risk-assessment*, *self-grooming* and the defecation rate should be recorded. A mouse is taken to have entered an arm when all four legs are on the arm. After each session the apparatus has to be cleaned. Testing should be take place during the dark phase, starting 1 h after the lights had been turned off. Dim illumination can be provided by a red light. Behavior should be video-recorded.

Object exploration

While many authors have emphasized the attractive properties of novelty, others have shown that an animal, if given the opportunity, will avoid novel stimuli (BARNETT 1975; VAN GAALEN and STECKLER 2000). In another study (MISSLIN and ROPARTZ 1981) the number of distinct contacts to a novel object in a familiar environment is significantly lower as if the animal is exposed to a novel object placed into a novel environment (COWAN 1976). Mice show in a familiar environment very few approach responses to a novel object and often push sawdust towards it (reason: contrast of familiar background and unfamiliar configuration). Thus, a novel object placed in a familiar environment releases avoidance responses in mice. But because of the attractive properties of novelty also *rearing* and *stretch-attend postures* play a role in the control of the responses towards the novel object (*stretch-attend posture*: directing the nose to the object within a distance of ≤ 2 cm and/or touching it with the nose – the body is stretched). In the object exploration task, the animal is confronted with two identical objects. After a habituation phase, one object is replaced by a novel object and the behavior of the mouse is recorded. Thus, this task investigates the emotionality (by placing a new object in a familiar environment) and the memory ability (by recognizing the novel object) of the animal.

Day 1: In this paradigm the mouse is first habituated to a conventional open field area. Habituation lasts for 10 min. Subsequently, the animal is briefly returned to its home cage.

Day 2-3: 24 h after the habituation trial without a novel object, the mouse is exposed to two identical objects, placed in opposite corners (quadrants) of the open field area, for 10 min. This procedure is repeated on the following day.

Day 4: The mouse is exposed again to two objects, one from the previous trial (familiar object) and a new object (novel object), which is different to the familiar object. The position of the two objects is counterbalanced and randomly permuted in the different treatment groups or genotypes.

In the habituation trial (day 1), the place preference of the animal is controlled by recording the entries into and the time spent in the different quadrants. Similar parameters are measured during the two trials with the presentation of two similar objects (day 2-3) and the last trial on day 4 with the novel object. The latency and frequency of *stretch-attend postures* and *rearings*, the latency to approach the objects, the time spent in exploration, and the overall time spent in the object quadrants is measured in the test trials (day 2-4).

Emotionality/anxiety of mice should be evaluated by at least two different tasks. The most common ones for mice are the open field test and the elevated plus maze test. Both paradigms depend on locomotion and therefore, the overall activity of the animals has to be measured in these schedules as well. If possible, additionally, the locomotion of the animals could be recorded in the hole board test. Importantly, mice should be tested standardized in the dark phase of the light cycle under dim red light to increase their activity and to avoid possible influences on the behavior by the mice's endogenous circadian rhythm. Furthermore, animals should be completely undisturbed during the last 24 h before the experiment and pre-experiences in other tests should be avoided. Finally, it must be kept in mind that most tests for emotionality/anxiety of rodents are based on the exposure to novelty. Therefore, repeated testing raises several unwanted problems with loss of reliability being probably most important.

2.2.5 Nociception

For the assessment of drug effects on nociception, mice could be screened using the tail flick and the hot plate task. It should be noted here that several other tests exist to measure pain perception (MILLAN MJ, 1999).

Tail flick

The tail flick test has been used widely as an experimental model to measure nociception, especially for the screening of analgesic drugs (DEWEY et al. 1969). The test is based on the withdrawal of the tail in response to a noxious cutaneous thermal stimulation (DOURISH et al.

1990; LEE and RODGERS 1990). This is a spinal reflex that requires both segmental connections and an ascending propriospinal connection from coccygeal and caudal sacral dorsal horn to motor neurons in the lumbar. The latency of the reflex is dependent upon 4 variables:

a) The time for activation of cutaneous nociceptors by the thermal stimulus.
b) The time for afferent conduction of the impulse to the spinal cord dorsal horn neurons.
c) The conduction within the central nervous system, or central delay.
d) The time for conduction of the impulse from the ventral horn to, and activation of tail muscles.

The tail flick reflex is modulated by supraspinal structures, which have excitatory or inhibitory effects on the activity of dorsal horn interneurons.

This kind of nociception task measures a simple spinal reflex to a sudden, painful thermal stimulus. A photo beam is used to apply a heat stimulus to the tail. Latency to flick the tail out of the path of the light beam is measured (NAVEILHAN et al. 2001). The photo beam is turned off if the tail is not flicked away within 20 s (to avoid tissue damage). Three consecutive determinations with a 5 min intertrial interval are averaged to obtain the tail flick response latency (MAIER et al. 1982). In order to minimize possible tissue damage, a different patch of the tail skin is stimulated in each trial.

Hot plate

The hot plate assay is one of the most commonly used tests for determining the analgesic efficacy of experimental drugs in rodents (PICK et al. 1991). The mouse is placed on the surface of a hot plate, which is maintained at 52.5 °C (VAN GAALEN and STECKLER 2000). A plastic frame consisting of Plexiglas encloses the surface so that the mouse cannot jump out. The mouse´s latency to *raise* or *lick hind paws*, to *flutter*, or to *jump up* is recorded (RUBINSTEIN et al. 1996; WIESENFELD-HALLIN et al. 1990). If the mouse has not responded within 15 s it is removed from the hot plate to prevent tissue damage.

There are several other paradigms besides the tail flick and the hot plate test for analyzing nociception, such as paw pressure experiments or acid-induced writhing tests. But these tests can induce severe injury in the animals and should be avoided – if possible - because of ethical considerations. Furthermore, the influence of the handling procedure on the mouse is stronger especially in paw pressure experiments and acid-induced writhing tests than in the hot plate task. Because of influences on the animal's behavior due to restraining the animal in the tail flick test, the hot plate task should be preferred when selecting only one paradigm for screening of drug effects on nociception.

2.2.6 Tests related to symptoms of human psychiatric disorders

Porsolt swim test

The method is based on the observation that a mouse, when forced to swim in a situation, from which there is no escape, will, after an initial period of vigorous activity, eventually make only those movements, which are necessary to held its head above the water. This identifiable behavioral *immobility* indicates a "state of despair", in which the mouse has learned that escape is impossible and resigns itself to the experimental conditions. The test is sensitive to antidepressants and is used as a model for aspects of human depression. The behavioral response to this paradigm might reflect "behavioral despair" and has been connected to a "learned helplessness" theory of depression (PORSOLT et al. 1978; SELIGMAN and MAIER 1967; SHERMAN et al. 1979).

A naive mouse is plunged into a vertical Plexiglas cylinder (height: 40 cm; diameter: 18 cm) containing 15 cm of water (so that the mice cannot balance on its feet or tail) maintained at 25 °C (PORSOLT et al. 1978; PORSOLT et al. 1977). The water surface is far enough away from the top of the cylinder, so that the animal cannot jump out. It is forced to swim inside the cylinder for 15 min. Each mouse, which does not swim or float, is immediately removed from the water. After 15 min in the water the mouse is removed and allowed to dry for 15 min in a heated enclosure (32 °C) before being returned to its home cage. This treatment produces long periods of *immobility* in the water (10-12 min total duration) and the mouse can be hypothermic and hypoactive for periods up to 30 min after the removal (therefore, this test is a great stressor for mice). The mouse is replaced in the cylinder 24 h later and the latency and total duration of *immobility* is measured during a 5 min test. The mouse is judged to be immobile whenever it remained floating passively in the water in a slightly hunched but upright position, its head just above the surface. It makes only those movements, which are necessary to keep the head above the water.

The duration of *immobility* is decreased by treatment with antidepressant drugs and therefore this paradigm is used as a depression model (KORZENIEWSKA-RYBICKA and PLAZNIK 1998). But other studies proved (BORSINI and MELI 1988; BORSINI et al. 1986) that a reduction of *immobility* time can also be observed in animals, which live in an enriched environment. Furthermore, there seems to be no relationship between inescapability and *immobility*, since it was shown that rats in an inescapable test design did not show longer *immobility* durations than rats in an escapable test design (O'NEILL and VALENTINO 1982). The prior exposure to the water alone resulted in longer *immobility*. Thus, familiarity with the environment rather

than "despair" may induce behavioral *immobility*. Perhaps a "life threatening experience" plays a role in inducing behavioral *immobility*.

Therefore, the Porsolt swim test does not provide a model resembling depressive illness in human beings (HAWKINS et al. 1978). Normally, antidepressive drugs have a chronic effect, but in the Porsolt swim test an acute effect of antidepressive treatment can be observed (decreased *immobility* duration). After all, this paradigm cannot be considered to be an adequate analog model for pathological depression.

Acoustic startle response and prepulse inhibition

The startle response is an unconditioned, reflexive response to a sudden environmental stimulus. Prepulse inhibition (a senorimotor gating reflex, similarly quantitated in rats, mice and humans) is a phenomenon, in which a weak prestimulus or prepulse suppresses the response to a startling stimulus. Deficits in prepulse inhibition (PI) are common in schizophrenic patients and may measure attentional dysfunctions that contribute to auditory hallucinations (BRAFF et al. 1978; BRAFF et al. 1999; CADENHEAD et al. 1993). The PI impairment observed in these neuropsychiatric patients is thought to reflect an underlying problem with inhibitory mechanism in neuronal systems used for senorimotor gating (SWERDLOW et al. 2000). This loss of auditory gating (as an aspect of schizophrenia) can be reproduced in an animal model (CAINE et al. 1992). Two tones are presented very close together. In most people it will not evoke a response to the second tone, but schizophrenic patients will respond to both tones (GRILLON et al. 1992). When a mild stimulus (prepulse) immediately preceded a startle stimulus, a healthy mouse will flinch less to the startle stimulus, although the prepulse does not startle the animal itself (PAYLOR and CRAWLEY 1997). So the prepulse depresses the startle response in humans and rodents. The prepulse inhibition is one of the few paradigms, in which humans and rodents are tested in a similar fashion (GEYER and SWERDLOW 1998). Key neuroanatomical and neuropharmacological substrates mediating PI have been analyzed in rats. Direct pharmacological injections and lesion studies indicate that structures contributing to prepulse inhibition include the nucleus accumbens, hippocampus, amygdala, medial prefrontal cortex, pedunclopontine tegmental nucleus, ventral and caudodorsal striatum, median and dorsal raphe nucleus, and the superior colliculus (SWERDLOW and GEYER 1998). Neurotransmitters affecting prepulse inhibition include dopamine, acetylcholine, serotonin, glutamate, and norepinephrine (KOCH 1999).

First, animals have to be habituated to the testing chambers for five consecutive days (5 min per day). Acoustic stimuli of 120/105 dB, a single prepulse interval (100 ms), and four different prepulse intensities (2/4/8/16 dB above background noise – i.e. white noise of 65

dB) are used. There are 5 min of acclimatization period (with background noise alone). Then the animal is presented with 72 startle trials, each trial consisting of one of three conditions:

a) 30 ms 120 dB noise burst presented alone;
b) 30 ms 120 dB noise burst preceded 100 ms by prepulses (30 ms noise bursts) that were 2/4/8/16 dB above the background noise;
c) No stimulus (background noise alone), which is used to measure baseline movement in the chamber;

These six trial types are each repeated six times in a pseudorandom order (36 trials), such that each trial type is presented once within a block of six trials. This block of six trials is then repeated using an acoustic stimulus of 105 dB. Analysis is based on the mean of the six trials for each trial type (CARTER et al. 1999).

In several studies, the Porsolt swim test is often discussed as an animal model for depression and the prepulse inhibition test as a model for schizophrenia. But obviously, each of these tests can only be used as one in a row of several additional experiments in order to finally draw a conclusion, which relates the finding to human psychiatric conditions. Some researchers even completely reject the possibility of developing an animal model for psychosis. In the same line, the effect of anti-depressant drugs in the Porsolt test should also be confirmed in other animal models for depression such as olfactory bulbectomy or even better postnatal maternal deprivation schedules and tests, which combine different stressful situations for animals (WILLNER 1990). It appears that the knowledge about the relationship between these animal models, the resulting behavior of rodents and their relevance for human psychiatric disorders has to be further developed before one or even a combination of such tasks can be considered as a valid model for this type of questions.

2.2.7 Aggression

Isolation-induced aggression

The adult male test animal is isolated for 14 days (i.e. kept single in a standard cage). After this period it is placed in a neutral cage together with a standard opponent mouse (BRAIN and POOLE 1974), which is an adult, male, weight-matched mouse of a defined genotype and a peaceful phenotype. The mice are placed simultaneously in opposite corners of the cage and fixed there for 5 s. Then the test starts, the mice can walk and interact freely in the cage. The latency and frequency of the agonistic behaviors *biting*, *tail rattling*, *sideway threats*, *upright postures*, and *aggressive grooming* and also of sociopositive behaviors like *nosing*, *anogenital sniffing*, *crawling over/under*, and *allo grooming* should be recorded during the next 10 min

(BRAIN and POOLE 1976; KOOLHAAS et al. 1980; LAGERSPETZ 1969). It is also possible to investigate the territorial behavior of the test animal by testing the mice in the home cage of the isolated mouse (BRAIN 1980).

Spontaneous aggression

If spontaneous aggression is to be measured, the adult male group-caged mouse is tested with a standard opponent in a neutral cage. Details of the procedure are similar to the methodology described above.

Territorial Aggression

Territorial aggression can be investigated by the Resident-Intruder paradigm. For this, the adult male, group-caged mouse is tested with a standard opponent in its home cage. The littermates of the test animal are removed from the home cage 10 min before the test. Then the standard opponent is taken to the test location. The resident mouse is fixed in one corner and the standard opponent intruder is placed in the opposite corner of the cage. After fixation for 2 s the test starts. The recorded parameters are similar compared to the isolation-induced aggression test (BLANCHARD et al. 1988).

For the characterization of aggressive behavior in mice, all three different test paradigms, which analyze different kinds of aggression in rodents, should be used (for an overview about further aggression tests see: MICZEK et al. 2001). Because of influences of fight-experiences on the behavior in consecutive aggression schedules, animals should be tested only once (BRAIN and POOLE 1974). The use of a defined, peaceful standard opponent (i.e. A/J mice) is as important (LAGERSPETZ 1969) as a high level of standardization during breeding (i.e. litter size, gender-ratio of litter, presence or absence of father). Since most strains of mice are per se very aggressive, this behavioral domain has also an important impact on other behavioral domains such as emotionality/anxiety. Furthermore, several studies have demonstrated that the behavioral performance of mice depends on whether these animals are dominant or not within their cohort.

2.3 Conclusion

The accurate evaluation of a behavioral phenotype and screening for toxic effects in rodents is an important step for evaluating novel drugs and compounds. The detection of specific drug-induced behavioral effects represents often a critical and far-reaching event in the development of novel drugs and compounds. In order to optimize current approaches and to obtain more reliable results, it is vital to exclude false positive and false negative results in

specific behavioral tests. These confounding factors result from previously undetected problems of the mouse in health and sensory performance. We therefore propose that an experimental design aimed at screening for unexpected drug effects should always be composed of monitoring general health, sensory functions, and motor abilities, right before specific behavioral domains are tested.

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2.6 Table

Table 1: Behavioral domains and the different corresponding experimental tasks – including relevant references.

Behavioral domain	Experimental task	References
Neurophysiology and health		CRAWLEY 1999; CRAWLEY and PAYLOR 1997; LIONE et al. 1999; PICCIOTTO and WICKMAN 1998
Motor functions	Pole test	OGAWA et al. 1985; SEDELIS et al. 2000
	Beam walking test	CARTER et al. 1999; PERRY et al. 1995
	Wirehang test	CRAWLEY 1999; MIYAKAWA et al. 2001
	Rotarod and accelerod	BARLOW et al. 1996; CARTER et al. 1999; CRAWLEY 1999; JONES and ROBERTS 1968; PICCIOTTO and WICKMAN 1998; SEDELIS et al. 2000
	Footprint test	BARLOW et al. 1996; CARTER et al. 1999
Learning and memory	Morris water maze	HODGES 1996; MORRIS 1981; OWEN et al. 1997

	Radial maze	BUBSER and SCHMIDT 1990; CRUSIO et al. 1987; HODGES 1996; HOLSCHER and SCHMIDT 1994; LANKE et al. 1993; MIYAKAWA et al. 1996; MORRIS 1981; OLTON and SAMUELSON 1976; PICCIOTTO and WICKMAN 1998; RAWLINS and DEACON 1993; SUZUKI et al. 1980; TESKEY et al. 1998; TROPP and MARKUS 2001; WINTER 1997
	Conditioned-fear paradigm	CRAWLEY 1985; CRAWLEY 1999; OWEN et al. 1997; VAN GAALEN and STECKLER 2000
	Active and passive avoidance	BOVET et al. 1969; CHAOULOFF et al. 1997; COSTALL et al. 1989; CRAWLEY and GOODWIN 1980; GROSSEN and KELLEY 1972; PICCIOTTO and WICKMAN 1998; STEWART et al. 1993
Emotionality/ Anxiety and exploration	Open field	BADISHTOV et al. 1995; BRITTON and BRITTON 1981; DEFRIES et al. 1966; DENENBERG 1969; PRUT and BELZUNG 2003; SATINDER 1982; TREIT and FUNDYTUS 1988; TRULLAS and SKOLNICK 1993; VAN GAALEN and STECKLER 2000; VON HORSTEN et al. 1998; WALSH and CUMMINS 1976; WHIMBLEY and DENENBERG 1967
	Hole board	LISTER 1987; PELLOW et al. 1985; VAN GAALEN and STECKLER 2000

	Elevated plus maze	BALDWIN and FILE 1986; CHAOULOFF et al. 1997; CRUZ et al. 1994; FERNANDES and FILE 1996; FILE 1986; FILE 1987; FILE 1993; HANDLEY and MITHANI 1984; HOGG 1996; KORTE and DE BOER 2003; LISTER 1987; MONTGOMERY 1958; MONTGOMERY and MONKMAN 1955; PELLOW et al. 1985; PELLOW and FILE 1986; RODGERS and COLE 1993; SHEILA and MITHANI 1984; TRULLAS and SKOLNICK 1993
	Object exploration	BARNETT 1975; COWAN 1976; MISSLIN and ROPARTZ 1981; TROPP and MARKUS 2001; VAN GAALEN and STECKLER 2000
Nociception	Tail flick and hot plate	CRAWLEY 1999; DEWEY et al. 1969; DOURISH et al. 1990; LECCI 2001; LEE and RODGERS 1990; MILLAN 1999; MIYAKAWA et al. 1996; NAVEILHAN et al. 2001; RUBINSTEIN et al. 1996; WIESENFELD-HALLIN et al. 1990
Tests related to symptoms of human psychiatric disorders	Porsolt swim test	BORSINI and MELI 1988; BORSINI et al. 1986; HAWKINS et al. 1978; HILAKIVI-CLARKE et al. 1991; KORZENIEWSKA-RYBICKA and PLAZNIK 1998; NIKULINA et al. 1991; NISHIMURA et al. 1988; O'NEILL and VALENTINO 1982; PORSOLT et al. 1978; PORSOLT et al. 1977; SELIGMAN and MAIER 1967; SHEARMAN et al. 2000; WIELAND et al. 1986; WILLNER 1990

	Acoustic startle response and prepulse inhibition	BRAFF et al. 1978; BRAFF et al. 1999; CADENHEAD et al. 1993; CADENHEAD et al. 2000; CAINE et al. 1992; GEYER and SWERDLOW 1998; GRILLON et al. 1992; KOCH 1999; PAYLOR and CRAWLEY 1997; SWERDLOW and GEYER 1998; SWERDLOW et al. 2000
Aggression	Isolation-induced aggression, spontaneous aggression, and territorial aggression	BLANCHARD et al. 1988; BRAIN and POOLE 1974; BRAIN 1980; BRAIN et al. 1987; BRAIN and POOLE 1976; KOOLHAAS et al. 1980; LAGERSPETZ 1969; MICZEK et al. 2001

Localization, transmission, spontaneous mutations, and variation of function of the *Dpp4* (Dipeptidyl-peptidase IV; CD26) gene in rats

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Abstract

Dipeptidyl-peptidase IV (DPPIV) is involved in endocrine and immune functions via cleavage of regulatory peptides with a N-terminal proline or alanine such as incretins, neuropeptide Y, or several chemokines. So far no systematic investigations on the localization and transmission of the *Dpp4* gene or the natural variations of DPPIV-like enzymatic function in different rat strains have been conducted. Here, we map the *Dpp4* gene to rat chromosome 3 and describe a semi-dominant mode of inheritance for *Dpp4* in a mutant F344/DuCrj(DPPIV-) rat substrain lacking endogenous DPPIV-like activity. This mutant F344/DuCrj(DPPIV-) rat substrain constantly exhibits a nearly complete lack of DPPIV-like enzymatic activity, while segregation of DPPIV-like enzymatic activity was observed in another DPPIV-negative F344/Crl(Ger/DPPIV-) rat substrain. Screening of twelve different inbred laboratory rat strains revealed dramatic differences in DPPIV-like activity ranging from 11 mU/µl (LEW/Ztm rats) to 40 mU/µl (BN/Ztm and DA/Ztm rats). A lack of DPPIV-like activity in F344 rats was associated with an improved glucose tolerance and blunted natural killer cell function, which indicates the pleiotropic functional role of DPPIV *in vivo*. Overall, the variations in DPPIV-like enzymatic activity probably represent important confounding factors in studies using rat models for research on regulatory peptides.

Keywords: F344 – Dipeptidyl-peptidase IV - CD26 – Gene mapping – Glucose tolerance – NK cell function

3.1 Introduction

Dipeptidyl-peptidase IV (DPPIV/CD26) is an ectopeptidase with a triple functional role. DPPIV is involved in catalyzing the release of *Xaa*-Pro or *Xaa*-Ala dipeptides from the N-terminus of circulating hormones and chemokines [1, 2]. Furthermore, the enzyme is involved in T cell dependent immune responses [3-5] and in cell adhesion processes [2, 6]. The biological activity of several hormones and chemokines can be abolished or modified by DPPIV *in vitro* and *in vivo* [2, 7]. Substrates of the DPPIV are neuropeptide Y (NPY), endomorphine, circulating peptide hormones like peptide YY, and chemokines like RANTES. However, it should be noted that cleavage of many of these substrates of DPPIV have been demonstrated only in *in vitro* studies, while the physiological relevance *in vivo* still remains unknown [2]. Additionally, cytokines and growth factors like IL-3, IL-10, and GM-CSF are characterized by a N-terminal structure with a proline in the second position. They are further potential substrates for this enzyme [2]. Thus, DPPIV is directly and indirectly involved in the regulation of endocrine, immune, and nervous functions [8].

In recent years a number of enzymes were identified expressing DPPIV-like activity [9, 10]. The fibroblast activating protein is a highly homologous protein to DPPIV, which possesses a gelatinase activity as well as a post-proline specific dipeptidyl-aminopeptidase activity. The protein was found on the surface of activated fibroblasts and on several cancer cells. It seems to play a role in cancer invasion and in angiogenesis [11-13]. DP8 and DP9 are also peptidases of the prolyl oligopeptidase family S9. For both a DPPIV-like activity could be proved [14, 15]. They are located intracellulary but physiological function remains unknown. Dipeptidyl-peptidase II (DP II, QPP) is a post-proline and post-alanine cleaving dipeptidyl-peptidase with an acidic pH-optimum [16]. It is located in intracellular vesicles, including lysosomes. Attractin shares no sequence homology with the other dipeptidyl-peptidases but a DPPIV-like activity was found [17] and could be confirmed by another study [18]. Low molecular weight substrates could not distinguish between these DPPIV-like activities. Only DP II could be measured at pH 5 were at least DPPIV and attractin but probably also the other enzymes are inactive. Recently, there were the first inhibitors described, which show a clear preference for DP II over DPPIV [19].

DPPIV knockout mice confirm the importance of DPPIV-like enzymatic activity in regulating blood glucose levels [20]. This effect of DPPIV-like enzymatic activity on glucose homeostasis is likely to be mediated via a prolonged action of the incretins glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), which potentiate

the glucose-stimulated insulin secretion [21-23]. GLP-1 and GIP possessing an alanine at the penultimate position are rapidly degraded and inactivated by DPPIV. The intact N-terminus is absolutely required for the biological activity of these incretins [24]. Thus, the truncated forms GLP-1_{9-36} and GIP_{3-42} are not insulinotropic any more [25, 26]. Despite these important and pleiotropic functions of DPPIV, so far, no systematic investigations on natural variations in DPPIV-like activity in different laboratory rat strains have been conducted. In contrast to humans and mice [27], also the genetics and the localization of the *Dpp4* gene in rats remained unknown.

Interestingly, and in addition to the above mentioned lack of knowledge on natural variation in DPPIV-like activity in rats, a sequence alteration (spontaneous mutation) of the *Dpp4* gene has been described in independent studies of F344 rat substrains from breeding colonies of Charles River Laboratories in Sulzfeld, Germany (laboratory code: Crl) [28] and in Atsugi, Japan (laboratory code: DuCrj) [29, 30]. These mutations result in an almost complete lack of enzymatic activity. In contrast, F344 rats from Charles River Laboratories (Crl) in Raleigh or Portage, USA, exhibit a normal DPPIV-like activity, but unfortunately carry the same laboratory code (Crl) as the animals from Germany (Sulzfeld). For the purpose of clearness, we therefore extended the official laboratory codes of these F344 rat substrains by adding the country or town of origin (i.e. “Ger” for Germany, “Ral” for Raleigh/USA, “Por” for Portage/USA), a description of the phenotype (i.e. “DPPIV-“ for lack of DPPIV-like activity), and if necessary, the year of receipt of these substrains from the commercial breeders (i.e. “98” for 1998 and “01” for 2001).

In F344/DuCrj(DPPIV-) rats a G to A transition at nucleotide 1897 in the *Dpp4* cDNA sequence leads to a substitution of Gly^{633} to Arg in the catalytic center of the enzyme (Gly^{629}-Trp-Ser-Tyr-Gly^{633}). The Ser^{631} is the active serine of rat DPPIV. As a result of this point mutation, DPPIV-like activity is deficient in plasma and other tissues of these F344 rats, although there is still evidence for mutant *Dpp4* mRNA [31]. In the other DPPIV-deficient F344 substrain from Germany [F344/Crl(Ger/DPPIV-)] the gene sequence has not been characterized but there is also evidence for non-active *Dpp4* mRNA [28]. Possibly, a mutation interrupts the translation of *Dpp4* mRNA in the Japanese F344/DuCrj(DPPIV-) substrain [31] as well as in the German F344/Crl(Ger/DPPIV-) substrain. Both DPPIV-deficient F344 substrains may provide an interesting model to further study the *in vivo* functional role of DPPIV for the cleavage of regulatory peptides and resulting various endocrine or immunological processes such as glucose tolerance or natural killer (NK) cell function. Likewise, differences in endogenous DPPIV-like activity in various rat strains may have an

important impact for specific experiments focusing on *in vivo* effects of regulatory peptides, which are substrates for the DPPIV.

Therefore, in the present study we mapped *Dpp4* using a gene linked SSLP marker, performed breeding experiments to identify the mode of inheritance of DPPIV-like activity, and screened several laboratory rat strains including F344 rat substrains from different breeding colonies obtained at different time points from Crl for their endogenous DPPIV-like activity. Furthermore, we determined the glucose tolerance and NK cell function in DPPIV-deficient and wildtype-like F344 rat substrains in order to exemplify the functional importance of spontaneous sequence variations in the *Dpp4* gene.

3.2 Materials and methods

Animals

All animals were housed at the Central Animal Facility of the Hannover Medical School (Ztm). Rats were maintained in a separated minimal barrier sustained facility and kept in Makrolon type III cages on standard bedding (Altromin GmbH, Lage, Germany). Food (Altromin Standard Diät 1320: Altromin GmbH) and water were available *ad libitum*. Environmental temperature was automatically regulated at 21 ± 2 °C, relative humidity at 55 ± 5% with an air change rate of 15 times per hour. The animal rooms were operated with a positive pressure of 0.6 Pa. Animals were maintained under 12:12 h light cycle, underwent routine animal care once a week and were microbiologically monitored according to FELASA recommendations [32]. All research and animal care procedures were approved by the district government, Hannover, Germany, and performed according to international guidelines for the use of laboratory animals.

Origin of different F344 substrains from Charles River Laboratories

The inbred F344 colony was originated by Curtis and Dunning at the Columbia University Institute for Cancer Research in 1920 [33]. In 1949 F344 animals were transfered to Heston (in F31) and in 1951 to the National Institute of Health (in F116). F344/Crl(Por) is derived from F344/Crl animals from the colony in Portage, which was established in 1998 with animals from the National Institute of Health subline. F344/Crl(Ral) were originated from F344/Crl animals from the colony in Raleigh (USA), which were derived from nucleus animals from Wilmington (New England) in 1985. The F344/DuCrj(DPPIV-) substrain was based on the colony of Curtis and Dunning in 1920. In 1960, the substrain was passed in generation F68 to Charles River Laboratories in Wilmington (Crl). After a rederivation via hysterectomy in the USA in 1965 (F81), the breeding was started in generation F110 at Charles River Laboratories in Japan in 1976 (Crj). F344/Crl(Ger/DPPIV-) rats were obtained as F344/Crl animals from Sulzfeld in Germany, which were derived from the nucleus-colony CDF from Wilmington in 1999.

Unfortunately, several of these F344 substrains carry the same laboratory code (Crl). For clarity, animal groups were coded dependent on country or town of origin and the DPPIV-related phenotype as followed: F344 rats derived from breeding colonies in Atsugi, Japan were designated as F344/DuCrj(DPPIV-), animals from breeding colonies in Sulzfeld, Germany, as F344/Crl(Ger/DPPIV-), wildtype-like rats obtained from colonies in Portage, USA, as F344/Crl(Por) and wildtype-like animals from Raleigh, USA, as F344/Crl(Ral). Additionally, the time point, at which the animals were obtained from Charles River

Laboratories (1998/2001) was indicated in our code ("98" for 1998 and "01" for 2001), whenever this appeared to be necessary.

***Dpp4* gene mapping**

Dpp4 maps to mouse chromosome 2 (MMU2) and the position of the homologous human gene (*DPP4)* is on human chromosome 2 (HSA2). Large parts of RNO3 are homologous to MMU2 and HSA2 (www.informatics.jax.org/menus/homology_menu.shtml).

In this study genomic rat sequences available on the NCBI Rat Genome Blast page (www.ncbi.nlm.nih.gov/genome/seq/RnBlast.html) were used to identify polymorphic short tandem repeats in close proximity to *Dpp4*. To determine the position of *Dpp4* in the rat genome, we analyzed 202 individual rats of a (LEW/Ztm-*ci2* x BN/Ztm)F1 x LEW/Ztm-*ci2* backcross (N2) for inheritance of simple sequence length polymorphisms. For tissue collection, animals were sacrificed (anesthetized with carbon dioxide followed by cervical dislocation). For DNA preparation genomic DNA was prepared from ear or tail tissue with the Nucleo Spin™ Tissue kit (Macherey-Nagel GmbH, Düren, Germany). We used Taq-DNA-polymerase (Peqlab Biotechnologie GmbH, Erlangen, Germany) and a PTC-200 thermal cycler (MJ Research Inc., Watertown, MA) for the polymerase chain reaction. Amplification was carried out in a 10 µl reaction with 1.5 mM $MgCl_2$, 75 µm of each dNTP, 0.17 µm of each primer, 100 ng genomic DNA and 0.5 unit polymerase. After an initial step at 95 °C for 4 min, 35 cyles of 15 s at 94 °C, 1 min at 55-56 °C and 2 min at 72 °C were performed, followed by 72 °C for 7 min. All PCR products were detected by gel electrophoresis using 3% NuSieve™ 3:1 agarose (Bio Wittaker Molecular Applications Inc., Rockland, Maine).

All N2 rats were genotyped with the following SSLP markers to create a genetic linkage map of rat chromosome 3 (RNO3): D3Mgh8, D3Mit12, D3Mit4, D3Mgh1. Primer sequences for all markers used were obtained from the Rat Genome Database. All oligonucleotide primer pairs were synthesized by Carl Roth GmbH (Karlsruhe, Germany). Selection of gene linked short tandem repeats followed this step of the analysis. The rat genomic sequence used for this study was derived from the CHORI-230 Rat BAC library sequencing project. The library was constructed with the DNA of a 3-months old female BN(BN/SsNHsd/MCW) rat (www.chori.org/bacpac/).

Rat genomic DNA sequence featuring identities to the rat cDNA sequence of *Dpp4* [34] were identified with the NCBI Rat Genome Blast program. Tandem repeats within the rat genomic sequence were found using the Mount Sinai/The Department of Biomathematical Science Tandem Repeats Finder. Primer pairs for the short tandem repeats found in the rat genomic sequence were chosen using Oligo4.0™ (Molecular Biology Insights, Inc., Cascade,

Colorado) software and synthesized by Carl Roth GmbH. Short tandem repeats displaying variant alleles for LEW/Ztm-*ci2* and BN/Ztm rat strains were used as gene linked SSLP markers.

Mode of inheritance of DPPIV-like activity

The mode of inheritance of DPPIV-like activity was determined using F344/DuCrj(98/DPPIV-), F344/Ztm, [F344/Ztm x F344/DuCrj(98/DPPIV-)]F1, and [F344/Ztm x F344/DuCrj(98/DPPIV-)]F2 rats. The enzyme activity of the parental strains, their F1, and their F2 generation was measured as described below.

Determination of DPPIV-like enzymatic activity

F344/DuCrj(98/DPPIV-), F344/DuCrj(01/DPPIV-), F344/Crl(Ger/98/DPPIV-), F344/Crl(Ger/01), F344/Crl(Por/98), F344/Crl(Por/01), and F344/Crl(Ral/01), as well as animals of various inbred rat strains maintained at Ztm (BDII, BDIX, BDE, BN, DA, E3, F344, LEW, LE, OM, WF, and WKY) were screened.

EDTA-plasma samples were kept at –80 °C until use. DPPIV enzyme activity of the different rat strains was determined using glycyl-prolyl-4-nitroaniline (Gly-Pro-pNA) as substrate. A volume of 30 µl plasma was diluted with 120 µl 0.9% NaCl and 600 µl of 0.5 M substrate solution in HEPES buffer pH 7.6 were added. Release of 4-nitroaniline were monitored at 37 °C and 390 nm up to 20 min using the UV1 spectrophotometer (ThermoSpectronics, Neuss, Germany). Activity (mU/ml) was calculated from the linear slope using a factor of 2193 µmol/l calculated from the molar absorption coefficient and the plasma dilution. One unit is defined as the DPPIV activity, which cleaves 1 µmol Gly-Pro-pNA per minute.

For determination of plasma activity of F344 rats during the inheritance study a more sensitive and faster microplate based fluorescence assay was used. The release of 4-Amino-7-Methylcoumarin (AMC) from the substrate Gly-Pro-AMC was monitored 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, 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.

Both assays result in different activities due to differences in assay conditions and the different substrates used. Direct comparison of the same samples in both assays demonstrate that the activity determined by the assay using Gly-Pro-pNA as substrate is approximately 1.9 times higher than the activity determined by the fluorescence assay (which was used for the mode of inheritance study). Both assays are selective for DPPIV-like activities. It has been

proven that the substrates are cleaved by DPPIV, by DP II and by attractin. Probably they are also substrates for DP8 and DP9. Importantly, the chromophores are not released by other proline-specific peptidases, such as prolidase, prolyl endopeptidase or aminopeptidase P.

Glucose tolerance

Animals of F344/Crl(Por/98), F344/DuCrj(98/DPPIV-), and F344/Crl(Ger/98/DPPIV-) substrains (n = 10) aging of 154 ± 5 days were used for this experiment. These animals were standardized in regard to their breeding conditions (littersize: n = 6, gender-ratio: 1:3 or 1:2), and number of animals per cage. Following an overnight fasting (12 h) 1 h after the onset of the light phase, animals (fasting) blood glucose levels were controlled. If the glucose concentration was < 7.8 mmol/l (< 140 mg/dl) the animals were orally administered with glucose (1.5 g glucose/kg) using a feeding tube [35, 36]. For this, rats were anesthetized shortly with Isofluran. Blood samples (10 µl) were collected from the tail vein of conscious unrestrained rats at 30, 60, 90, and 120 min following the oral glucose load and the glucose level was measured by a glucometer (Bayer AG, Leverkusen, Germany). Criteria for the definition of the glucose tolerance of the animals were blood glucose concentrations after 120 min: < 7.8 mmol/l (< 140 mg/dl): normal glucose tolerance; 7.8-11.1 mmol/l (140-200 mg/dl): impaired glucose tolerance; > 11.1 mmol/l (> 200 mg/dl): *Diabetes mellitus*.

Quantification of NK cell cytotoxicity in spleens of different F344 substrains

A single cell suspension of splenocytes was prepared for each subject of F344/Crl(Por/98), F344/DuCrj(98/DPPIV-), and F344/Crl(Ger/98/DPPIV-) rats by gently pressing spleen tissue using the ends of stamps from sterile plastic syringes (n = 3 per each F344 substrains in three independent experiments). After erythrolysis and two washes in PBS, cells were re-suspended in exactly 10 ml RPMI 1640. Leukocyte numbers were determined using a Coulter cell counter and the splenocyte concentrations adjusted to 6×10^6 cells/ml with supplemented RPMI 1640 containing 10% fetal bovine serum. NK cytotoxicity was measured in classical ^{51}Cr-release assays using MADB106 target cells [37, 38], which were derived from standard cell culture conditions, as previously described [27]. Splenocytes, prepared through Ficoll-Hypaque gradient [39], were used as effectors and effector-to-target (E:T) ratios of 12.5:1, 25:1, 50:1, 100:1 were obtained. Co-incubation of effector and target cells was carried out either for 4 h with addition of 1000 U/ml IL-2 (EuroCetus, Amsterdam, The Netherlands) or for 18 h incubation, respectively [38]. Control wells containing only labelled targets were also plated to determine the spontaneous release. For determination of maximal possible release, the targets in one set of control wells were lyzed with Triton X-100. The spontaneous release was always less than 10% of the maximum release. Plates were centrifuged for 4 min prior to

incubation (37 ˚C, 5% CO_2) and again prior to harvesting 75 µl of the supernatant for determining ^{51}Cr release in a gamma counter. The specific cytotoxity was calculated by means of the following formula: [(experimental release) – (spontaneous release)] / [(maximal release) – (spontaneous release)] × 100.

Statistical analysis

The position of all RNO3 SSLP markers obtained from the Rat Genome Database as well as the position of our gene-linked marker were calculated using JoinMap™2.0 (Centre for Plant Breeding and Reproduction Research; CPRO-DLO, Wageningen, The Netherlands) software. MapChart™2.0 (CPRO-DLO) software was applied for drawing a linkage map of RNO3. The analysis of the blood plasma DPPIV-like activity was assessed by one-way ANOVA followed by the Fisher-PLSD-test for post hoc comparison, if appropriate. The analyses of the blood glucose level and NK cell mediated specific cytotoxicity were assessed by analysis of variance (ANOVA) for repeated measures followed by two-way and one-way ANOVA and the Fisher-PLSD-test as post hoc test, if appropriate. Differences were regarded as statistically significant if $p < .05$. In the figures and tables, all data are displayed as means ± standard error of the mean (SEM) and significant post hoc effects versus the wildtype-like control animals of the F344/Crl(Por/98) substrain are indicated by asterisks (*$p < .05$ - **$p < .01$ - ***$p < .001$).

3.3 Results

Dpp4 gene mapping

Rat specific DNA sequences derived from BAC clones were useful for this investigation. We screened rat BAC sequence data for the presence of both a tandem repeat and identity to the cDNA of rat *Dpp4*. BAC clone CH230-34P1/AC1257121 contains the genomic sequence of *Dpp4* as well as a $(CT)_{36}$ tandem repeat. According to the sequence of this BAC clone the tandem repeat is located within an intron of rat *Dpp4*. This tandem repeat displayed variant alleles for LEW/Ztm-*ci2* and BN/Ztm rats and was used as a gene linked SSLP marker (*D3Ztm1*). We used TGG GGG ATT ATA CTA ATT CAG TCC CCA (5´-3´) as upper primer and ACT TCC CTT GCA AGC ACA GAA AAC (5´-3´) as lower primer for amplification of *D3Ztm1*. The calculated product size (BN rats) of this gene linked SSLP marker is 299 base pairs.

Linkage analysis revealed that the four D3 SSLP markers described by the Rat Genome Database map to RNO3. The maximum distance between *D3Mgh8* and *D3Mgh1* is 114.0 centimorgan (cM) as calculated for the N2 population studied. Since *D3Ztm1* showed significant linkage to these four RNO3 markers it was integrated into the scaffold previously created by those microsatellites. The position of *Dpp4/D3Ztm1* was determined as 29.0 cM on our RNO3 genetic linkage map (Fig. 1).

Mode of inheritance of DPPIV-like activity

DPPIV-like activity in male F344/DuCrj(98/DPPIV-) rats was 3.0 ± 0.4 mU/ml, while female F344/Ztm rats exhibit an average DPPIV-like activity of 19.1 ± 0.7 mU/ml. In the [F344/Ztm x F344/DuCrj(98/DPPIV-)] F1 generation an average enzyme activity of 9.3 ± 0.4 mU/ml could be measured. The F2 progeny of the F344/Ztm x F344/DuCrj(98/DPPIV-) cross could be subdivided into three phenotypic groups. The first group had a low DPPIV-like activity of 2.2 ± 0.1 mU/ml and was therefore considered homozygous for the mutant *Dpp4* allele. A high endogenous DPPIV-like activity of 20.6 ± 1.4 mU/ml was found in the second group. These animals appear to be homozygous for the *Dpp4* wildtype-like allele. The third group represents F344 rats with an intermediate DPPIV-like activity as this has been shown for the F1 generation. The mean value in this group was 10.8 ± 0.3 mU/ml, displaying an intermediate phenotype suggestive for a semi-dominant mode of inheritance.

DPPIV-like enzymatic activity

The different F344 substrains showed significant overall differences in the DPPIV-like activity (one-way ANOVA: $p < .0001$). The F344/Crl(Por/98) substrain ($n = 27$) exhibited a

normal DPPIV-like activity whereas the DPPIV-deficient substrains F344/DuCrj(98/DPPIV-) (n = 32) and F344/Crl(Ger/98/DPPIV-) (n = 31) showed a dramatic reduction in the DPPIV-like activity (Fig. 2).

The F344 animals ordered from the different breeding colonies of Charles River Laboratories in Germany, Japan, and the USA in 2001 showed a unique pattern of DPPIV-like activity and differed from the previously in 1998 obtained animals. Male and female animals of the colony in Raleigh, F344/Crl(Ral/01), and surprisingly also from Sulzfeld, F344/Crl(Ger/01) exhibited a considerable high DPPIV-like activity (Table 1). The latter was opposite to the extreme reduction of DPPIV-like activity in the F344/Crl(Ger/98/DPPIV-) rats subsequently bred in our laboratory. The animals of the Japanese substrain, F344/DuCrj(01/DPPIV-) did still lack DPPIV-like activity (one-way ANOVA: p = 0.02; Table 1). The results found in the F344 substrain from the colony in Portage, F344/Crl(Por/01) were gender-dependent. Female rats exhibited a normal DPPIV-like activity, while males nearly completely lacked DPPIV-like activity (one-way ANOVA: p < .0001). This potent gender-dependent phenotype was different to the F344/Crl(Por/98) animals bred in our laboratory (Fig. 2).

Furthermore, we also found a wide range of DPPIV-like activity in several other inbred rat strains maintained in the Ztm (Table 2). Among those strains LEW/Ztm exhibited the lowest (11.5 mU/ml) while DA/Ztm and BN/Ztm showed the highest DPPIV-like activity (DA/Ztm: 39.8 mU/ml and BN/Ztm: 39.7 mU/ml).

Glucose tolerance

The fasting blood glucose levels of the three F344 substrains were not different (Fig. 3). The DPPIV-negative substrains F344/DuCrj(98/DPPIV-) and F344/Crl(Ger/98/DPPIV-) showed a significant lower blood glucose level during the oral glucose tolerance test in comparison to F344/Crl(Por/98), which were taken as a control group. This was shown by ANOVA for repeated measures (p < .0001) and one-way ANOVA for the blood glucose levels split by time at 30 min (p = 0.004), 60 min (p = 0.001), and 90 min (p = 0.01) after the oral administration of glucose. Although at the critical time point of 120 min after the administration the blood glucose tolerance of all three substrains was normal (< 7.8 mmol/l), the tolerance of the two DPPIV-deficient substrains during the schedule was improved compared to the wildtype-like animals.

NK cell cytotoxicity in spleens of different F344 substrains

NK specific lysis of MADB106 tumour cells using a classical *ex vivo* NK cell functional assay is shown in Fig. 4. In the DPPIV-deficient F344/Crl(Ger/98/DPPIV-) and F344/DuCrj(98/DPPIV-) substrains, NK cell mediated lysis against MADB106 tumor targets

is significantly decreased compared to wildtype-like F344/Crl(Por/98) rats. This effect was observed in both assays (4 h co-incubation in the presence of IL2; Fig. 4A; and 18 h incubation; Fig. 4B). Two-way ANOVA showed a significant effect of "substrain" ($p < .001$) and "E:T ratio" ($p < .01$) in the 4 h assay. Similarly, two-way ANOVA of the 18 h assay data revealed a significant effect of "substrain" ($p < .001$) and "E:T ratio" ($p < .05$). Furthermore, post hoc analysis of the 4 h assay data (Fig. 4A) revealed a significantly reduced NK cytotoxicity in the F344/Crl(Ger/98/DPPIV-) substrain when compared with the F344/DuCrj(98/DPPIV-) rats.

3.4 Discussion

DPPIV-like enzymes specifically cleave several regulatory peptides including GLP-1, NPY, substance P, and chemokines, which are characterized by a N-terminal alanine or proline. Biological research on *in vivo* functions of these regulatory peptides is often carried out in rats or in rat derived biomaterial. The present study demonstrates that the *Dpp4* gene is located on rat chromosome 3 and that it is inherited in a semi-dominant fashion. Furthermore, we show that laboratory inbred strains of rats exhibit obvious differences in DPPIV-like enzymatic activity, which probably affects degradation and half-life of regulatory peptides, which are substrates of the DPPIV. Even more important for researchers using F344 rats, we also observed that the commercially available F344/DuCrj(DPPIV-) rat substrain from a breeding colony in Japan (Atsugi) constantly exhibits a nearly complete lack of DPPIV-like enzymatic activity, while rats of the F344/Crl(Ger) substrain from breeding colonies in Germany (Sulzfeld) only occasionally showed a lack of DPPIV-like enzymatic activity. In regard to the physiological relevance of this enzymatic system, we furthermore demonstrated that a lack of DPPIV-like activity is associated with an improved glucose tolerance and a decreased NK cell mediated lysis of tumor cells.

Using an SSLP marker located within the sequence of rat *Dpp4* we were able to define the position of this gene on RNO3. This finding suggests that the gene content is conserved between this segment of RNO3 and the corresponding parts of MMU2 and HSA2, where the homologous orthologous genes of rat *Dpp4* are located. Further on, we provide information about the distance of *Dpp4* to anonymous microsatellite markers, which are generally used for linkage mapping of loci defined by phenotype alone. This should be a valuable prerequisite for further analysis of the influence of *Dpp4* on the phenotypes of different rat strains.

Since the present study demonstrates that F344/DuCrj(DPPIV-) rats, which are homozygous for the mutant *Dpp4* allele, lack the endogenous DPPIV-like activity our data about the mode of inheritance provide strong evidence that the wildtype-like DPPIV remains active on an intermediate level in heterozygous F344 rats. Therefore, out- and intercrosses between F344/DuCrj(DPPIV-) and F344/Ztm might be a valuable tool to examine the impact of DPPIV-like activity also on other DPPIV-dependent physiological parameters.

Furthermore, we screened different F344/Crl breeding colonies in Portage and Raleigh (USA), in Atsugi (Japan), and in Sulzfeld (Germany) in 1998 and 2001. The two independent sets of F344 rats from Japan F344/DuCrj(98/DPPIV-) and F344/DuCrj(01/DPPIV-)

constantly exhibit a nearly complete lack of DPPIV-like activity. In contrast, we found a variation between the F344/Crl(Ger/98/DPPIV-) colony, which we continued to breed in our laboratory, and another set of animals from the same colony from Crl, which we obtained 3 years later [F344/Crl(Ger/01)]. While F344/Crl(Ger/98/DPPIV-) rats show a nearly complete loss of DPPIV-like activity, F344/Crl(Ger/01) are indistinguishable from the wildtype-like F344/Crl(Por/98) and F344/Ztm substrains. Surprisingly, rats recently obtained from the Crl colony in Portage, USA [F344/Crl(Por/01)], show a gender-dependent difference in the DPPIV-like enzymatic activity. Males have a dramatic reduction in the enzymatic activity, while females exhibit a wildtype-like phenotype. The differences in the DPPIV-like activity among the F344 rats from different breeding colonies of a world-wide operating vendor at different intervals clearly indicates a persisting segregation for the *Dpp4* gene. Furthermore, the laboratory code of the substrains from the different breeding colonies in the USA and Germany is - unfortunately - identical. Overall, these findings on variation in DPPIV-like enzymatic activity indicate that scientists, who obtain F344 rats from this vendor or who work on DPPIV-dependent physiological processes in the rat should screen their animals in regard to their DPPIV-like activity.

In addition, the present study shows that there is a considerable variance in the DPPIV-like activity in different inbred strains of rats. High levels are found especially in the DA/Ztm and BN/Ztm, while LEW/Ztm animals show a low activity. In spite of the microsatellite *D3Ztm1* being polymorphic between BN/Ztm and LEW/Ztm, it still remains unclear whether the various DPPIV-like activities can be attributed to differences among coding regions of the *Dpp4* genes or to modifier genes of the respective genetic background. Regarding the F344 substrains F344/DuCrj(98/DPPIV-) and F344/Crl(Ger/98/DPPIV-) we assume that the deficiency in DPPIV-like activity can be attributed to a previously described spontaneous mutation [31], which lead to a null allele. Modifier genes can be most likely excluded due to the fact that the genetical background of the different F344 substrains might be similar. Nevertheless, these findings might have important implications for research focusing on endocrine and immunological aspects directly or indirectly related to DPPIV/CD26 such as research on diabetes, T cell function, or cell migration in the rat.

To our knowledge, only a few studies so far used DPPIV-deficient F344/DuCrj(DPPIV-) rats to investigate influences of DPPIV-like activity on glucose tolerance [35, 36, 40]. Because of partly contradictory findings regarding the glucose tolerance in these F344/DuCrj(DPPIV-) animals and since the DPPIV-deficient F344/Crl(Ger/DPPIV-) colony has not been tested, we investigated the glucose homeostasis in the three F344 substrains bred in our laboratory since

1998. The results of the study provide strong evidence that DPPIV plays an essential role in the physiological control of blood glucose. This is reflected by an improved glucose tolerance in the DPPIV-deficient F344/DuCrj(98/DPPIV-) and F344/Crl(Ger/98/DPPIV-) rats. These findings are in agreement with studies in F344/DuCrj(DPPIV-) rats [35, 40] and in DPPIV knockout mice [20]. The lack of DPPIV-like activity in the two DPPIV-deficient substrains seems to be responsible for the improved glucose tolerance.

GLP-1 is involved in glucose homeostasis by its multifaceted actions, which include stimulation of insulin gene expression, increase of glucose-stimulated insulin secretion [22, 23], and inhibition of glucagon secretion, all of which contribute to normalize elevated blood glucose levels [41]. Administration of GLP-1 functions as an antidiabetic in patients with type 2 diabetes [35] and GLP-1 receptor antagonists induce glucose intolerance in rats [42]. Thus, active GLP-1 has a powerful influence on glucose tolerance. Interestingly, inhibition of DPPIV-like activity is effective to suppress the degradation of exogenously administered or endogenously circulating incretins like GLP-1 [41, 43]. Besides DPPIV-inhibition by valine-pyrrolidide or isoleucyl-thiazolidide (*Ile-Thia*) improve the glucose tolerance [23, 44] and *Ile-Thia* also enhances the insulin secretion [45]. Obviously, inhibition of DPPIV results in increased levels of active GLP-1 and GIP [24] and an improved glucose tolerance. We conclude that a lack in DPPIV-like activity in the mutant F344 rats improves the glucose tolerance probably by an incretin-mediated mechanism [23]. Manipulation of plasma incretin concentrations by acute inhibition of DPPIV could be a therapeutic approach for improving glucose tolerance and could prevent transition to type 2 diabetes. Therefore, different F344 substrains may represent a useful tool for research focusing on glucose homeostasis.

The finding on a blunted NK cell mediated cytotoxicity against syngenic tumor cell targets suggests that DPPIV/CD26 is involved in mediating specific aspects of NK cell function. Previous work on the role of DPPIV/CD26 on NK cells has demonstrated that IL-2 stimulation increases DPPIV/CD26 expression on a subpopulation of these cells [4, 46]. However, NK cell cytotoxicity of DPPIV/CD26-positive NK cells was not different compared to DPPIV/CD26-negative NK cells [46]. Since in addition DPPIV/CD26 inhibitors had no effect on NK cell function but instead suppressed DNA synthesis and cell cycle progression of NK cells, it was concluded that DPPIV/CD26 is involved in the regulation of NK cell proliferation, whereas natural cytotoxicity seems to be regulated independently [46]. The present finding that the mutant substrains exhibited a differential suppression of their NK cell function might suggest that either a lack of DPPIV/CD26 enzymatic activity or a differential

DPPIV/CD26 expression or both factors may mediate the decrease in NK cell mediated tumor lysis.

However, important for the *in vivo* situation, it should be noted here that other partly completely different enzymes also mediate a DPPIV-like enzymatic activity [namely, fibroblast activation protein (FAP), DP8 and DP9, which like DPPIV belongs to the prolyl oligopeptidase protease family and the lysosomal enzyme DP II]. All of these peptidases possess a high homology around the active site. Furthermore, the structural unrelated protein attractin was found to express post-proline dipeptidyl-peptidase activity. At least for DP II and for attractin we could demonstrate that the Gly-Pro substrates are cleaved by these enzymes. Theoretically, a cleavage of substrates with a N-terminal penultimate proline is also possible by a subsequent action of aminopeptidase P and a prolyl aminopeptidase activity. This could be widely excluded for the measured plasma activities because control experiments demonstrates that the measured activity could be nearly completely inhibited be competitive DPPIV inhibitor *Ile-Thia*. But this small inhibitor may also be effective for inhibition of all other DPPIV-like enzymes, as it was proven for DP II and for attractin.

In conclusion, we present a gene linked SSLP marker, which allowed us to map the semi-dominant inherited *Dpp4* to rat chromosome 3. Screening of DPPIV-like enzymatic activity in commercially available F344/DuCrj(DPPIV-) rats exhibited a nearly complete lack of DPPIV-like enzymatic activity, while F344/Crl(Ger) and F344/Crl(Por) rats obtained in different years (1998 and 2001) apparently segregate or show a gender-associated difference. In addition, different rat inbred strains revealed considerable differences in enzymatic activity. DPPIV deficiency of F344 substrains was found to be associated with an improved glucose tolerance and a decreased NK cell function. Overall, the variations in DPPIV-like enzymatic activity reported here could act as confounding factors in biomedical research using rats not screened (genetically monitored) for this factor beforehand and probably should be considered when selecting rat strains for studies on regulatory peptides, which are substrates for the DPPIV.

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3.7 Tables and figures

Table 1:

	Male		Female	
	n	DPPIV-like activity	**n**	DPPIV-like activity
F344/Crl (Por/01)	3	6.6 ± 1.0	6	20.2 ± 0.4
F344/Crl (Ral/01)	3	18.1 ± 1.2	6	16.8 ± 3.5
F344/DuCrj(01/DPPIV-)	3	3.5 ± 0.1	6	6.7 ± 0.7
F344/Crl (Ger/01)	3	18.0 ± 0.2	6	17.8 ± 0.8

Gender-dependent DPPIV-like activity [mU/ml] in the different F344 rat substrains obtained from Crl in 2001 [F344/Crl(Por/01), F344/Crl(Ral/01), F344/DuCrj(01/DPPIV-), and F344/Crl(Ger/01)]. Data represent means ± SEM.

Table 2:

Rat strain (Ztm)	DPPIV-like activity
LEW	11.5 ± 1.1
WF	14.0 ± 0.3
OM	14.4 ± 1.5
WKY	14.6 ± 1.3
BDII	18.4 ± 1.7
F344	19.1 ± 0.7
BDE	20.9 ± 1.2
LE	23.1 ± 1.0
E3	24.7 ± 0.4
BDIX	31.0 ± 0.8
BN	39.7 ± 3.3
DA	39.8 ± 0.9

DPPIV-like activity [mU/ml] in different inbred rat strains obtained from the Ztm in 2001. Data represent means ± SEM ($n \geq 3$ rats).

Fig. 1: Genetic linkage map of RNO3 including gene linked SSLP marker (*D3Ztm1*).

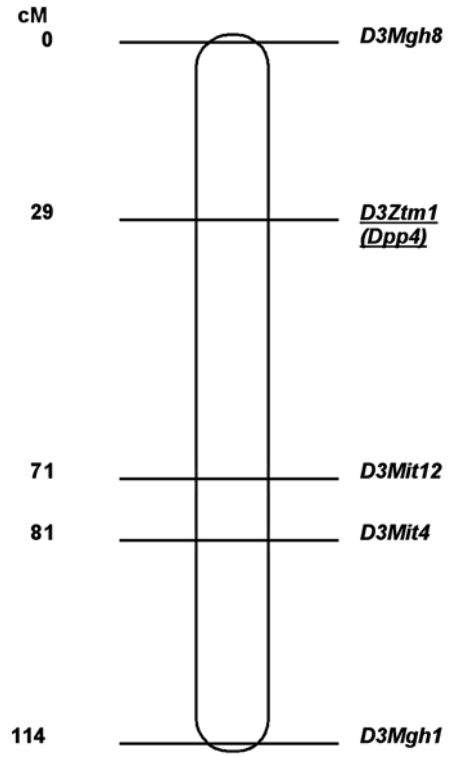


Fig. 2: DPPIV-like activity [mU/ml] in three different F344 rat substrains obtained from Crl in 1998 [F344/Crl(Por/98), F344/DuCrj(98/DPPIV-), and F344/Crl(Ger/98/DPPIV-)]; blood samples were taken from the tail vein. Data represent means + SEM. Significant post hoc effects versus the wildtype-like animals of the F344/Crl(Por/98) substrain are indicated by asterisks (***$p < .001$).

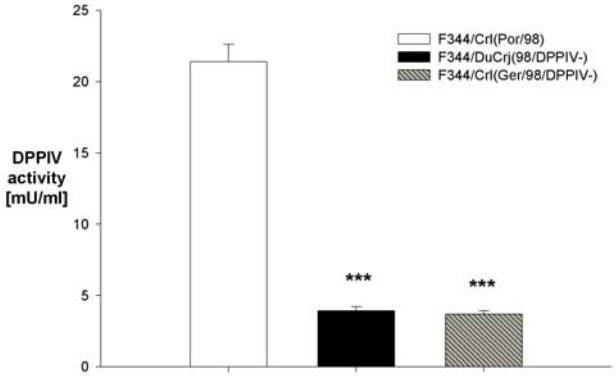


Fig. 3: Glucose tolerance [mmol/l] in three different F344 rat substrains obtained from Crl in 1998 [F344/Crl(Por/98), F344/DuCrj(98/DPPIV-), and F344/Crl(Ger/98/DPPIV-)] following an overnight fast; administration of oral glucose (1.5 g glucose/kg) in animals, which were anesthetized shortly with Isofluran. Blood samples (10 µl) were collected at 30, 60, 90, and 120 min following the oral glucose load. Data represent means + SEM. Significant post hoc effects versus the wildtype-like animals of the F344/Crl(Por/98) substrain are indicated by asterisks (*$p < .05$ - **$p < .01$ - ***$p < .001$).

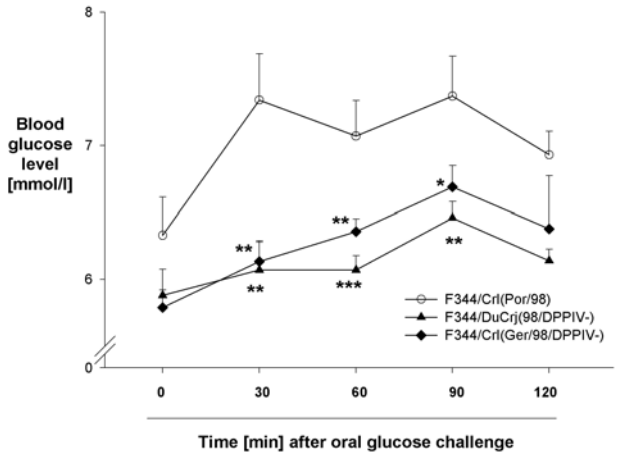
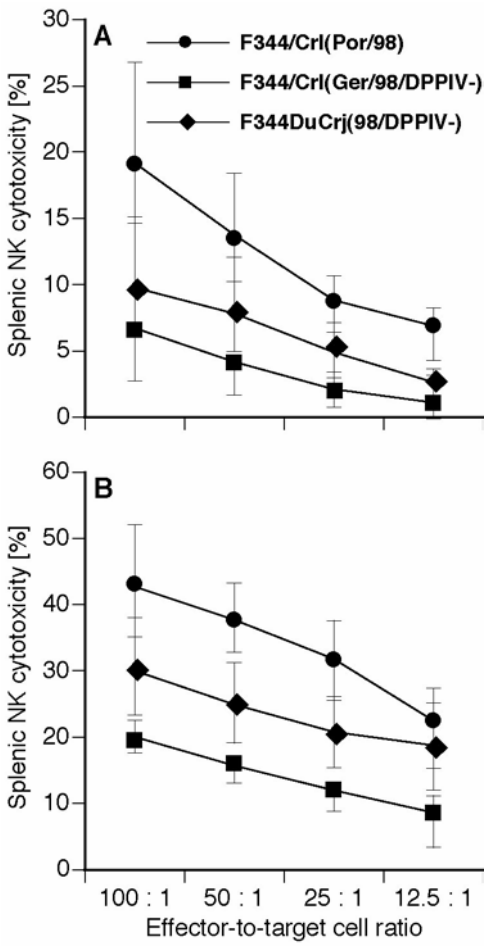


Fig. 4: Splenic NK cytotoxicity against MADB106 cells in DPPIV-deficient F344 rat substrains [F344/Crl(Ger/98/DPPIV-) and F344/DuCrj(98/DPPIV-)] compared to wildtype-like F344/Crl(Por/98) rats [%]; NK cytotoxicity was measured in an assay for ^{51}Cr release from labelled target cells after (A) 4 h incubation with IL2 and (B) 18 h incubation using syngeneic MADB106 tumor cells as targets. Assays were repeated twice. Data represent means ± SEM.



Extreme reduction of dipeptidyl-peptidase IV activity in F344 rat substrains is associated with various behavioral differences

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Abstract

The enzyme and binding protein dipeptidyl-peptidase IV (DPPIV; CD26) has a unique enzymatic specificity in cleaving dipeptides from neuropeptides, chemokines, and hormones. Thus, DPPIV is potentially involved in the regulation of functions of the nervous, endocrine, and immune systems. In the present study we compared DPPIV-deficient, mutant Japanese [F344/DuCrj(DPPIV-)] and German [F344/Crl(Ger/DPPIV-)] F344 rat substrains with a wildtype-like F344 substrain [F344/Crl(Por)] from the USA in a multi-tiered strategy using a great number of different behavioral tests. General health, neurological and motor functions, and sensory abilities of the different F344 substrains were not different. A reduced body weight and a reduced water consumption was observed in mutant animals. DPPIV-deficient rats exhibited increased pain sensitivity in a non-habituated hot plate test, indicative of a reduced stress-induced analgesia. In line with this finding, reduced stress-like responses in tasks like the open field, social interaction, and the passive avoidance test were found. Differences in DPPIV-like activity appear to be involved in neurophysiological processes because DPPIV-deficient animals were less susceptible to the sedative effects of ethanol. The varying phenotypes of the F344 substrains are likely to be mediated by differential degradation of DPPIV substrates such as substance P, glucagon-like peptide 1 (GLP-1), enterostatin, and especially neuropeptide Y. Potentially, DPPIV-deficient substrains represent an important tool for biomedical research, focusing on the involvement of DPPIV and its substrates in behavioral and physiological processes.

Keywords: Dipeptidyl-peptidase IV - F344 rats – Substrain comparison - Basic behavioral phenotyping – Motor function - Nociception - Anxiety - Memory - Ethanol - Porsolt swim test - Prepulse inhibition – Feeding behavior

4.1 Introduction

The enzyme and binding protein dipeptidyl-peptidase IV (DPPIV), which is identical to CD26, was discovered in 1966 (31) and is a member of the class of membrane-associated peptidases. Most vertebrate tissues contain this ectopeptidase, which is also in close contact with circulating hormones in the blood. In the adult nervous system, DPPIV cleaves neuropeptides mainly in the cerebrospinal fluid since it is found primarily in the circumventricular organs and on leptomeningeal cells in the central nervous system (CNS) (50). However, other DPPIV-like enzymes such as attractin may also contribute to these processes. DPPIV has a unique enzymatic specificity, cleaving dipeptides from peptides and proteins carrying *Xaa*-Pro or *Xaa*-Ala in their penultimate position (14). Many neuropeptides, chemokines and hormones have proline residues at specific positions in their amino acid sequence. Known substrates for DPPIV are growth-hormone-releasing-factor, glucagon-like peptide 1 (GLP-1), GLP-2, kentsin, enterostatin, substance P, peptide YY, and trypsinogen. Relevant substrates in immune reactions are chemokines as well as cytokines such as IL-2 (28). In addition, it is known that DPPIV is involved in T-cell dependent immune responses (34) and in cell adhesion (50). The rapid degradation of Tyr-Pro dipeptides from the neurotransmitter neuropeptide Y (NPY) with high turnover rates by DPPIV (50) is of particular importance, because NPY is the most abundantly expressed neuropeptide within the CNS. NPY is involved in the central regulation of various neuroendocrine and behavioral functions like feeding, nociception, anxiety, and memory in rodents (35, 38, 85). Thus, DPPIV is both directly and indirectly involved in the regulation of nervous, endocrine, and immune functions. Despite these pleiotropic effects of DPPIV, no systematic investigations on the role of the DPPIV-like activity in the CNS have been conducted so far.

Interestingly, a spontaneous mutation of the *Dpp4* gene has been described in F344 rat substrains from breeding colonies of Charles River Laboratories (Crl) in Atsugi, Japan (77, 84), and Sulzfeld, Germany (75). This mutation results in an almost complete loss of DPPIV activity in contrast to the wildtype-like F344 rats from Crl breeding colonies in Portage, USA (75, 79). Therefore, we considered the mutant Japanese and German F344 rat substrains to be similar to DPPIV "germline" knockout animals. Recently we were able to demonstrate an involvement of DPPIV in the substrains' glucose homeostasis, which is most likely fine-tuned via DPPIV-dependent cleavage of the incretin GLP-1 in the periphery. These results validate

the relevance of the DPPIV-mutant F344 substrains for studying DPPIV-dependent processes (36). Obviously, these DPPIV-deficient F344 substrains may provide an interesting model to study the functional role of DPPIV in various other physiological processes, including various CNS-dependent behavioral processes. Specifically, the different enzymatic activity of DPPIV should result in a differential degradation of DPPIV substrates such as enterostatin or NPY, thereby having pronounced effects on the behavior of animals.

Therefore, we compared DPPIV-deficient F344 substrains with the wildtype-like F344 substrain. We applied a multi-tiered strategy for a behavioral and physiological characterization of an unknown phenotype in mice (11, 37) and modified this concept to the characterization of rats. General health, neurological and motor functions, and the sensory abilities of the rats were monitored. Furthermore, the effects of differential endogenous DPPIV-like activity on specific CNS-related behavioral domains such as feeding behavior, nociception, anxiety, learning and memory, and ethanol preference were investigated. Additionally, the neurophysiological role of DPPIV was evaluated in experiments screening for the sedative effect of ethanol and for symptoms related to human psychiatric disorders such as schizophrenia and depression. Since single tests for certain behavioral domains might not detect a specific defect, a large series of different tests for various behavioral domains were applied (Table 1).

4.2 Materials and methods

Animals

To ensure clarity of nomenclature, we coded animal groups as previously described (36): F344 rats derived from a breeding colony in Atsugi, Japan were coded as F344/DuCrj(DPPIV-), animals from a breeding colony in Sulzfeld, Germany, as F344/Crl(Ger/DPPIV-), and wildtype-like rats obtained from a colony in Portage, USA, as F344/Crl(Por). All animals were obtained from Charles River Laboratories in 1998.

All F344 rats of the three different substrains were housed and bred at the Central Animal Facility of the Hannover Medical School (Ztm). Animals were maintained in a separated minimal barrier sustained facility and kept in Makrolon type III cages with standard bedding (Altromin GmbH, Lage, Germany). Food (Altromin Standard Diät 1320: Altromin GmbH) and water were available *ad libitum*. Environmental temperature was automatically regulated at 21 ± 2 °C and relative humidity was 55 ± 5% 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 (light onset at 4am) being further standardized in regard to their breeding conditions (littersize: 6, gender-ratio: 1:5 or 2:4), and number of animals per cage. Routine animal care was carried out once a week. Routine microbiologic monitoring according to FELASA recommendations (64) 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.

Test sets of animals and phenotyping by determination of DPPIV-like activity

To avoid major influences from the high number of different behavioral test paradigms applied to the animals, three independent sets of age-matched F344 rats of the three substrains were used for the present study (details see Fig. 1). One set of animals (experimental set II) was single-housed, whereas the two other sets of animals were group-caged (2 - 3 animals). Prior to the final experiments, these sets were single-housed for the ethanol-self-administration task (experimental set I) and for the second feeding behavior task (feeding II –

experimental set III). All animals were characterized in regard to their DPPIV-like enzymatic activity as previously described (36) and were tested in the various behavioral paradigms in the animal room.

Experimental design of the behavioral test paradigms

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 different F344 substrains as described previously (11, 12, 37). Neuromuscular strength of the animals was tested in the wirehang test (69). Visual abilities were controlled by observing the response of the rat to an unknown object, a modified visual cliff task (59), and the so-called ring paradigm, in which the rat was dangled by the tail into the middle of a plastic ring (diameter: 20 cm) with three convexities. The occurrence of the rat´s reaching behavior with the forepaws in direction of one of the ring´s convexities was measured.

Determination of motor functions

This test of neuromotor performance is based on a motor-driven, rotating rod (33). An Ugo Basile accelerating rotarod (model 7750) for rats, supplied by Technical & Scientific Equipment GmbH (TSE GmbH), Bad Homburg, Germany, was used. During the four days of training session (onset 1 h after onset of the dark phase) animals were placed onto the apparatus at a constant speed of 20 rotations per minute (rpm) for 120 s. In the following four days of rotarod test session the animals were subjected for 1 min each to various increasing rotation speed levels (between 4 - 40 rpm) twice a day (intertrial interval: 1 h) for 1 min each. Afterwards, rats were tested for another four days (twice a day – intertrial interval: 1 h) in the accelerod test session, in which the rotation speed was constantly increased over a time period of 5 min. In both rotarod and accelerod test sessions, the latency to fall off the rod and the actual rotation speed were recorded (7).

Feeding behavior

The animals were housed either in groups (“Feeding I”) or singly in a cage (“Feeding II”). The observation period lasted 78 h each. The daily food and water consumption was measured. The consumption of the group-caged animals was recorded at the onset of the light and the dark cycle and 2 h after the onset of the dark cycle. The feeding behavior of the single-housed animals (“Feeding II”) was measured once a day at the onset of the light cycle. Multiple pairs of water tubes were monitored on empty cages to control for evaporation and accidental spillage (43).

Screening for differences in nociception

Tail flick

This test is based on the withdrawal of the tail in response to a noxious, cutaneous, thermal stimulation (17). A tail flick unit (TSE GmbH) was used. The rat was held on the apparatus top panel so that its tail received the infrared energy. Latency to flick the tail out of the path of the light beam was measured. In order to minimize possible tissue damage, different patches of tail skin were stimulated per trial (cut-off time: 15 s) (66).

Hot plate

The hot plate assay is one of the most commonly used tests for determining the analgesic efficacy of experimental drugs in rodents. A 30x30 cm hot plate analgesia meter (Columbus Instruments, Columbus, USA), maintained at 52.5 °C, was used for this experiment. Latency to *lick* or *raise hindpaws* was recorded. To prevent any tissue damage, the rat was removed from the hot plate if the animal did not respond within 20 s (56, 70). Animals were tested in a non-habituated task ("Hot plate I") and after 5 days of habituation (placing the rat onto the inactivated apparatus daily for 3 min) in a habituated task ("Hot plate II").

Evaluation of anxiety

In early studies various authors defined the open field and the elevated plus maze as tests for emotionality (1, 16, 83). The construct "emotionality" was defined by Denenberg with high defecation scores and low ambulation in the open field being indicators of high "emotionality" and *vice versa*. In more recent years, the same paradigms are discussed in regard to their potential to provide indicators of anxiety and the construct "emotionality" is less frequently used (24, 49, 63). This may mainly be due to the fact that the construct "emotionality" is likely to be confounded by effects, which primarily affect locomotor activity only. Therefore, we use the term "anxiety-like behavior" in regard to these tests.

Open field (OF)

Locomotor activity and anxiety of rats can be evaluated by placing the animal in an open field arena (16). This paradigm mimics the natural conflict between the tendency to explore a novel environment and the tendency to avoid a brightly lit, open area (15). The exploratory activity, the anxiety-like reactivity, or both different behavioral dimensions can influence the animal´s behavior (37). In this task an inverse relationship between exploration/defecation and the anxiety (emotionality) of rodents is described (16, 80). The apparatus used in this experiment has been described previously (80) and was dimly illuminated (10 lux). 1 h after onset of the dark phase animals were placed into the center of the open field arena (start area) and observed for 10 min in 2 min intervals. The latency to leave the start area, the latency and

frequency of *self-grooming*, *rearing*, and *rearing at the wall*, distance traveled (number of square entries), the ratio of distance traveled and time spent in the center and the periphery of the arena, and the defecation score were recorded (83). Exploration was measured by ambulation, latency and frequency of *rearing* and *rearing at the wall*, and latency to leave the start area. We used an increase in the ratio of traveled distance, in the time spent in the center of the arena and in ambulation and a decrease in the defecation score as indicators for anxiolytic-like behavioral responses (63), although the defecation score is discussed controversially in the literature (26, 68, 83). To avoid cohort removal effects, one animal per cage per day was tested (39).

Elevated plus maze (EPM)

The pharmacologically validated EPM deals with the preference of rodents for a dark, enclosed, small space over a brightly lit, large, open space (45, 54). The open and closed arms generate exploratory behavior and the avoidance of the elevated, open arms is an indication of the intensity of anxiety (29, 45). An elevated plus maze (TSE GmbH) was used as previously described (4). Illumination was dim (10 lux). 1 h after onset of the dark phase the animal was placed onto the center platform facing an open arm. In the following 5 min the time spent on open arms as well as the percentage of open arm entries were recorded. The number of closed and total arm entries were considered to provide indices of general motor activity (58) and an increase in the time spent on open arms and in the percentage of open arm entries is used as an indication of anxiolysis (22, 49, 58). In addition, *rearing*, *self-grooming*, and the defecation score were measured. To avoid cohort removal effects, one animal per cage per day was tested.

Social interaction

The rat social interaction test is used widely to measure anxiety-like behavior (21) and to detect anxiogenic and anxiolytic-like effects of drugs (39). Active social interaction (SI) time is inversely related to the anxiety of the animals (23), which is confirmed by the observation that the maximum active SI time is found when rats are tested in a familiar test arena with a low level of illumination (23). Furthermore, a decrease in SI time is correlated with an increase in other anxiety-like behaviors: defecation, *freezing*, and displacement activity (i.e. eating of non-edible objects by rats that are not food deprived). Therefore, the decrease in social behaviors is consistent with behavioral indications of increased arousal or anxiety and is not explained by any changes in other competing behaviors such as exploration (20, 21).

The apparatus used for this study has been previously described (39). 1 h after onset of the dark phase two weight-matched rats of the similar substrain but from different cages were

exposed to the arena, which was brightly illuminated (180 lux). The total duration and the frequency of different behaviors like *anal sniffing*, *following*, *allo grooming, walking over*, *crawling under*, and *nosing* were recorded and summed during 10 min. Additionally, the motor activity of the rats was analyzed by recording distance traveled (counting the number of entries into squares).

Learning and memory

Radial maze

The radial maze test, in which rodents are trained to visit a pattern of arms to receive a food reward (precision pellets for rodents: Campden Instruments LTD, Loughborough, England), is specific to measure spatial memory – which includes working and reference memory (71). We used an octagonal alley maze (automated radial arm maze: TSE GmbH), made of grey PVC. The apparatus was placed in a well-lit room (180 lux) containing 4 completely different structured walls (with different geometrical cues painted on each wall, which served as external cues). Food deprivation started one week before the experiment. The body weight was kept at 85-95% of the pre-test body weight by presenting food after the daily trials for only 2 h (13, 53). Animals were tested during the light phase as previously described (30, 81):

Day 1-3: habituation of rats to the maze and baited food cups for 10 min;

Day 4-11: the same four randomly chosen arms were baited in the naive sessions; animals were placed into alternate non-baited start arms and were tested twice a day for 5 min or until all pellets were collected; entries in already visited arms were recorded as working memory errors, whereas entries in never baited arms were counted as reference memory errors;

Day 12-16: the previously unbaited arms were baited (and *vice versa*) in order to test the ability of the rats to relearn (reversal learning);

Day 17: in the intratrial delay the rats were retained in the second visited arm after entering for 30 s to control the rats´ ability to bridge delays, which seems to rely on a functioning hippocampus and/or basal ganglia;

Day 23: in this intertrial delay session (after 5 days without testing the animals in the radial maze) similar arms were baited, as conducted six days prior; the animals´ ability to remember the prior baited arms is analyzed during this session.

The number of working and reference memory errors, arm entries and right choices, the latency to leave the start area, the total duration for collecting all pellets, the sequence of arm entries, and the defecation score were analyzed.

Passive avoidance

The passive avoidance task measures the response resulting from an associative learning process (8). The apparatus used was a Shuttle box system (TSE GmbH). In the training session (1 h after onset of the dark phase), the rat was placed for 10 s into a lightened chamber, after which the door to the dark compartment was opened. Instantly after entering the dark chamber, the door of the dark chamber was closed and a 0.8 mA footshock was delivered for 1 s. The animal remained in the dark compartment for further 10 s to allow the formation of an association between the location/surrounding and the footshock. In the retention test session 24 h later, the rat was placed into the light chamber again and the door to the dark chamber remained opened. The latency to enter the dark chamber (light-dark transversion time) and the defecation score were measured (10) in the training and the retention test session (cut-off time: 300 s). Differences in the light-dark transversion time between the substrains in the retention session could be a hint for a different ability of the animals to remember the aversive stimulus received in the dark chamber 24 h earlier. In the training session the light-dark transversion time is discussed to be affected by anxiety, because rats exhibit an aversion of a brightly lit compartment (10) and diazepam decreased the latency to enter such a compartment (8). Therefore, we interpreted differences in the light-dark transversion time in the training session as an indicator of anxiety in animals.

Tests related to symptoms of human psychiatric disorders

Prepulse Inhibition (PPI)

Prepulse inhibition of a startle response is the phenomenon, in which a weak prepulse suppresses the response to a startling stimulus (57). Deficits in prepulse inhibition are common in schizophrenic patients (72). An automated startle system (TSE GmbH) was used. Animals were habituated to the startle chambers on five consecutive days for 5 min each. The test schedule was based on that used by Caine and coworkers (6), with minor modifications, and the animals were tested 1 h after the onset of the dark phase in two sessions (startle stimulus: 90/120 dB) with an intertrial interval of 48 h. The rat´s startle amplitude was recorded for each trial.

Porsolt swim test

Rats, when forced to swim, will eventually only make those movements, which are necessary to hold the head above the water (*behavioral immobility*). This *behavioral immobility* is taken as a state of "behavioral despair" and can be reduced by antidepressant treatment (3, 60). In this task the rat was plunged into a vertical plexiglass cylinder (height: 40 cm; diameter: 18 cm) containing 15 cm of water maintained at 25 °C. The animal had to swim for 10 min. The

animal was then removed and allowed to dry for 15 min in a heated enclosure (32 °C). 24 h later the rat was tested again for 5 min. The latency and total duration of *behavioral immobility* was measured during both trials.

Ethanol consumption and sensitivity

Ethanol self-administration

The two-bottle choice technique of assessing ethanol self-administration was established in 1940 (65). Animals were housed individually. Two bottles were presented, one bottle containing tap water, while the other contained ethanol [96% (v/v)] of increasing concentrations [for three days each: 3%, 6%, 10%, and 20% (v/v)]. Daily fluid consumption was recorded 4 h after onset of the light phase. The bottles´ positions were alternated each day. Multiple pairs of water and ethanol tubes were monitored on empty cages to control evaporation and accidental spillage. Consumption of ethanol was expressed relative to total fluid consumption (ethanol preference ratio) and relative to body weight.

Sedative effect of ethanol

The time to regain the righting reflex after an ethanol injection was measured (73). Rats received an intraperitoneal (i.p.) injection of ethanol [2.37 g/kg body weight, 20% (v/v) mixed in isotonic saline]. At the onset of the ethanol-induced sedation (defined as loss of righting reflex: loss of the ability to stand on all four paws after being placed on the back; three times within 30 s) rats were placed on their back onto bedding material. The time that elapsed between the loss and the regain of the righting reflex was recorded.

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” (for the “between-subject effects”) and different continuous response variables within successive measurements were used as the “within-factors” (e.g. body weight over time; for the “within-subject effects”). In case of significant differences in regard to the “between factor”, this was followed by one-way ANOVAs (factor: “substrain”); split by the dimension of the continuous response variable. One-way ANOVAs were followed by the Fisher-PLSD-test for post hoc comparison to evaluate pairwise differences among levels of main effects. Differences were regarded as statistically significant if $p < .05$. The number of animals per substrain (n) was 10. Presenting the *degrees of freedom* indicates exceptions from this. Significant post hoc effects versus the control animals of the

F344/Crl(Por) substrain are indicated by asterisks (*$p < .05$; **$p < .01$; ***$p < .001$), whereas significant differences between the two DPPIV-deficient rat substrains F344/DuCrj(DPPIV-) and F344/Crl(Ger/DPPIV-) are shown by rhombs (#$p < .05$ - ##$p < .01$ - ###$p < .001$). All data are presented as means ± standard error of the mean (SEM).

4.3 Results

DPPIV-like activity

One-way analysis of variance of the DPPIV-like activity of the test animals from the three different substrains revealed significant differences in the enzymatic activity [$F(2, 55) = 111.4$; $p < .001$; Fig. 2]. A nearly 5-fold lower level of DPPIV-like activity was evident in the two mutant substrains F344/DuCrj(DPPIV-) and F344/Crl(Ger/DPPIV-) when compared to the enzymatic activity found in the wildtype-like rats from the F344/Crl(Por) substrain.

Behavioral test paradigms

General health and neurological examination

Screening for general health, neurological reflexes, and sensory abilities established the good health, normal status of reflexes, good neuromuscular strength, and normal sensory abilities in all animals of all substrains (statistical analysis by one-way ANOVA). Interestingly, the body weight was significantly decreased in animals of the mutant substrains (Fig. 3) that were aged between 42 and 70 days [repeated measures ANOVA; "between factor": $F(2, 15) = 9.5$; $p = 0.02$], which was confirmed by separate one-way ANOVAs split by day [day 42: $F(2, 15) = 13.4$; $p = 0.0005$; day 49: $F(2, 15) = 8.2$; $p = 0.004$; day 56: $F(2, 15) = 6.3$; $p = 0.01$; day 63: $F(2, 15) = 10.8$; $p = 0.001$; day 70: $F(2, 15) = 9.1$; $p = 0.003$]. Importantly, screening of body weight in older rats of all three substrains directly before each behavioral test did not reveal any significant differences in body weight anymore.

Determination of motor functions

No differences were found in neuromotor performance in the rotarod and accelerod paradigm (data not shown).

Feeding behavior

Monitoring the feeding behavior of group-caged rats ("Feeding I") revealed no significant differences in food intake between the three different F344 substrains but a trend toward reduced fluid consumption in the DPPIV-deficient F344 substrains (data not shown). In single-housed rats ("Feeding II") we again found no significant differences in food consumption (data not shown). However, significant differences in water intake were found [repeated measures ANOVA; "between factor": $F(2, 26) = 9.5$; $p = 0.0008$ - followed by one-way ANOVA: day 1: $F(2, 26) = 4.5$; $p = 0.02$; day 2: $F(2, 26) = 7.8$; $p = 0.002$; day 3: $F(2,$

26) = 5.0; p = 0.02; Fig. 4) with a reduced fluid consumption in F344/DuCrj(DPPIV-) and F344/Crl(Ger/DPPIV-].

Screening for differences in nociception

No significant differences in tail flick latency were observed in this type of pain measurement (data not shown). However, in the hot plate, repeated measures ANOVA revealed a significant substrain effect ["between factor": $F(2, 27) = 15.2$; $p < .001$] and additionally, a significant substrain × habituation interaction [$F(2, 27) = 16.7$; $p < .001$; Fig. 5]. This interaction was due to the fact that in the first non-habituated hot plate ("Hot plate I") the latency to respond reflected a "hyperalgesic-like response" (i.e. reduced latency to respond by approximately 40%) in the two mutant substrains [one-way ANOVA: $F(2, 27) = 20.2$; $p < .001$]. However, these differences in pain sensitivity disappeared [one-way ANOVA: $F(2, 27) = 2.0$; $p = 0.1$] when the same animals were habituated to the apparatus for five consecutive days ("Hot plate II").

Evaluation of anxiety

Open field

The distance traveled (number of square entries), the ratio of center to total square entries, and the defecation score of the three different F344 substrains did not differ significantly. Interestingly, a significantly decreased latency to rear up [one-way ANOVA: $F(2, 24) = 9.1$; $p = 0.001$; Fig. 6A] and a significantly increased frequency of this "exploratory-like" behavior [one-way ANOVA: $F(2, 24) = 7.5$; $p = 0.003$; Fig. 6B] was found in the DPPIV-deficient rats of both substrains.

Elevated plus maze

The ratio of open to total arm entries was similar in the three different F344 substrains [F344/Crl(Por): 27.2 ± 16.9; F344/DuCrj(DPPIV-): 21.0 ± 13.8 and F344/Crl(Ger/DPPIV-): 21.7 ± 11.7]. We also found no significant differences regarding the motor activity (number of closed and total arm entries) of the animals (data not shown).

Social interaction

One-way ANOVA proved that the overall time spent on behaviors like *anal sniffing*, *following*, *allo grooming*, *walking over*, *crawling under*, and *nosing* was significantly increased in the F344/Crl(Ger/DPPIV-) animals compared to the wildtype-like F344/Crl(Por) rats [$F(2, 27) = 4.5$; $p = 0.02$]. The summed social interaction time of the F344/DuCrj(DPPIV-) substrain was not significantly increased (Fig. 7) compared to the control animals. Furthermore, one-way ANOVA exhibited a significant increase in the frequency of *nosing* [$F(2, 27) = 5.0$; $p = 0.02$] in the F344/Crl(Ger/DPPIV-) animals (32.1 ±

3.5) compared to the F344/DuCrj(DPPIV-) (21.6 ± 3.9) substrain and the wildtype-like F344/Crl(Por) animals (18.4 ± 2.1).

Learning and memory

Radial maze

In the complex radial maze task, the learning and memory of the F344 rat substrains were not significantly different in the naive, reversal, and intratrial sessions (data not shown). In the intertrial session on day 23, the two DPPIV-deficient substrains showed a decreased retention ability compared with the wildtype-like substrain regarding the number of reference memory errors [F344/Crl(Por): 2 ± 0.3; F344/DuCrj(DPPIV-): 3 ± 0.2 and F344/Crl(Ger/DPPIV-): 2 ± 0.1], although this failed to be statistically significant using parametric analysis.

Passive avoidance

In the passive avoidance task no differences in associative learning of the different substrains were found. Interestingly, we found a significant substrain × light-dark transversion time interaction [two-way ANOVA: $F(2, 23) = 3.4$; $p = 0.05$], which was due to the increased latency in the mutant F344/DuCrj(DPPIV-) (113.5 ± 40.9 s) and F344/Crl(Ger/DPPIV-) (79.6 ± 33.6 s) substrain compared to the control substrain F344/Crl(Por) (24.7 ± 4.3 s) in the training session. The difference was significant between F344/DuCrj(DPPIV-) and control rats in the training session [one-way ANOVA: $F(2, 23) = 4.4$; $p = 0.03$; Fisher-PLSD-test: $p = 0.02$]. Additionally, none of the wildtype-like rats entered the dark compartment in the retention session. Furthermore, only the wildtype-like animals defecated in the training session [F344/Crl(Por): 2.2 ± 1.4].

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All animals showed a clear startle response, indicating normal hearing abilities. However, no significant differences in prepulse inhibition were found (data not shown). Also, the Porsolt swim test revealed no differences in the duration of *behavioral immobility* between the different F344 substrains (data not shown).

Ethanol consumption and sensitivity

The different substrains did not differ in their ethanol preference in the voluntary ethanol consumption task of different ethanol concentrations (data not shown). Interestingly, all substrains showed a significantly higher preference for ethanol than for tap water when ethanol concentrations of 3% [repeated measures ANOVA: $F(2, 26) = 15.4$; $p = 0.0002$] and 6% [repeated measures ANOVA: $F(2, 26) = 11.5$; $p = 0.001$] were presented (Table 2). The sedative effect of ethanol, quantified by the duration of the loss of righting reflex, was

significantly shortened in both mutant substrains [one-way ANOVA: $F(2, 15) = 5.5$; $p = 0.02$; Fig. 8].

4.4 Discussion

The systematic phenotyping of F344 substrains with or without endogenous DPPIV-like enzymatic activity demonstrated an important involvement of the ectopeptidase DPPIV in several behavioral and physiological processes. The two DPPIV-deficient F344 substrains [F344/DuCrj(DPPIV-) and F344/Crl(Ger/DPPIV-)] exhibited a significantly reduced body weight and water intake, a significant hyperalgesia in the non-habituated hot plate task, and a significantly differential response to the habituation for this experiment. In the OF we observed significantly increased exploratory-like behavior in these rats, while anxiety was significantly reduced in the F344/Crl(Ger/DPPIV-) rats in the social interaction test and in F344/DuCrj(DPPIV-) rats in the passive avoidance task. Interestingly, a reduced susceptibility to the sedative effect of ethanol was evident in the mutant F344 substrains.

The observed differences in body weight in the different F344 substrains could be based on elevated endogenous levels of the DPPIV substrate enterostatin in young DPPIV-deficient rats. Enterostatin, which is stabilized by DPPIV inhibition with val-pro-asp-pro-arg (VPDPR) (50), produces a dose-dependent reduction in fat intake and a chronic decrease in body weight and body fat levels (19), which could be responsible for the initial differences in the body weight between the mutant and wildtype-like rats.

DPPIV is the principal metabolizing enzyme for GLP-1. Studies with DPPIV knockout mice (48) and F344/DuCrj(DPPIV-) rats (42, 55) described the inactivating effect of DPPIV on active GLP-1 levels *in vivo*. GLP-1 functions as a potent inhibitor of water intake in rodents after intracerebroventircular (i.c.v.) administration, and stimulates urinary excretion of water. Furthermore, both i.p. and i.c.v.-injection of GLP-1 inhibit both basal- and angiotensin II-induced drinking behavior (18). Therefore, the decreased water intake of DPPIV-deficient animals could be mediated via elevated levels of non-cleaved GLP-1 in both mutant substrains compared to the control animals.

The hyperalgesic phenotype of F344/DuCrj(DPPIV-) and F344/Crl(Ger/DPPIV-) rats in the hot plate test could be related to the involvement of DPPIV substrates such as substance P and NPY in pain sensitivity. Analgesia in the hot plate task is proven to be elicited by low doses of intraventricularly or intrathecal administered substance P (61). On the other hand, high doses of this substance produce hyperalgesia (46). Therefore, differences in the degradation of substance P between the mutant and wildtype-like substrains could be involved in the

decreased pain threshold of the DPPIV-deficient F344/DuCrj(DPPIV-) and F344/Crl(Ger/DPPIV-) rats. Interestingly, the DPPIV substrate NPY is also involved in the mediation of nociception (2, 32). The neurotransmitter is known to increase the pain threshold in rats in the hot plate paradigm (5, 51) and selective NPY receptor agonists [Y_2 agonist: N-acetyl(Leu^{28}, Leu^{31})NPY$_{24-36}$ and Y_1 agonist: (Leu^{31}, Pro^{34})NPY] decrease thermal hyperalgesia (78). A further hypothesis for the differential pain sensitivity in the non-habituated hot plate test is due to the fact that the wildtype-like F344/Crl(Por) exhibited a higher stress-associated behavioral response in tests for anxiety compared to DPPIV-deficient rats (as indicated by an increased SI time in the social interaction test, a high ambulation in the open field, and an increased light-dark transversion time and decreased defecation score in the passive avoidance task). Since a powerful stress response increases NPY release in the CNS (9, 62), the endogenous NPY level of the wildtype-like animals could be higher than the NPY levels of the mutant animals and therefore lead to a decrease in pain sensitivity in the control animals. Additionally, the higher stress response could increase the pain threshold of the control animals by the so-called stress-induced hypoalgesia (41, 52). This is confirmed by the significant differential response to the hot plate habituation between the substrains. In contrast, we were unable to find differences in the tail flick test. This could be based on differences between the two nociception schedules: the measurement of nociception in the hot plate test is significantly influenced by processes in the CNS, whereas the tail flick test measures predominantly spinal reflexes (44). Furthermore, we speculate, that only in the tail flick task the animal´s behavior is strongly influenced by the necessary handling procedure (fixation of the animal).

In the open field we recorded earlier and more often exploratory-like behavior (*rearing*) of the mutant substrains than in the wildtype-like animals. It is commonly assumed that low ambulation indicates high anxiety and *vice versa* (1). In additional tests for anxiety the lower anxiety of the DPPIV-deficient substrains is confirmed by a significantly increased social interaction time of the F344/Crl(Ger/DPPIV-) animals in the social interaction test and an increased light-dark transversion time of the F344/DuCrj(DPPIV-) rats in the passive avoidance task. Furthermore, the wildtype-like animals did not enter the dark compartment during the retention session in the second task (and defecated more often than the mutant animals). The pattern of these findings hints to a reduced behavioral stress response in the mutant F344 substrains. These differences in the level of arousal and/or anxiety between the different F344 substrains could be based on a differential degradation of NPY, the best peptide substrate for DPPIV (14), in the mutant and control animals, which has been reported

to reduce stress responsiveness in transgenic NPY overexpressing rats (76). Cleavage of NPY to NPY_{3-36} by DPPIV leads to a loss of Y_1 receptor specificity. NPY_{3-36} has a considerably reduced activity at the NPY Y_1 receptor subtype but still a high activity at the Y_2 and Y_5 receptor subtypes (25). Therefore, the non-degraded, native NPY in DPPIV-deficient rats is a more potent activator of the Y_1 receptor subtype than the degraded NPY_{3-36} in the wildtype-like animals. Importantly, NPY administration increases the preference for open arms in an elevated plus maze test (5) and decreases anxiety in the social interaction test (40, 67). Furthermore, rats, antisense-treated to reduce NPY Y_1 receptor density, show anxiogenic-like behavior in the elevated plus maze test (27, 82), suggesting that the anxiolytic-like effect of NPY is primarily mediated via the Y_1 receptor subtype. Since the NPY catabolism of our mutant substrains and the wildtype-like animals is probably linked to DPPIV, the reduced anxiety of the DPPIV-deficient F344/DuCrj(DPPIV-) and F344/Crl(Ger/DPPIV-) animals could be based on elevated levels of non-cleaved NPY. The reduced stress-response of our DPPIV-deficient animals is also found in NPY overexpressing transgenic rats (76), which could be a further hint for the involvement of NPY in the reduced anxiety of our mutant F344 substrains. Interestingly, DPPIV-cleaved cytokines like IL-2 could also be involved in the increased exploration of the mutant animals (86). The differences within the two DPPIV-deficient substrains F344/DuCrj(DPPIV-) and F344/Crl(Ger/DPPIV-) in both the social interaction test and passive avoidance task could be due to different compensatory mechanisms during ontogeny, but further studies on this phenomenon are required.

NPY-deficient mice showed increased ethanol consumption and are less susceptible to the sedative effect of ethanol, whereas NPY overexpressing mice exhibited an opposite phenotype (74). DPPIV-deficient rats should, in comparison to control animals, behave like NPY overexpressing mice because of higher levels of non-cleaved endogenous NPY. In addition to a comparable ethanol consumption of mutant and control F344 rats, the DPPIV-deficient substrains exhibited a reduced susceptibility to the sedative effect of ethanol. This may be explained by several processes, but in regard to a possible involvement of the NPY system in this phenomenon, further investigations have to be conducted. Finally, we did not find significant differences between the mutant and wildtype-like animals in prepulse inhibition and the Porsolt swim test, although an influence of DPPIV on psychiatric and psychosomatic diseases has recently been proposed (47).

In conclusion, the DPPIV-deficient F344/DuCrj(DPPIV-) and F344/Crl(Ger/DPPIV-) rats exhibited a reduced behavioral stress response in tests like the hot plate, social interaction, and passive avoidance. These findings suggest an involvement of DPPIV-like activity in the

regulation of anxiety-like behaviors, and these effects are possibly mediated by differential degradation of as yet unspecified/alternative DPPIV substrates. Due to the pattern of behavioral differences between mutant and control animals, which are similar to transgenic NPY overexpressing rats, it seems as if a differential cleavage of NPY could especially explain several, but not all, of the described differences herein. We have demonstrated that significant variations in DPPIV-like activity among commercially available F344 rats from different breeding colonies of Crl appear to be associated with significant differences in the behavioral, anxiety-like, exploratory, and neurophysiological phenotype. The existence of substrains among the F344 genotype should be of considerable interest for many researchers. Our F344 substrains provide an interesting model to study the functional role of DPPIV in various physiological and behavioral processes. Overall, the present approach for detecting distinct differences in a rat´s phenotype is based on a systematic behavioral and physiological phenotyping. This includes a broad range of different behavioral tests, which are necessary for an extensive characterization and for detecting specific behavioral differences. This strategy for analysing mutant rodents is becoming an increasingly important and useful tool in biomedical research.

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4.7 Tables and figures

Table 1:

Behavioral/Physiological domains	Experimental tasks
General health and neurological examination	Response to an unknown approaching object
	Modified visual cliff
	Several reflexes (e.g. eye blink and ear twitch)
Motor functions	Rotarod and accelerod
Feeding behavior	Food and fluid consumption
Nociception	Tail flick
	Hot plate
Anxiety	Open field
	Elevated plus maze
	Social interaction
	(Passive avoidance)
Learning and memory	Radial maze
	Passive avoidance
Tests related to symptoms of human psychiatric disorders	Prepulse inhibition (schizophrenic-like)
	Porsolt swim test (depression-like)
Ethanol consumption and sensitivity	Ethanol self-administration
	Sedative effect of ethanol

Behavioral/Physiological domains and the different corresponding experimental tasks

Table 2:

Ethanol concentration	F344/Crl(Por)	F344/DuCrj(DPPIV-)	F344/Crl(Ger/DPPIV-)
3%	64.9 ± 6.2	69.3 ± 6.5	67.2 ± 5.9
6%	60.5 ± 8.4	60.0 ± 7.5	61.4 ± 8.4
10%	43.1 ± 6.7	47.8 ± 6.8	45.9 ± 6.1
20%	23.6 ± 5.6	22.5 ± 4.6	32.9 ± 6.5

Ethanol self administration; ethanol preference ratio of single-housed rats was recorded for different ethanol concentrations (3%/6%/10%/20%) as total ethanol consumption relative to total fluid consumption (mean ± SEM) [%].

Fig. 1: Test setting; test biography of the three different experimental sets of age-matched F344 rats (n = 10 per substrain); additionally, the animals´ age in the various behavioral tests is shown.

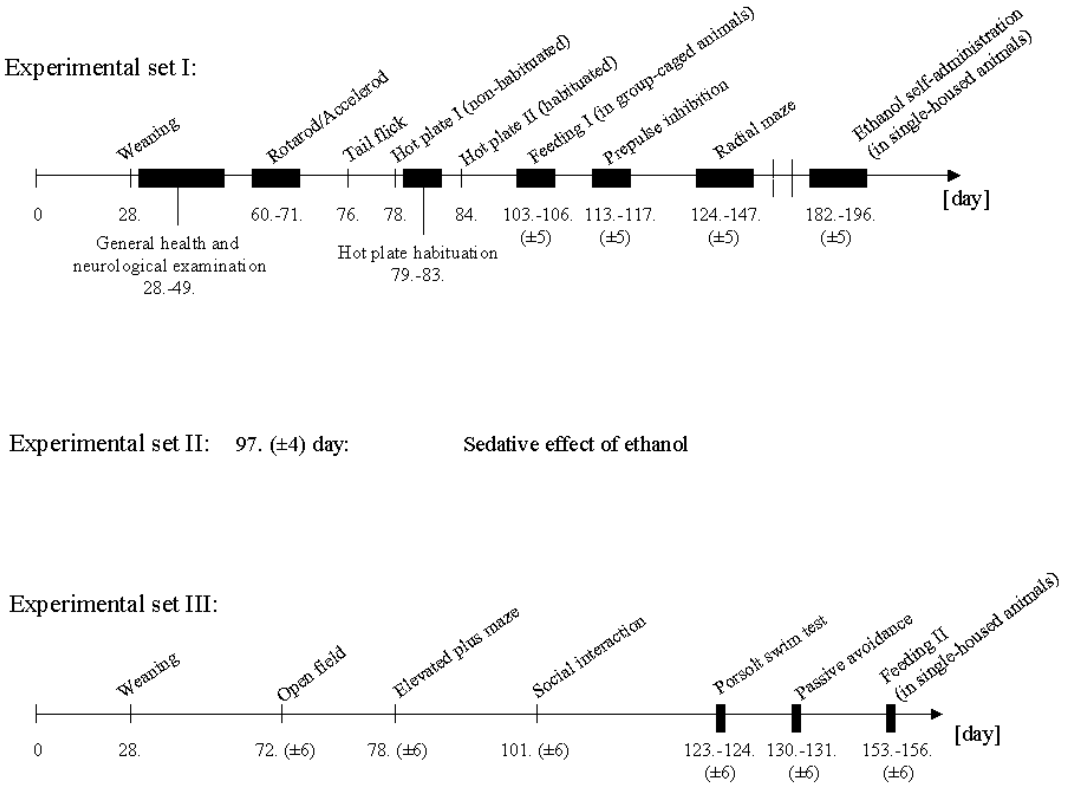


Fig. 2: DPPIV-like activity [mU/ml] of all test animals was screened; blood from the tail vein was sampled and analyzed. Data represent means + SEM and provide the *p*-values of the corresponding post hoc test (Fisher-PLSD-test). Significant effects versus the wildtype-like animals are indicated by asterisks (***$p < .001$).

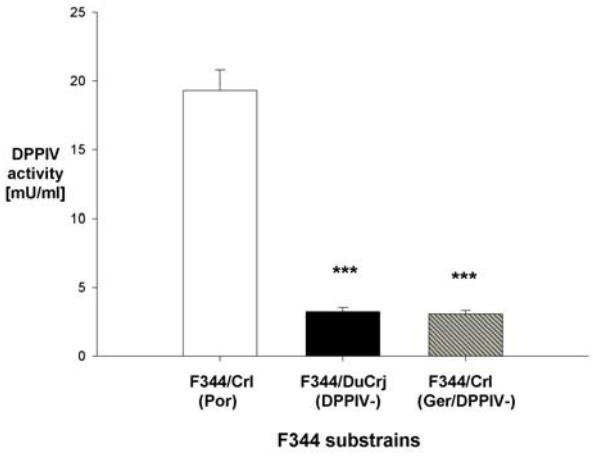


Fig. 3: Body weight development [g] of the different substrains, aged between 42-70 days. Data represent means + SEM and provide the *p*-values of the corresponding post hoc test (Fisher-PLSD-test). Significant effects versus the wildtype-like animals are indicated by asterisks (*$p < .05$; **$p < .01$; ***$p < .001$).

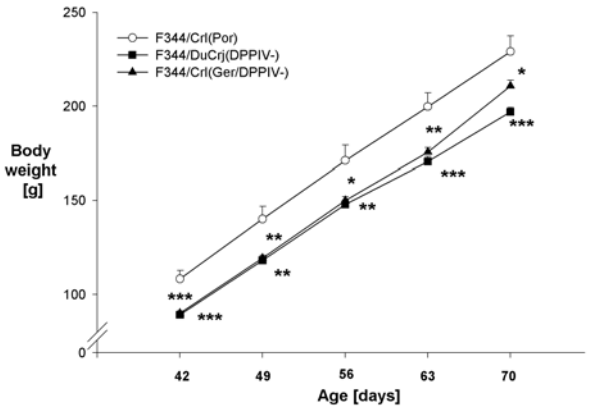


Fig. 4: Water intake; the water intake was measured in single-housed animals for 3 consecutive days; the consumption [ml] was recorded at the onset of the light phase of the light cycle. Data represent means + SEM and provide the *p*-values of the corresponding post hoc test (Fisher-PLSD-test). Significant effects versus the wildtype-like animals are indicated by asterisks (*$p < .05$; **$p < .01$; ***$p < .001$).

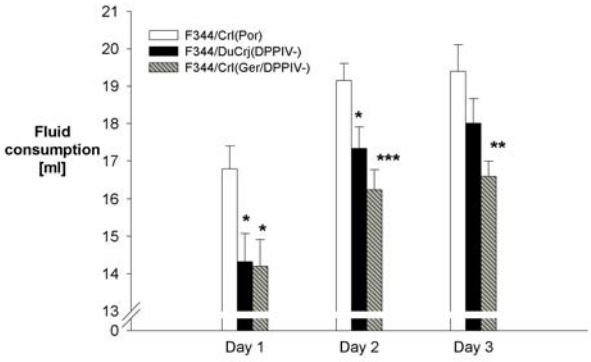


Fig. 5: Nociception was analyzed in the hot plate task; the non-habituated or habituated rat was placed onto the surface of the apparatus, which was maintained at 52.5 °C, and the latency to respond (*lick* or *raise hindpaws)* was recorded [s]. Data represent means + SEM and provide the *p*-values of the corresponding post hoc test (Fisher-PLSD-test). Significant effects versus the wildtype-like animals are indicated by asterisks (**$p < .01$; ***$p < .001$).

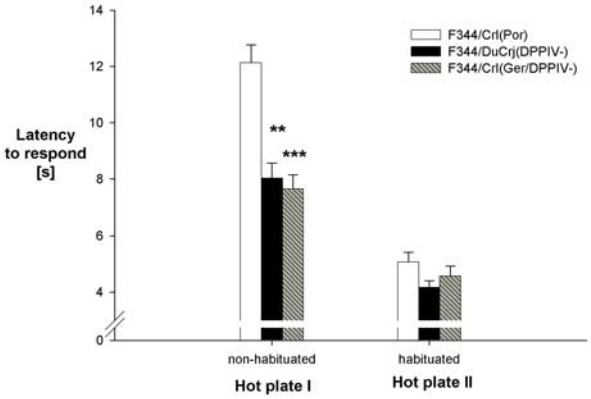


Fig. 6A: Exploratory behavior in the OF; the latency of *rearing* [s] was recorded during a 10 min session; the task took place 1 h after onset of the dark phase; the group-caged animals were tested on two consecutive days to test only one animal per cage per day. Data represent means + SEM and provide the *p*-values of the corresponding post hoc test (Fisher-PLSD-test). Significant effects versus the wildtype-like animals are indicated by asterisks (**$p < .01$; ***$p < .001$).

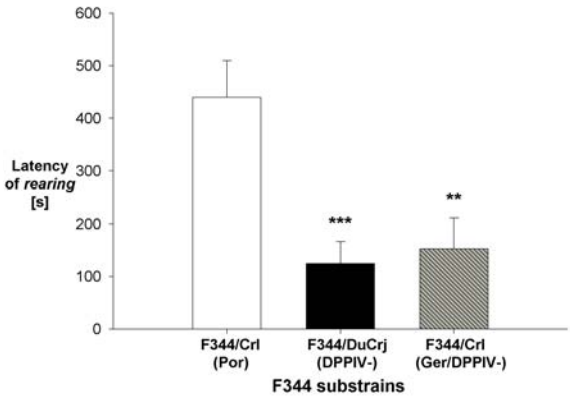


Fig. 6B: Exploratory behavior in the OF; the frequency of *rearing* [n] was recorded during a 10 min session; the task took place 1 h after onset of the dark phase; the group-caged animals were tested on two consecutive days to test only one animal per cage per day. Data represent means + SEM and provide the *p*-values of the corresponding post hoc test (Fisher-PLSD-test). Significant effects versus the wildtype-like animals are indicated by asterisks (*$p < .05$; ***$p < .001$).

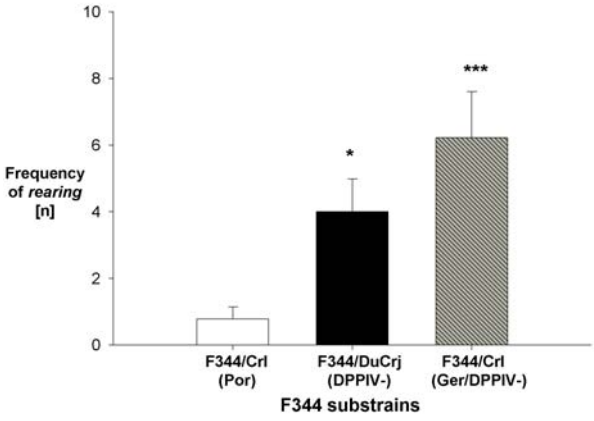


Fig. 7: Anxiety; the time spent in active social interaction was recorded in the social interaction test [s]; two unfamiliar weight-matched rats of the same substrain were exposed to an open field 1 h after onset of dark phase for 10 min. Data represent means + SEM and provide the *p*-values of the corresponding post hoc test (Fisher-PLSD-test). Significant effects versus the wildtype-like animals are indicated by asterisks (**$p < .01$).

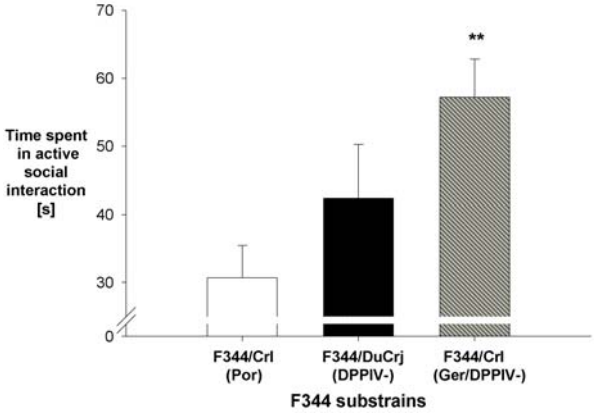
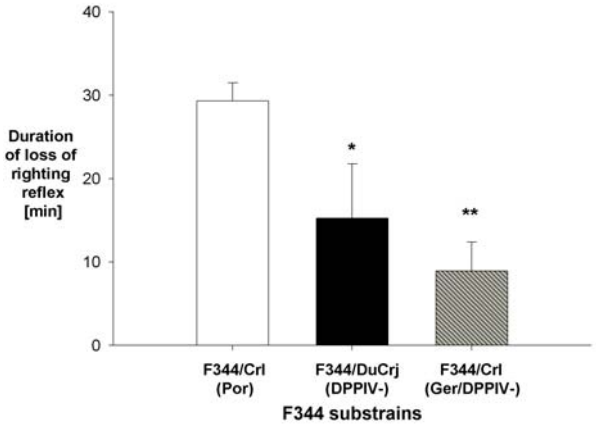


Fig. 8: Sedative effect of ethanol; the time to regain the righting reflex after an ethanol injection was measured [min]; rats received an intraperitoneal (i.p.) injection of ethanol [2.37 g/kg body weight, 20%(w/v) mixed in isotonic saline]. Data represent means + SEM and provide the *p*-values of the corresponding post hoc test (Fisher-PLSD-test). Significant effects versus the wildtype-like animals are indicated by asterisks (*$p < .05$; **$p < .01$).



Behavioral effects of neuropeptide Y in F344 rat substrains with a reduced dipeptidyl-peptidase IV activity

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Abstract

Dipeptidyl-peptidase IV (DPPIV; CD26) is involved in several physiological functions by cleavage of dipeptides with a *Xaa*-Pro or *Xaa*-Ala sequence of regulatory peptides such as neuropeptide Y (NPY). Cleavage of NPY by DPPIV results in NPY_{3-36}, which lacks affinity for the Y_1 but not for other NPY receptor subtypes. Among other effects, the NPY Y_1 receptor mediates anxiolytic-like effects of NPY. In previous studies with F344 rat substrains lacking endogenous DPPIV-like activity we found a reduced behavioral stress response, which might be due to a differential degradation of NPY. Here we tested this hypothesis and administered intracerebroventricularly two different doses of NPY (0.0, 0.2, 1.0 nmol) in mutant and wildtype-like F344 substrains. NPY dose-dependently stimulated food intake and feeding motivation, decreased motor activity in the plus maze and social interaction test, and exerted anxiolytic-like effects. More important for the present hypothesis, NPY administration was found to be more potent in the DPPIV-negative substrains in exerting anxiolytic-like effects (increased social interaction time in the social interaction test) and sedative-like effects (decreased motor activity in the elevated plus maze). These data demonstrate for the first time a differential potency of NPY in DPPIV-deficient rats and suggest a changed receptor-specificity of NPY, which may result from a differential degradation of NPY in this genetic model of DPPIV deficiency. Overall, these results provide direct evidence that NPY-mediated effects in the central nervous system are modulated by DPPIV-like enzymatic activity.

Keywords: Dipeptidyl-peptidase IV – CD26 – Neuropeptide Y – F344 — Social interaction test - Anxiety – Elevated plus maze - Sedation - Food intake - Feeding motivation

5.1 Introduction

The enzyme and binding protein dipeptidyl-peptidase IV (DPPIV) belongs to a class of membrane-associated peptidases (De Meester et al., 1999). The ectopeptidase is identical to the leukocyte differentiation marker CD26 and is involved in T-cell dependent immune responses (Kahne et al., 1999) and in cell adhesion (Mentlein, 1999; Shingu et al., 2003). Due to its unique ability to liberate *Xaa*-Pro and *Xaa*-Ala dipeptides from the N-terminus of regulatory peptides, important substrates include neuropeptides such as neuropeptide Y (NPY), peptide YY (PYY), and endomorphin (Hildebrandt et al., 2000; Mentlein, 1999). Further substrates are glucagon-like peptide 1 (GLP-1), GLP-2, enterostatin, substance P, and various chemokines (De Meester et al., 2000). In the adult nervous system, DPPIV is found primarily in the circumventricular organ and on leptomeningeal cells. The enzyme has direct contact to neuropeptides such as NPY in the cerebrospinal fluid (Mentlein, 1999) and also to endothelial cells of blood vessels including those contributing to the blood brain barrier (Hildebrandt et al., 2000). In addition, a soluble form of DPPIV exists in the blood plasma (Mentlein, 1999).

Because of these pleiotropic effects of DPPIV, we previously investigated the role of the DPPIV-like activity on behavioral and physiological processes in a mutant rat model of DPPIV deficiency. Spontaneous mutations in the *Dpp4* gene of F344 substrains 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) result in an almost complete lack of DPPIV-like activity in these animals. This DPPIV deficiency does not exist in wildtype-like F344 rats from Crl breeding colonies in Portage, USA (Karl et al., 2003a). In a systematical behavioral and physiological characterization of the different substrains, we observed differences in the mutant, DPPIV-deficient F344 rats showing increased water intake, improved glucose homeostasis, blunted natural killer cell function, reduced anxiety-like behaviors, and increased sensitivity for the sedative effect of ethanol (Karl et al., 2003a; Karl et al., 2003b). We hypothesized that at least some of these differences may be due to a differential metabolism of NPY.

The neurotransmitter NPY was discovered in 1982 (Tatemoto et al., 1982) and is a member of the pancreatic family of peptides. Significant NPY levels were found in most brain regions including the cerebral cortex, hippocampus, thalamus, hypothalamus, and brainstem (Allen et

al., 1983; Colmers and Wahlestedt, 1993). The neuropeptide is a 36 amino acid peptide with a large number of tyrosine residues. Based on this amino acid sequence, NPY is cleaved by DPPIV. It has a high affinity to the enzyme and is also metabolized in human serum by DPPIV (De Meester et al., 2000). Among several other physiological responses NPY especially affects feeding behavior and anxiety (Kalra et al., 1999; Kask et al., 2002; Wettstein et al., 1995). The neurotransmitter binds to several NPY receptor subtypes (Y_1, Y_2, Y_4, and Y_5) in rats, which belong to the large superfamily of G-protein-coupled receptors. They are widely distributed in the brain (Blomqvist and Herzog, 1997) and mediate regulatory effects of NPY (Parker and Herzog, 1999). The NPY Y_1 receptor is one of the major receptor subtypes expressed in the rat brain (Dumont et al., 1998) and is particularly involved in the regulation of anxiety (Kask et al., 2002). Importantly, DPPIV removes the first two N-terminal amino acid residues (Tyr-Pro) of NPY with high turnover rates and generates the C-terminal fragment NPY_{3-36}. NPY_{3-36} has a markedly reduced affinity to the NPY Y_1 receptor subtype, while being as potent as the native peptide on the NPY Y_2 and Y_5 receptor subtypes. Possibly, the previously described behavioral and physiological differences of the two DPPIV-deficient F344 substrains from Japan, F344/DuCrj(DPPIV-), and Germany, F344/Crl(Ger/DPPIV-), on the one hand, and the wildtype-like substrain from USA, F344/Crl(Por), on the other hand (Karl et al., 2003b), could be due to a faster degradation of endogenous NPY to NPY_{3-36} in the control substrain. This would result in a relatively reduced endogenous NPY Y_1 receptor-like tone. Such a mechanism could sufficiently explain the reduced anxiety-like behaviors in the mutant, DPPIV-deficient substrains.

To test this hypothesis, it was investigated whether NPY applied intracerebroventricularly (i.c.v.) produces differential effects in the DPPIV-deficient substrains. Thus, we characterized the effect of i.c.v. administration of different doses of NPY in the F344/DuCrj(DPPIV-), F344/Crl(Ger/DPPIV-), and F344/Crl(Por) rats on various behavioral tasks aiming at a characterization of feeding behavior (food intake) and anxiety-like behaviors.

5.2 Materials and methods

Animals

For clarity, animal groups obtained in 1998 were coded as previously described (Karl et al., 2003a): F344 rats derived from breeding colonies of Crl in Atsugi (Japan) were called F344/DuCrj(DPPIV-), animals from breeding colonies in Sulzfeld (Germany) F344/Crl(Ger/DPPIV-), and wildtype-like rats obtained from colonies in Portage (USA) F344/Crl(Por).

All F344 rats of the three different substrains were housed and bred at the Central Animal Facility of the Hannover Medical School as previously described (Karl et al., 2003a). Animals were maintained in a separated minimal barrier sustained facility and kept in Macrolon type III cages with a standard bedding (Altromin GmbH, Lage, Germany). Food (Altromin Standard Diät 1320: Altromin GmbH) and water were available *ad libitum*. Environmental temperature was automatically regulated at 21 ± 2 °C and relative humidity was 55 ± 5% 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 (light onset at 4am). They underwent routine animal care 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.

Determination of DPPIV-like enzymatic activity

All test animals were characterized in regard to their DPPIV-like enzymatic activity as previously described (Karl et al., 2003a). For determination of plasma activity of F344 rats a microplate based fluorescence assay was used. EDTA-plasma samples were kept at –80 °C until use. DPPIV enzyme activity of the different rat substrains was determined by monitoring the release of 4-Amino-7-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 DPPIV-like activities. It has been proven that the substrate is cleaved by DPPIV, by DP II, and by attractin. Probably, they are also substrates for DP8 and DP9. Importantly, the chromophores are not released by other proline-specific peptidases, such as prolidase, prolyl endopeptidase, or aminopeptidase P.

Surgery of i.c.v. cannulation

For surgery, animals were anesthetized with intramuscular (i.m.) 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 that outlined in a previous report (von Horsten et al., 1998a). After placement of the rat in a 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-ointment (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) was used to verify that the guide cannula was positioned just above the ventricular system. The guide cannula was then fitted with a dummy cannula (Plastics one) of the same length to prevent leakage of cerebrospinal fluid. Animals were housed individually after surgery. The anatomical position of the cannula was verified by post mortem i.c.v. dye application (Berlin blue) and inspection of the stained third ventricle in randomly chosen rats. The animals of the three F344 substrains F344/DuCrj(DPPIV-), F344/Crl(Ger/DPPIV-), and F344/Crl(Por) were operated at an age of 95 (±5) days. The observation of the animals' behavior in different behavioral test paradigms (an overview is given in Fig. 1) commenced after a recovery phase of at least 10 days.

Drugs, i.c.v. injections, and dosages

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 the different experiments.

For the 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 was injected i.c.v. in 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), which allowed the animal to move freely during the i.c.v. injection. Then the internal cannula was replaced by the dummy cannula again and the rat was returned into the home cage. All experiments started 15 min after the administration procedure. For the procedure of i.c.v.-injection, animals were habituated to experimental handling daily within 7 days prior to the start of the first experiment. During this habituation phase the handling procedure was exactly the same except for the application of the compound.

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 divided into three treatment groups each (n = 7), which were treated with 0.9% saline (vehicle: 0.0 nmol/5 μl), or two different doses of NPY (0.2 nmol/5 μl, or 1.0 nmol/5 μl).

Experimental designs of the behavioral test paradigms

Feeding behavior (food intake)

The feeding response (latency to start eating and the overall food consumption) following an i.c.v administration of NPY were measured for 2 h. Two experiments were performed: in experiment I, the feeding response after i.c.v. treatment was recorded in the light phase; in experiment II, 60 h later, the same response was measured 1 h after onset of the dark phase (Kushi et al., 1998; Marsh et al., 1998).

Anxiety

In early studies various authors defined tasks like the open field and the elevated plus maze as tests for emotionality (Archer, 1973; Denenberg, 1969; Walsh and Cummins, 1976). In more recent years, the same paradigms have been discussed in regard to their potential to provide indicators of anxiety, and the construct “emotionality” has been less frequently used (File and

Seth, 2003; Mechiel Korte and De Boer, 2003; Prut and Belzung, 2003). Therefore, we use the term "anxiety-like behavior" in regard to these tests.

Elevated plus maze

The elevated plus maze (EPM) represents the natural conflict of rats between the tendency to explore a novel environment and the tendency to avoid a brightly lit, open area (Handley and Mithani, 1984; Montgomery, 1958). The behavior is also influenced by thigmotaxis and the fear of heights (Treit and Fundytus, 1988). The time spent on open arms of the EPM as well as the percentage of open arm entries (ratio of open to total arm entries) are inversely related to anxiety (Hogg, 1996; Pellow et al., 1985; Pellow and File, 1986). The number of enclosed and total arm entries reflects the general motor activity (File, 1986). An automated EPM (TSE GmbH, Bad Homburg, Germany) with ledges (Fernandes and File, 1996) for rats was used. The animal was placed onto the center platform facing an open arm. In the following 5 min the entries onto open/enclosed arms, the time spent on open/enclosed arms or on the center platform, behaviors like *rearing* and *self-grooming*, and the defecation score were recorded on video (Baldwin and File, 1986; Lister, 1987). Illumination was dim (10 lux) and the experiment started 1 h after onset of the dark phase. A rat was taken to have entered an arm when all four paws were on this arm. After each session the apparatus was cleaned with 70% ethanol. To avoid influences of the animals´ endogenous rhythm on behavioral activity, tests were performed on three consecutive days with the animals of all substrains being grouped by dose of i.c.v. NPY treatment.

Social interaction

Pairs of rats, placed in a novel environment, engage in active social interactions, which include a variety of social behaviors (File, 1988; File and Hyde, 1978). The rat social interaction test is used widely to measure anxiety-like behaviors (File, 1988) and to detect anxiogenic and anxiolytic-like effects of drugs (Kask et al., 2001a). The overall active social interaction (SI) time is inversely related to the anxiety of the animals (File, 1980; File and Hyde, 1978; Karl et al., 2003c), which is confirmed by the observation that the maximum active SI time is found when rats are tested in a familiar test arena with a low level of illumination (File and Hyde, 1978). Furthermore, a decrease in SI time is correlated with an increase in other behaviors indicating increased anxiety: defecation, *freezing*, and displacement activity (i.e. eating of non-edible objects by rats that are not food deprived or *self-grooming*). Therefore, the decrease in social behaviors is consistent with behavioral indications of increased arousal or anxiety and is not explained by any changes in other competing behaviors such as exploration (File, 1980; File, 1988). The apparatus used was a

squared steel open field (50x50x50 cm) that was placed inside a sound isolation box. The floor was divided into 25 squares by a cross grid (Kask et al., 2001a). Two unfamiliar rats of the similar substrain and NPY treatment were exposed to the area. In the following 10 min the total duration and the frequency of the different behaviors *anal sniffing*, *following*, *allo grooming*, *walking over*, *crawling under*, and *nosing* of each rat were recorded. Additionally, the frequency of *self-grooming* and the motor activity (traveled distance - by recording the total number of square entries) were analyzed. The behavior was monitored online using a video camera placed above the open field inside the isolation box. The arena was brightly illuminated (180 lux) and experiments started 1 h after onset of the dark phase. After each session the apparatus was cleaned with 70% ethanol. As in the EPM test, rats were tested on three consecutive days to avoid influences of the rats´ endogenous rhythm.

Statistical analysis

The analysis of the various behavioral data was assessed using a two-way analysis of variance (ANOVA; factor: "substrain" × "treatment") and by one-way ANOVA (factor: "substrain" or "treatment" - split by the corresponding factors) followed by the Fisher-PLSD-test for post hoc comparison, if appropriate. Differences were regarded as statistically significant if $p < .05$. In the results section present the *degrees of freedom*, *F*-values, and *p*-values of two- and one-way ANOVAs are given, while in the figures and tables the *p*-values of the corresponding post hoc tests (Fisher-PLSD-test) are provided. In most cases the number of animals per substrain and treatment group was $n = 7$. Presenting the *degrees of freedom* indicates exceptions from this. Significant post hoc effects for the factor "substrain" versus the control animals of the F344/Crl(Por) substrain are indicated by asterisks (*$p < .05$; **$p < .01$; ***$p < .001$), whereas significant differences between the two DPPIV-deficient rat substrains F344/DuCrj(DPPIV-) and F344/Crl(Ger/DPPIV-) are shown by rhombs (#$p < .05$; ##$p < .01$; ###$p < .001$). Significant post hoc effects for the factor "treatment" versus the vehicle-treated animals are indicated by crosses (+$p < .05$; ++$p < .01$; +++$p < .001$), whereas significant differences between the two different NPY doses 0.2 nmol and 1.0 nmol are shown by "x" (x$p < .05$; xx$p < .01$; xxx$p < .001$). All data are displayed as means ± standard error of the mean (SEM).

5.3 Results

DPPIV-like activity

One-way analysis of variance of the DPPIV-like activity of all animals exhibited significant differences between substrains (Fig. 2). An almost complete lack of enzymatic-like activity was found in the two mutant substrains [F344/DuCrj(DPPIV-) and F344/Crl(Ger/DPPIV-)] and a wildtype-like pattern of DPPIV-like activity was found in the rats from the F344/Crl(Por) substrain [F (2; 61) = 400.4; $p < .0001$].

Behavioral test paradigms

Feeding behavior (food intake)

Two-way ANOVA revealed a significant treatment effect on food intake in the dark phase [factor "treatment": F (2; 55) = 54.7; $p < .0001$] as well as in the light phase [factor "treatment": F (2; 55) = 116.8; $p < .0001$]. Each of the different substrains exhibited a dose-dependent increase of overall food consumption after i.c.v. administration of NPY (data not shown). Neither significant effects for the factor "substrain" nor any significant interactions were observed, although mutant, DPPIV-negative rats showed a trend for reduced food intake stimulation in the light phase. Similarly, two-way ANOVA revealed that i.c.v. treatment shortened significantly the latency to eat dose-dependently [factor "treatment": in dark phase: F (2; 55) = 13.1; $p < .0001$; one-way ANOVA for F344/DuCrj(DPPIV-): F (2; 18) = 17.6; $p < .0001$ - in light phase: F (2; 55) = 26.9; $p < .0001$; one-way ANOVA for F344/Crl(Por): F (2; 18) = 10.1; $p = 0.001$; one-way ANOVA for F344/DuCrj(DPPIV-): F (2; 18) = 4.8; $p = 0.02$; one-way ANOVA for F344/Crl(Ger/DPPIV-): F (2; 19) = 13.6; $p = 0.0002$]. However, neither significant differences for the factor "substrain" nor significant "substrain" × "treatment" interactions were found (Table 1).

Anxiety

Elevated plus maze

Two-way ANOVA revealed no significant effect for the factor "substrain" on the ratio between open and total arm entries as an indicator of anxiety [F (2; 51) = 1.1; $p = 0.3$; Fig. 3], although this ratio increased dose-dependent significantly after i.c.v. treatment with NPY (factor "treatment": F (2; 51) = 10.5; $p < .0001$). Additionally, NPY administration increased significantly the time spent on open arms [two-way ANOVA; factor "treatment": F (2; 51) =

8.6; $p = 0.0006$; data not shown]. But since in both parameters no significant "substrain" × "treatment" interactions or significant substrain differences were found, these results indicate that the three substrains did not respond differentially to the anxiolytic-like effects of i.c.v. NPY as determined by the EPM.

However, the motor activity (total arm entries) was significantly reduced in the mutant F344/DuCrj(DPPIV-) and F344/Crl(Ger/DPPIV-) animals [two-way ANOVA; factor "substrain": F (2; 51) = 6.4; $p = 0.003$] compared to the wildtype-like rats after i.c.v. administration of 1 nmol NPY [one-way ANOVA for 1 nmol: F (2; 16) = 10.2; $p = 0.001$; Fig. 4A]. Overall, NPY treatment significantly decreased motor activity, especially in the DPPIV-deficient rats of both substrains [two-way ANOVA; factor "treatment": F (2; 51) = 4.9; $p = 0.01$]. In addition, the frequency of enclosed arm entries was significantly reduced after i.c.v. treatment with NPY [two-way ANOVA; factor "treatment": F (2; 51) = 12.1; $p < .0001$; one-way ANOVA for F344/DuCrj(DPPIV-): F (2; 18) = 5.8; $p = 0.01$; one-way ANOVA for F344/Crl(Ger/DPPIV-): F (2; 19) = 4.7; $p = 0.02$; Table 2], although the differences between the substrains failed to be statistically significant [two-way ANOVA; factor "substrain": F (2; 51) = 2.3; $p = 0.11$]. Furthermore, a significant "substrain" × "treatment" interaction regarding the time spent on open arms per entry was found [two-way ANOVA; "substrain" × "treatment": F (2; 51) = 2.6; $p < .05$; Fig. 4B]. Although one-way ANOVA revealed no significant differences regarding the factor "substrain" within the differential NPY treatments, the animals, which lack DPPIV-like activity, were found to stay longer on the open arms per entry than the control F344/Crl(Por) rats (Fig. 4B). NPY treatment significantly increased the time spent on open arms per entry [two-way ANOVA; factor "treatment": F (2; 51) = 7.7; $p = 0.0012$].

Social interaction

Recording the time spent in active social interaction (SI) in pairs of rats of the same substrain and treatment as an indicator of anxiety revealed that the time spent in various behaviors was increased in F344/DuCrj(DPPIV-) rats (trend, not significant) as well as significantly increased in the F344/Crl(Ger/DPPIV-) animals of the 0.2 nmol NPY treatment group [two-way ANOVA; factor "substrain": F (2; 48) = 7.8; $p = 0.001$; one-way ANOVA for 0.2 nmol: F (2; 16) = 9.4; $p = 0.002$; Fig. 5A]. Furthermore, two-way ANOVA revealed a significant NPY-dependent increase in the social interaction time of all animals [factor "treatment": F (2; 48) = 11.3; $p < .0001$; Fig. 5A]. Additionally, we found a significant "substrain" × "treatment" interaction in the frequency of *anal sniffing* [two-way ANOVA; "substrain" ×

"treatment" interaction: F (2; 48) = 3.0; p = 0.03], which was significantly increased in the F344/Crl(Ger/DPPIV-) rats after treatment with 0.2 nmol NPY [two-way ANOVA; factor "substrain": F (2; 48) = 3.5; p = 0.04; one-way ANOVA for 0.2 nmol: F (2; 16) = 10.9; p = 0.001; Fig. 5B]. The significantly decreased frequency of *self-grooming* in the DPPIV-deficient substrains disappeared after i.c.v. treatment with 1 nmol NPY [two-way ANOVA; factor "substrain": F (2; 48) = 7.6; p = 0.001; one-way ANOVA for vehicle: F (2; 18) = 4.2; p = 0.03; one-way ANOVA for 0.2 nmol: F (2; 16) = 4.2; p = 0.03; Fig. 5C]. Additionally, two-way ANOVA revealed a significant reducing effect of NPY administration on the frequency of *self-grooming* [factor "treatment": F (2; 48) = 3.8; p = 0.03]. Interestingly, the sum of frequencies of the various social behaviors (*anal sniffing*, *following*, *allo grooming*, *walking over*, *crawling under*, and *nosing*) exhibited no significant differences between the substrains [two-way ANOVA; factor "substrain": F (2; 48) = 2.6; p = 0.09; Fig. 6], although the overall motor activity of the rats (measured by number of square entries) was significantly reduced by NPY [two-way ANOVA; factor "treatment": F (2; 48) = 55.2; $p < .0001$].

5.4 Discussion

This study shows for the first time a differential response in rats that differ in their endogenous DPPIV-like enzymatic activity to i.c.v. administration of NPY. Namely, the different doses of NPY induced significantly more pronounced sedative-like effects on EPM behavior and anxiolytic-like effects on the rat social interaction test in the DPPIV-deficient F344 rats. The study provides further evidence for the orexigenic, potent sedative-, and anxiolytic-like effects of NPY and confirms previously described spontaneous mutation in the *Dpp4* gene in the two F344 rat substrains, which leads to an extreme reduction of DPPIV-like activity in the F344/DuCrj(DPPIV-) and the F344/Crl(Ger/DPPIV-) animals (Karl et al., 2003a; Tsuji et al., 1992).

NPY, as a physiological appetite transducer (Kalra et al., 1999), is the only known peptide that can cause animals to eat until they are obese (Inui, 1999). Injections of NPY either in the third ventricle (Jolicoeur et al., 1991; Levine and Morley, 1984), the hypothalamus (Schwartz et al., 2000; Stanley et al., 1986), and especially into the paraventricular nucleus (PVN) (Brief et al., 1992; Merlo Pich et al., 1992) exert a powerful stimulatory effect on feeding and drinking behavior of rats. All animals of the different substrains exhibited a dose-dependent increase in food intake after i.c.v administration of NPY. In addition, for the first time this study proved a stimulating effect of i.c.v. administrated NPY on the latency to start eating, which could be discussed as an influence of NPY on the feeding motivation of rodents. However, in contrast to the proposed hypothesis, no pronounced differences in feeding behavior were found between the wildtype-like and mutant F344 substrains. This may be due to the fact that active DPPIV in the wildtype-like animals cleaves NPY to NPY_{3-36}, which results in a loss of Y_1 receptor subtype but not Y_5 (and Y_2) receptor subtype affinity. Importantly, the feeding behavior of rodents is very likely mediated via Y_1 and Y_5 receptors (Inui, 1999; Kalra et al., 1999; Turnbull et al., 2002) and possibly also via Y_2 receptors (Sainsbury et al., 2002). Y_5 agonists stimulate food intake (Bischoff and Michel, 1999) and inhibition of NPY-stimulated food intake has been observed upon central administration of antisense oligonucleotides directed against the Y_5 receptor. Additionally, Y_5 receptor mRNA was detected in abundance in the PVN and the lateral hypothalamus, areas that have been implicated in the control of feeding behavior (Gerald et al., 1996). Antisense oligonucleotides directed against the Y_1 receptor were also reported to inhibit NPY-stimulated food intake (Bischoff and Michel, 1999), and treatment with Y_1 receptor antagonists like BIBO3304 or

BIBP3226 blocked NPY-induced food intake (Bischoff and Michel, 1999; Inui, 1999). Also the Y_2 receptor subtype seems to be involved in feeding and body weight regulation (Inui, 2000). Y_2 receptor knockout mice developed increased food intake and body weight (Naveilhan et al., 1999), which indicates an inhibitory role for this receptor subtype in the central regulation of these parameters. After all, the stimulating effect of NPY on feeding seems to be mediated via the Y_1, Y_5, and perhaps the Y_2 receptor, although NPY, Y_1, and Y_5 receptor knockout mice did not show expected impaired feeding and body weight loss (Erickson et al., 1996; Marsh et al., 1998; Pedrazzini et al., 1998). Therefore, it is not surprising that the food intake of all F344 substrains is similar because endogenous native NPY in the mutant substrains and cleaved NPY_{3-36} in the wildtype-like substrain are both potent mediators for feeding behavior.

NPY is deeply involved in anxiety-like behaviors of rodents (Kask et al., 2002). I.c.v. administration of NPY and Y_1 agonists decreased anxiety in the EPM (Broqua et al., 1995; Heilig et al., 1989) and the open field (von Horsten et al., 1998b). Furthermore, bilateral microinjections of NPY into the dorsocaudal septum increased the social interaction time in the social interaction test (Kask et al., 2001b). In addition, rats treated with antisense oligodeoxynucleotides blocking Y_1 receptors displayed behavioral signs of anxiety (Wahlestedt et al., 1993) and the Y_1 receptor antagonists BIBO3304 and BIBP3226 blocked anxiolytic-like effects of exogenously administered NPY (Kask et al., 2001b; Kask et al., 1996; von Horsten et al., 1998b). These findings strongly suggest a role for the Y_1 receptor in mediating the anxiolytic-like action of NPY (Heilig et al., 1989; Kask and Harro, 2000). But anxiolytic-like effects of NPY are not only mediated via NPY Y_1 receptors in the amygdala (Sajdyk et al., 1999) and the dorsal periaqueductal gray matter (Kask et al., 1998a) but probably also via Y_2 receptors in the locus coeruleus (Kask et al., 2001b; Kask et al., 1998b) and via Y_5 receptors (Sajdyk et al., 2002). Importantly, also a dose-dependent sedative effect of NPY on locomotor activity in the plus maze has been reported (Broqua et al., 1995; Heilig et al., 1989). In addition, central NPY administration suppressed activity in the open field and in the homecage (Heilig and Murison, 1987), which was interpreted as sedation (Fuxe et al., 1983). Also other studies proved a dose-dependent decrease in the motor activity of rats after i.c.v. administration of NPY (Jolicoeur et al., 1995) and NPY antibody treatment increased motor activity (Yamada et al., 1996). This suppression of activity is supposed to be mediated by Y_1 receptors (Heilig et al., 1988; Kask et al., 1999; von Horsten et al., 1998b), which are also involved in the NPY-induced sensitization to sedation (Naveilhan et al., 2001).

In our study the NPY-treated mutant animals of F344/DuCrj(DPPIV-) and F344/Crl(Ger/DPPIV-) exhibited a dose-dependent reduction in motor activity in the EPM. The wildtype-like F344/Crl(Por) rats were unaffected by the sedative effect of NPY, probably because of differences in the NPY catabolism between the F344 substrains with a DPPIV-dependent cleavage of NPY to the Y_1 receptor unspecific NPY_{3-36} only in control rats. Also the dose-dependent increased time spent on open arms per entry in the mutant animals supports a probably Y_1-mediated sedative-like effect of NPY. The differential response of the three F344 substrains to the different doses of NPY in regard to this parameter supports our hypothesis regarding the DPPIV-dependent differential NPY catabolism in wildtype-like and mutant animals. In addition, NPY had an anxiolytic-like effect in all substrains, which was detected by an increase in the ratio of open arm to total arm entries and in the total time spent on open arms. Notably, these possible anxiolytic-like effects of NPY on the total time spent on open arms could also be influenced by the sedative-like effect of NPY. At least in the F344 genetic background, importantly, a very narrow pharmacological window between sedative-like and anxiolytic-like effects of NPY especially in the EPM has to be considered in order to avoid false positive results. Therefore, not only the time spent on open arms should be recorded as a parameter for detecting anxiolytic-like behaviors in the EPM. Furthermore, various behavioral tests for anxiety have to be used for a complete screening of anxiety-like behaviors in rodents. Therefore, we also applied the social interaction test for recording anxiolytic-like effects of NPY. Interestingly, in this task, the DPPIV-deficient rats exhibited an increased anxiolytic-like response to the NPY administration compared to the wildtype-like animals by an increased SI time. This is supported by the frequency of *anal sniffing* with a significant differential response of the substrains regarding the different NPY treatments. To check for possible sedative-like influences of NPY on the anxiety-like behaviors in this task, we analyzed the overall motor (number of square entries) and social-like (summed frequency of social behaviors) activity of our animals. Motor activity was dose-dependently reduced by NPY treatment, but despite the sedative effect on ambulatory activity, social-like behaviors remained unaffected by NPY. Thus, NPY exerts a clear effect on anxiety as exemplified by an increased SI time in the rat social interaction test in the mutant animals, which was further substantiated by a significantly reduced frequency of *self-grooming* (as a measurement for displacement activity) in the mutant substrains, which disappeared dose-dependently after NPY treatment.

Overall, these data show the suggested differences between the wildtype-like and mutant F344 substrains regarding their NPY catabolism because of the more potent Y_1 receptor-

mediated anxiolytic-like and sedative-like effects of the neurotransmitter in the mutant, DPPIV-deficient animals. The differences within the two DPPIV-deficient substrains F344/DuCrj(DPPIV-) and F344/Crl(Ger/DPPIV-) in the social interaction test could be due to different compensatory mechanisms during ontogeny, but further studies are necessary focusing on this phenomenon.

In conclusion, this study shows for the first time a differential potency and specificity of NPY between wildtype-like and DPPIV-deficient rat substrains and thereby supports the concept that these animals provide a useful model to study the various behavioral and physiological effects associated with DPPIV-enzymatic activity. Therefore, F344 rat substrains F344/Crl(Por), F344/DuCrj(DPPIV-), and F344/Crl(Ger/DPPIV-) indicate an excellent natural animal model to study the "DPPIV-NPY-axis". In particular, the mutant animals exhibited an increased responsiveness to the sedative-like and anxiolytic-like effect of i.c.v.-administered NPY, which is very likely mediated via a Y_1 receptor-dependent mechanism. Since pharmacological inhibition of DPPIV-like activity has been demonstrated to potentiate the effects of NPY administration *in vivo* (Dimitrijevic et al., 2002), it seems possible that DPPIV inhibitors specifically targeting the CNS are useful modifiers of the centrally mediated effects of NPY.

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5.7 Tables and figures

Table 1:

Latency to eat during the light phase [s]			
NPY	**F344/Crl(Por)**	**F344/DuCrj(DPPIV-)**	**F344/Crl(Ger/DPPIV-)**
Vehicle	77.1 ± 20.4	60.3 ± 21.2	85.7 ± 18.2
0.2 nmol	12.7 ± 2.3++	12.7 ± 1.1+	17.5 ± 3.3+++
1.0 nmol	10.6 ± 1.6+++	13.3 ± 4.3+	8.0 ± 0.8+++
Latency to eat during the dark phase [s]			
NPY	**F344/Crl(Por)**	**F344/DuCrj(DPPIV-)**	**F344/Crl(Ger/DPPIV-)**
Vehicle	51.6 ± 18.5	80.6 ± 15.3	54.3 ± 16.1
0.2 nmol	39.8 ± 14.8	17.5 ± 3.4+++	38.2 ± 14.0
1.0 nmol	10.3 ± 1.5	12.3 ± 1.3+++	14.2 ± 1.4

Latency to start eating in F344 substrains; animals´ feeding behavior (food intake) was recorded [s] after receiving an i.c.v. injection of vehicle or NPY (0.2 nmol/1.0 nmol). Data represent means ± SEM. Significant post hoc effects for the factor "treatment" versus the vehicle-treated animals are indicated by crosses (+$p < .05$; ++$p < .01$; +++$p < .001$).

Table 2:

NPY	F344/Crl(Por)	F344/DuCrj(DPPIV-)	F344/Crl(Ger/DPPIV-)
Vehicle	4.3 ± 0.75	3.9 ± 0.77	3.8 ± 0.87
0.2 nmol	3.1 ± 0.51	2.0 ± 0.85	2.7 ± 0.68
1.0 nmol	2.4 ± 0.41	0.6 ± 0.31++	1.0 ± 0.38++

Motor activity in the EPM [n]; the number of enclosed arm entries as an additional parameter for motor activity is presented [n]; animals were tested after receiving an i.c.v. injection of vehicle or NPY (0.2 nmol/1.0 nmol). Data represent means ± SEM. Significant post hoc effects for the factor "treatment" versus the vehicle-treated animals are indicated by crosses (++$p < .01$).

Fig. 1: Test setting; test biography of rats of the three different substrains F344/Crl(Por), F344/DuCrj(DPPIV-) and F344/Crl(Ger/DPPIV-). The animals´ age in the various tests is shown.

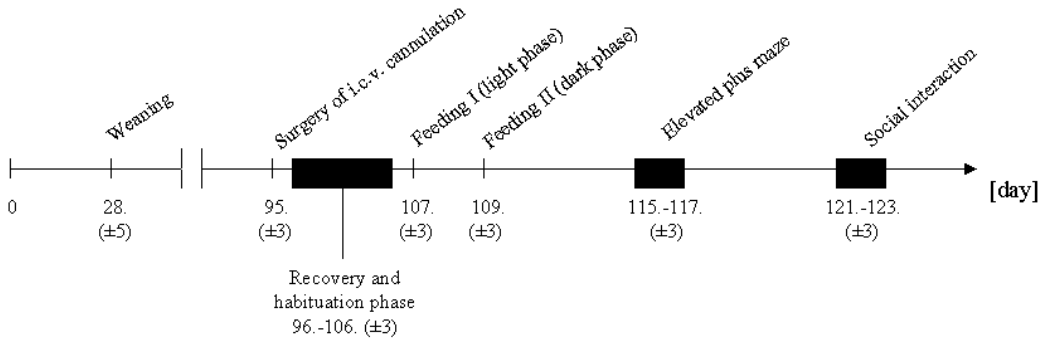


Fig. 2: DPPIV-like activity; DPPIV-like activity [mU/ml] of all test animals was screened; blood from the tail vain was sampled and analyzed as described above. Data represent means + SEM. Asterisks (***p < .001) reflect significant differences versus F344/Crl(Por) – detected in the Fisher-PLSD-test.

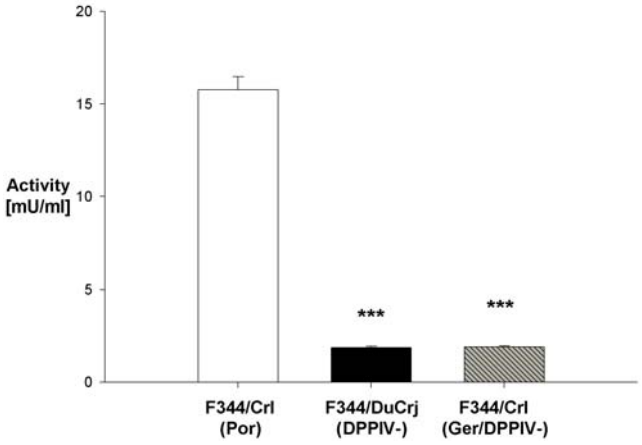


Fig. 3: Anxiolytic-like effects in the EPM; the number of arm entries of the test animal into open and enclosed arms were recorded for 5 min; the ratio of open arm entries to total arm entries serves as a measure for anxiety [%]; animals were tested after receiving an i.c.v. injection of vehicle or NPY (0.2 nmol/1.0 nmol). Data represent means + SEM.

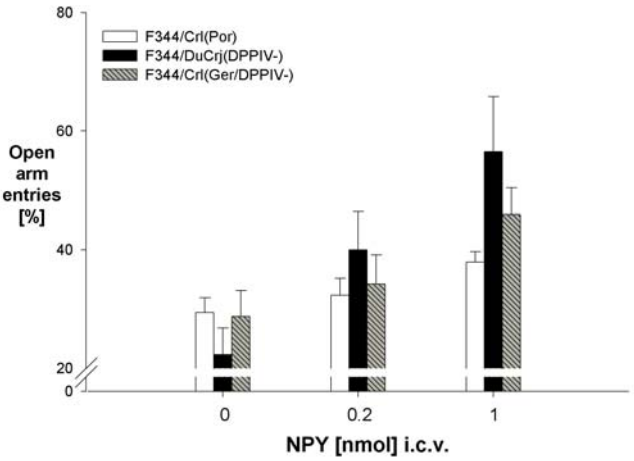


Fig. 4A: Motor activity in the EPM; the total number of arm entries of the test animal was recorded for 5 min; the number of total arm entries serves as a measure for motor activity [n]; animals were tested after receiving an i.c.v. injection of vehicle or NPY (0.2 nmol/1.0 nmol). Data represent means + SEM. Asterisks (***p < .001; **p < .01) reflect significant differences versus F344/Crl(Por) – detected in the Fisher-PLSD-test.

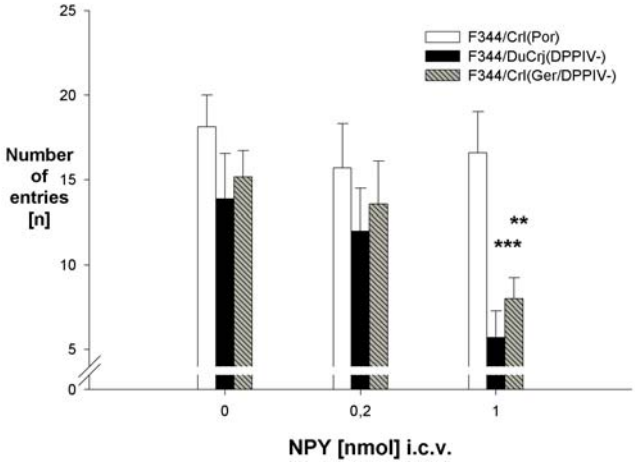


Fig. 4B: Sedation in the EPM; the time spent on open arms per entry was recorded [s]; additionally, the overall time spent on open arms per entry in each substrain is presented; animals were tested after receiving an i.c.v. injection of vehicle or NPY (0.2 nmol/1.0 nmol). Data represent means + SEM. Asterisks (**$p < .01$) reflect significant differences versus F344/Crl(Por) – detected in the Fisher-PLSD-test.

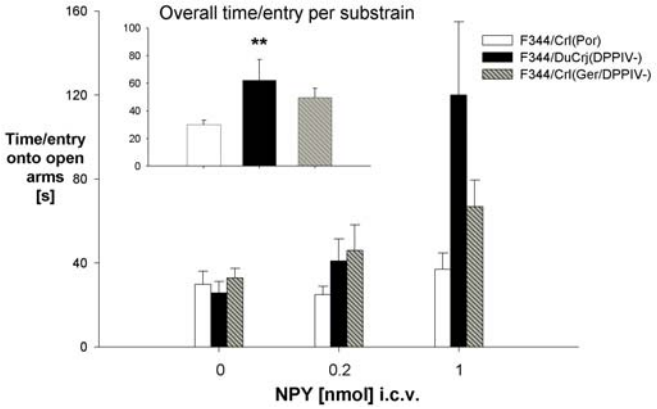


Fig. 5A: Anxiolytic-like effects in the social interaction test; the time spent in active social interaction (SI) was recorded [s]; additionally, the overall effect of different doses of NPY on SI is presented; animals were tested after receiving an i.c.v. injection of vehicle or NPY (0.2 nmol/1.0 nmol). Data represent means + SEM. Asterisks (***$p < .001$) reflect significant differences versus F344/Crl(Por), whereas double crosses (#$p < .05$) display significant differences between F344/DuCrj(DPPIV-) and F344/Crl(Ger/DPPIV-) – detected in the Fisher-PLSD-test. Significant post hoc effects for the factor "treatment" versus the vehicle-treated animals are indicated by crosses (+p < .05; +++$p < .001$), whereas "x" (x$p < .05$) reflects significant differences between the two different NPY doses 0.2 nmol and 1.0 nmol - detected in the Fisher-PLSD-test.

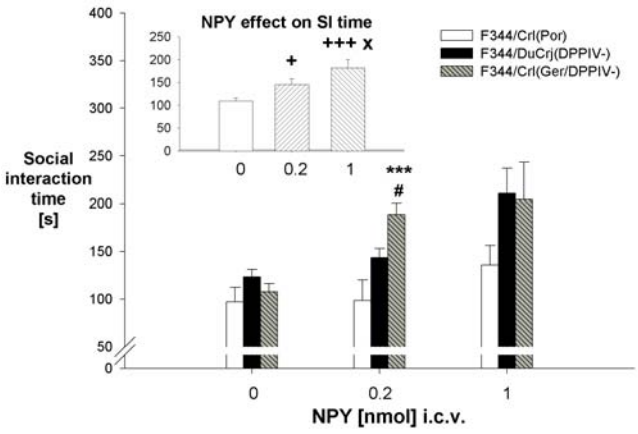


Fig. 5B: *Anal sniffing* in the social interaction test; the frequency of *anal sniffing* was recorded during 10 min of observation [n]; rats of different substrains but of the same treatment group were compared; animals were tested after receiving an i.c.v. injection of vehicle or NPY (0.2 nmol/1.0 nmol). Data represent means + SEM. Asterisks (**$p < .01$) reflect significant differences versus F344/Crl(Por), whereas double crosses (###$p < .001$) display significant differences between F344/DuCrj(DPPIV-) and F344/Crl(Ger/DPPIV-) – detected in the Fisher-PLSD-test.

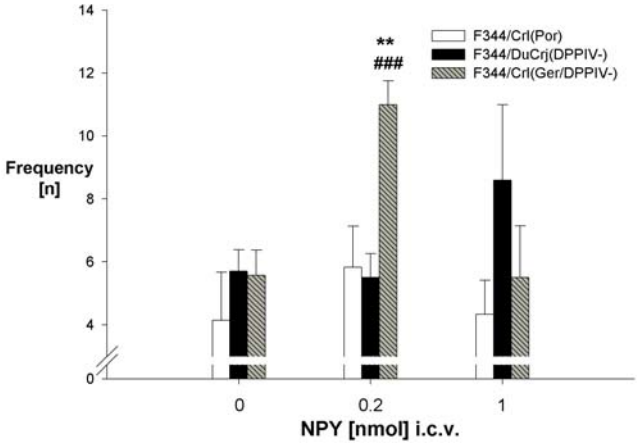


Fig. 5C: *Self-grooming* in the social interaction test; the frequency of *self-grooming* was recorded during 10 min of observation [n]; rats of different substrains but of the same treatment group were compared; animals were tested after receiving an i.c.v. injection of vehicle or NPY (0.2 nmol/1.0 nmol). Data represent means + SEM. Asterisks (*$p < .05$) reflect significant differences versus F344/Crl(Por) – detected in the Fisher-PLSD-test.

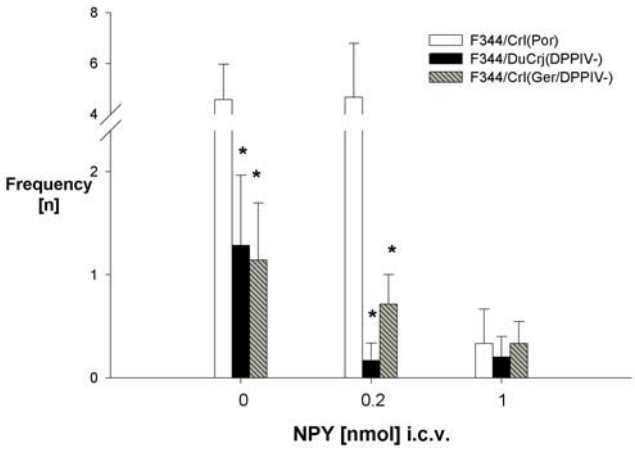
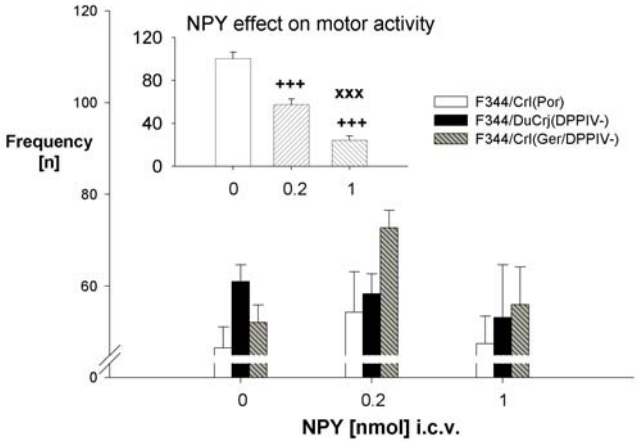


Fig. 6: Motor activity and social-like behaviors in the social interaction test; the summed frequency of social-like behaviors was recorded [n]; additionally, the overall effect of NPY on motor activity is presented by the total number of square entries after receiving an i.c.v. injection of vehicle or NPY (0.2 nmol/1.0 nmol). Data represent means + SEM. Significant post hoc effects for the factor "treatment" versus the vehicle-treated animals are indicated by crosses (+++$p < .001$), whereas "x" (xxx$p < .001$) reflects significant differences between the two different NPY doses 0.2 nmol and 1.0 nmol - detected in the Fisher-PLSD-test.



Effects of neuropeptide Y on nociception in dipeptidyl-peptidase IV-deficient F344 rat substrains

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Abstract

Dipeptidyl-peptidase IV (DPPIV; CD26) is involved in several physiological functions via cleavage of dipeptides with a *Xaa*-Pro or *Xaa*-Ala sequence of regulatory peptides such as neuropeptide Y (NPY). Cleavage of NPY by DPPIV results in NPY_{3-36}, which lacks affinity for the Y_1, but not for other NPY receptor subtypes. Among other effects, the NPY Y_1 receptor seems to be involved in analgetic-like effects of NPY. In this study we investigated whether intracerebroventricular (i.c.v.) administration of different doses of NPY (vehicle, 0.2, or 1.0 nmol) or of the DPPIV inhibitor isoleucyl-thiazolidide (*Ile-Thia*) (vehicle, 0.5, or 5.0 nmol) modulates the behavioral response in the hot plate paradigm in wildtype-like and DPPIV-negative F344 substrains. Untreated non-habituated DPPIV-deficient animals, which display an anxiolytic-like phenotype, exhibited a reduced latency to respond to an aversive thermal stimulus in the hot plate, suggesting a process of reduced stress-induced analgesia. I.c.v. administration of NPY decreased the pain sensitivity of these animals but not of the wildtype-like F344 rats. DPPIV inhibition resulted in an increased nociception of the wildtype-like animals in the same paradigm. The differential response to i.c.v. administration of NPY confirms the recently described differential NPY catabolism within the different F344 substrains. Furthermore, the study demonstrates for the first time that the modulation of nociception by NPY can be modified by DPPIV-like enzymatic activity. The differences in pain sensitivity are based on a more potent Y_1 receptor-like tone in the DPPIV-deficient rats, which mediates the analgetic-like effect.

Keywords: Dipeptidyl-peptidase IV – CD26 – Neuropeptide Y - Isoleucyl-thiazolidide – F344 rats — Nociception – Stress-induced analgesia – Hot plate

6.1 Introduction

The enzyme and binding protein dipeptidyl-peptidase IV (DPPIV) belongs to the class of membrane-associated peptidases (De Meester et al., 1999). The ectopeptidase is identical to the leukocyte differentiation marker CD26 and is involved in T-cell dependent immune responses (Kahne et al., 1999) and in cell adhesion (Mentlein, 1999; Shingu et al., 2003). Due to its unique ability to liberate *Xaa*-Pro and *Xaa*-Ala dipeptides from the N-terminus of regulatory peptides, important substrates include neuropeptides such as neuropeptide Y (NPY), peptide YY (PYY), and endomorphin (Hildebrandt et al., 2000; Mentlein, 1999). Further substrates are glucagon-like peptide 1 (GLP-1), GLP-2, enterostatin, substance P, and various chemokines (De Meester et al., 2000). Endomorphin-2, substance P, and especially NPY are discussed in regard to their analgetic-like effects in tests for nociception.

We previously investigated the role of the DPPIV-like activity on behavioral and physiological processes in a mutant rat model for DPPIV deficiency. Spontaneous mutations in the *Dpp4* gene of F344 substrains 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), result in an almost complete lack of DPPIV-like activity in these animals. This DPPIV deficiency does not exist in wildtype-like F344 rats from Crl breeding colonies in Portage, USA (Karl et al., 2003a). In a systematical behavioral and physiological characterization of the different substrains, we observed differences in the mutant, DPPIV-deficient F344 rats showing reduced anxiety-like behaviors and increased nociception (Karl et al., 2003b). In another study (Karl et al., 2003c) we showed that the anxiolytic-like phenotype of the DPPIV-deficient substrains is based on a differential degradation of NPY, strongly suggesting that a more potent NPY Y_1 receptor-like tone in the DPPIV-deficient F344 substrains causes these differences.

Among several other physiological responses NPY especially affects anxiety and nociception (Kalra et al., 1999; Kask et al., 2002; Naveilhan et al., 2001; Wettstein et al., 1995) via the NPY Y_1 receptor subtype, although the effect on pain sensitivity is discussed controversially. Some studies described an analgetic effect after NPY administration (Hua et al., 1991; Merlo Pich et al., 1990), some showed a hyperalgesic effect of the same neuropeptide (Broqua et al., 1996; von Horsten et al., 1998b) and others did not find any influence of NPY on nociception processes (Heilig et al., 1993; Jolicoeur et al., 1991). We hypothesize that the hyperalgesic

phenotype of the two DPPIV-deficient F344 substrains from Japan [F344/DuCrj(DPPIV-)] and Germany [F344/Crl(Ger/DPPIV-)] (Karl et al., 2003b) compared to the wildtype-like rats could be due to a differential stress response along with a differential stress-induced analgesia. This could be based on the recently proven differential NPY metabolism within the different substrains (Karl et al., 2003c). Furthermore, we postulate an antinociceptive effect of NPY on pain sensitivity. Such processes could sufficiently explain the increased pain sensitivity in the mutant, DPPIV-deficient substrains.

To test this hypothesis, we re-investigated the phenomenon of a differential stress-induced analgesia in different F344 substrains and tested whether NPY applied intracerebroventricularly (i.c.v.) produces differential effects in the different F344 substrains in regard to their pain sensitivity. Thus, we characterized the effect of i.c.v. administration of different doses of NPY in the F344/DuCrj(DPPIV-), F344/Crl(Ger/DPPIV-), and F344/Crl(Por) rats in the hot plate paradigm. To prove our hypothesis that the various behavioral and physiological differences between the different F344 substrains – especially the hyperalgesic phenotype of the mutant animals – is based on the DPPIV deficiency we also investigated pharmacologically the effect of i.c.v. DPPIV inhibition on the hot plate response of these animals.

6.2 Materials and methods

Animals

For clarity, animal groups were coded as previously described (Karl et al., 2003a): F344 rats derived in 1998 from breeding colonies of Crl in Atsugi (Japan) were called F344/DuCrj(DPPIV-), animals from breeding colonies in Sulzfeld (Germany) F344/Crl(Ger/DPPIV-), and wildtype-like rats obtained from colonies in Portage (USA) F344/Crl(Por).

All F344 rats of the three different substrains were housed and bred at the Central Animal Facility of the Hannover Medical School as previously described (Karl et al., 2003a). Animals were maintained in a separated minimal barrier sustained facility and kept in Macrolon type III cages with a standard bedding (Altromin GmbH, Lage, Germany). Food (Altromin Standard Diät 1320: Altromin GmbH) and water were available *ad libitum*. Environmental temperature was automatically regulated at 21 ± 2 °C and relative humidity was 55 ± 5% 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 (light onset at 4am). They underwent routine animal care 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.

Determination of DPPIV-like enzymatic activity

All test animals were characterized in regard to their DPPIV-like enzymatic activity as previously described (Karl et al., 2003a). For determination of plasma activity of F344 rats a microplate based fluorescence assay was used. EDTA-plasma samples were kept at –80 °C until use. DPPIV enzyme activity of the different rat substrains was determined by monitoring the release of 4-Amino-7-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 DPPIV-like activities. It has been proven that the substrate is cleaved by DPPIV, by DP II, and by attractin. Probably, they are also substrates for DP8 and DP9. Importantly, the chromophores are not released by other proline-specific peptidases, such as prolidase, prolyl endopeptidase or aminopeptidase P.

Surgery of i.c.v. cannulation

For surgery, animals were anesthetized with intramuscular (i.m.) 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 that outlined in a previous report (von Horsten et al., 1998a). After placement of the rat in a 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-ointment (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) was used to verify that the guide cannula was positioned just above the ventricular system. The guide cannula was then fitted with a dummy cannula (Plastics one) of the same length to prevent leakage of cerebrospinal fluid. Animals were housed individually after surgery. The anatomical position of the cannula was verified by post mortem i.c.v. dye application (Berlin blue) and inspection of the stained third ventricle in randomly chosen rats. The animals of the three F344 substrains F344/DuCrj(DPPIV-), F344/Crl(Ger/DPPIV-), and F344/Crl(Por) were operated at an age of 95 (±5) days. The observation of the animals' behavior in the hot plate test commenced after a recovery phase of at least 10 days.

Drugs, i.c.v. injections, and dosages

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. A stock solution of the DPPIV inhibitor isoleucyl-thiazolidide (*Ile-Thia*) (Probiodrug AG, Halle, Germany) was adjusted under sterile conditions to final concentrations (0.5 nmol/5 µl and 5.0 nmol/5 µl) using 0.9% saline. The final concentrations of NPY and *Ile-Thia* were made 24 h before the different experiments.

For the i.c.v. administration animals were taken out of the home cage and the dummy cannula was replaced by the internal cannula. Peptide, inhibitor or 0.9% saline was injected i.c.v. in 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), which allowed the animal to move freely during the i.c.v. injection. Then the internal cannula was replaced by the dummy cannula again and the rat was returned into the home cage. All experiments started 15 min after the administration procedure. For the procedure of i.c.v.-injection, animals were habituated to experimental handling daily over 7 days prior to the start of the first experiment. During the habituation phase the handling procedure was exactly the same except for the application of the compound.

Two different doses of NPY/*Ile-Thia* were used for this study and a 3 (substrain) × 3 (treatment) experimental design was set up. Thus, F344 rats of each substrain were divided into three treatment groups each (n = 7), which were treated with 0.9% saline (vehicle: 0.0 nmol/5 µl), or two different doses of NPY (0.2 nmol/5 µl, or 1.0 nmol/5 µl) or of *Ile-Thia* (0.5 nmol/5 µl, or 5.0 nmol/5 µl).

Nociception

A 30x30 cm hot plate analgesia meter (Columbus Instruments, Columbus, USA) was used for this experiment, which was carried out during the light phase. The experiments were performed as previously described (Karl et al., 2003b; Stephan et al., 2002). 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 tissue damage, the rat was removed from the hot plate if the animal did not respond within 20 s (Naveilhan et al., 2001; von Horsten et al., 1998b). Non-habituated animals were tested twice in the hot plate paradigm. In the first test (Hot plate I) rats (age: 111 ± 3 days) received an i.c.v injection of NPY (vehicle, 0.2, or 1.0 nmol/5 µl). Fifteen days later (Hot plate II) animals were tested

once more in this paradigm but this time rats were treated with the DPPIV inhibitor *Ile-Thia* (vehicle, 0.5, or 5.0 nmol/5 µl).

Statistical analysis

The analysis of the various behavioral data was assessed using a two-way analysis of variance (ANOVA; factor: "substrain" × "treatment") and by one-way ANOVA (factor: "substrain" or "treatment" - split by the corresponding factors) followed by the Fisher-PLSD-test for post hoc comparison, if appropriate. Differences were regarded as statistically significant if $p < .05$. In the results section the *degrees of freedom*, *F*-values, and *p*-values of two- and one-way ANOVAs are given, while in the figures the *p*-values of the corresponding post hoc tests (Fisher-PLSD-test) are provided. In most cases the number of animals per substrain and treatment group was $n = 7$. Presenting the *degrees of freedom* indicates exceptions from this. Significant post hoc effects for the factor "substrain" versus the control animals of the F344/Crl(Por) substrain are indicated by asterisks (*$p < .05$; **$p < .01$; ***$p < .001$), whereas significant differences between the two DPPIV-deficient rat substrains F344/DuCrj(DPPIV-) and F344/Crl(Ger/DPPIV-) are shown by rhombs (#$p < .05$; ##$p < .01$; ###$p < .001$). All data are displayed as means ± standard error of the mean (SEM).

6.3 Results

DPPIV-like activity

One-way ANOVA of the DPPIV-like activity of all animals exhibited significant differences between substrains (Fig. 1). An almost complete lack of enzymatic-like activity was found in the two mutant substrains F344/DuCrj(DPPIV-) and F344/Crl(Ger/DPPIV-) and a wildtype-like pattern of DPPIV-like activity was found in the rats from the F344/Crl(Por) substrain [F (2; 61) = 400.4; $p < .0001$].

Nociception

In the first hot plate experiment (Hot plate I) two-way ANOVA revealed a significantly reduced latency in the mutant F344/DuCrj(DPPIV-) and F344/Crl(Ger/DPPIV-) substrains to *lick* or *raise a hindpaw* [factor "substrain": F (2; 55) = 10.1; p = 0.0002] after treatment with vehicle. This significant hyperalgesic effect of DPPIV deficiency in the mutant substrains [one-way ANOVA; factor "substrain" - vehicle-treated: F (2; 19) = 6.9; p = 0.006; Fig. 2A] disappeared after i.c.v. administration of NPY. In the second experiment (Hot plate II) using the DPPIV-inhibitor *Ile-Thia*, again, vehicle-treated DPPIV-deficient animals of F344/DuCrj(DPPIV-) and F344/Crl(Ger/DPPIV-) exhibited a significantly shortened latency to respond to the thermal stimulus of the hot plate [two-way ANOVA; factor "substrain": F (2; 44) = 4.9; p = 0.01; one-way ANOVA; factor "substrain" – vehicle-treated: F (2; 18) = 3.6; p = 0.049]. Injections of 0.5 nmol or 5.0 nmol *Ile-Thia* attenuated the differences in nociception between the different substrains. DPPIV-inhibition increased the pain sensitivity in control animals of the F344/Crl(Por) substrain (Fig. 2B).

6.4 Discussion

The DPPIV-deficient animals of the F344/DuCrj(DPPIV-) and F344/Crl(Ger/DPPIV-) substrains exhibited hyperalgesia compared to control animals of the F344/Crl(Por) substrain. I.c.v. treatment with NPY had a dose-dependent hypoalgesic effect only in the mutant rats, whereas i.c.v. administration of the DPPIV inhibitor *Ile-Thia* had a hyperalgesic effect only in the wildtype-like animals.

The effect of NPY on nociception is discussed controversially, although the presence of NPY-immunoreactivity in some areas involved in pain modulation, such as the periaqueductal gray (PAG), locus coeruleus, amygdala, thalamus, or the dorsal horn of the spinal cord, suggests a role for NPY as a putative regulator of pain transmission and perception (Broqua et al., 1996; Jolicoeur et al., 1991). In the hot plate test i.c.v. administration of NPY to spontaneously hypertensive rats induced an elevation in the nociceptive threshold in the hot plate paradigm (Merlo Pich et al., 1990) and intrathecal administration of NPY produced a dose-dependent elevation in the nociceptive threshold (Hua et al., 1991). In other studies i.c.v. administration of very low doses of NPY induced hyperalgesia (Broqua et al., 1996; von Horsten et al., 1998b), while some studies did not find any effect of NPY on nociception (Heilig et al., 1993; Heilig et al., 1992; Heilig et al., 1989; Jolicoeur et al., 1991). In our study an analgetic effect of i.c.v. administered NPY in the DPPIV-deficient F344 substrains in the hot plate test is obvious. Y_1 receptor knockout mice develop hyperalgesia to acute thermal, cutaneous, and visceral chemical pain (Naveilhan et al., 2001). Therefore, it is concluded that the Y_1 receptor is required for central physiological and pharmacological NPY-induced analgesia (Broqua et al., 1996; Wang et al., 2000), although another study described a Y_2 receptor-mediated effect of NPY on thermal hyperalgesia (Tracey et al., 1995). Wildtype-like F344/Crl(Por) rats with a normal DPPIV-like activity lose the Y_1 receptor subtype-specific affinity of endogenously released NPY more rapidly by DPPIV-dependent cleavage processes. This is based on the cleavage of NPY to the C-terminal fragment NPY_{3-36}, which has a high affinity for the NPY Y_2 and Y_5 receptor subtypes but only a low affinity for the Y_1 receptor subtype. This differential NPY catabolism could increase the pain sensitivity in these animals. Surprisingly, and in contrast to this hypothesis, hyperalgesia was detected in the vehicle-treated DPPIV-deficient F344/DuCrj(DPPIV-) and F344/Crl(Ger/DPPIV-) animals. This phenotype could be based on recently reported reduced anxiety levels in the mutant animals (Karl et al., 2003b;

Karl et al., 2003c). Simililar to our wildtype-like animals, NPY-deficient mice appear to be hypoalgesic in the hot plate paradigm (Bannon et al., 2000) and exhibit an anxiogenic-like phenotype. This phenotype seems to be based on an extremely reduced Y_1 receptor-like tone in the DPPIV-deficient rats and NPY-deficient mice. The increased anxiety of both mice and rats could increase the pain threshold in these animals by stress-induced analgesia, which is associated with or even triggered by a release of analgesia-inducing endogenous opioid peptides from central and peripheral sites (Kelley, 1986). In addition, stress based on anxiety increases the endogenous NPY-release so that the endogenous NPY level in our less anxious DPPIV-deficient rats could be reduced compared to the wildtype-like substrain. Therefore, a potent stress-induced NPY release in the wildtype-like animals paired with stress-induced analgesia could lead to the observed hypoalgesic phenotype. After i.c.v. administration of NPY the pain sensitivity in the mutant rats decreased dose-dependently, which is a strong indication of the antinociceptive effect of NPY. The effect is only present in DPPIV-deficient rats, which manifests the recently observed differences in the NPY catabolism between the mutant and wildtype-like substrains (Karl et al., 2003c). In the control animals NPY had no further analgetic effect, probably because of anxiogenic-induced already high endogenous levels of NPY in F344/Crl(Por). Furthermore, substance P, another substrate of the DPPIV, is involved in nociception. Similar to NPY, it could be cleaved differentially in the DPPIV-deficient and the wildtype-like F344 substrains. This difference in substance P metabolism could influence the pain sensitivity of these animals as already shown for the DPPIV substrate NPY in our study. It has to be mentioned that another substrate of the DPPIV, endomorphin-2, also seems to be involved in nociception processes.

In addition, we investigated the effect of i.c.v. DPPIV inhibitor treatment in the F344 substrains. As expected, no effects were observed in the DPPIV-deficient animals, but in the control animals DPPIV inhibition led to hyperalgesia. This could be based on DPPIV inhibition-caused reduced anxiety levels of the wildtype-like animals, which could be linked to a decreased endogenous NPY-release and a reduced stress-induced analgesia.

Overall, these data confirm the suggested differences between the wildtype-like and mutant F344 substrains regarding their NPY catabolism because of the more potent Y_1 receptor-mediated analgetic-like effects of the neurotransmitter in the DPPIV-deficient animals. Furthermore, the various behavioral and physiological differences between the wildtype-like and DPPIV-deficient F344 substrains are exclusively based on the DPPIV deficiency because pharmacological inhibition of DPPIV in the control substrain results in a phenotype similar to the DPPIV-deficient animals. Most likely, the “DPPIV-NPY” axis represents an interesting

system for specific pharmacotherapy including but not limited to pain syndromes. Thus, this bidirectional up- and down-regulation of the latency to respond in the hot plate test by either NPY or DPPIV inhibitor in either the DPPIV-deficient or the wildtype-like animals strongly suggests a specific role of the “DPPIV-NPY” axis in the central pain perception and processing systems.

6.5 References

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6.6 Figures

Fig. 1: DPPIV-like activity; DPPIV-like activity [mU/ml] of all test animals was screened; blood from the tail vain was sampled and analyzed as described above. Data represent means ± SEM. Asterisks (***p < .001) reflect significant differences versus F344/Crl(Por) – detected in the Fisher-PLSD-test.

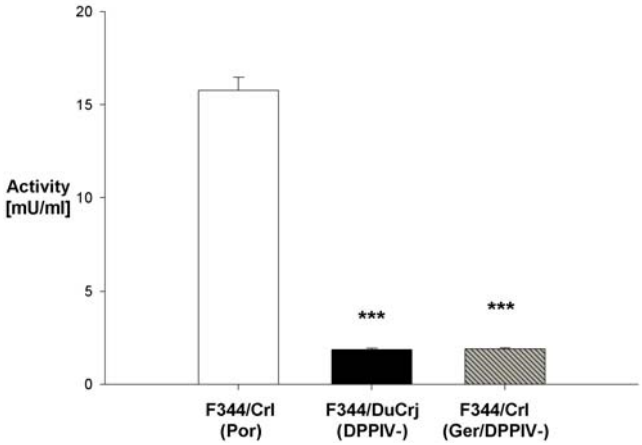


Fig. 2A: Nociception; pain sensitivity [s] was analyzed in the hot plate test; animals were tested after receiving an i.c.v. injection of vehicle or NPY (0.2 nmol or 1.0 nmol); the latency to respond (*lick* or *raise a hindpaw*) was recorded (Hot plate I). Data represent means ± SEM. Asterisks (*$p < .05$; **$p < .01$) reflect significant differences versus F344/Crl(Por) – detected in the Fisher-PLSD-test.

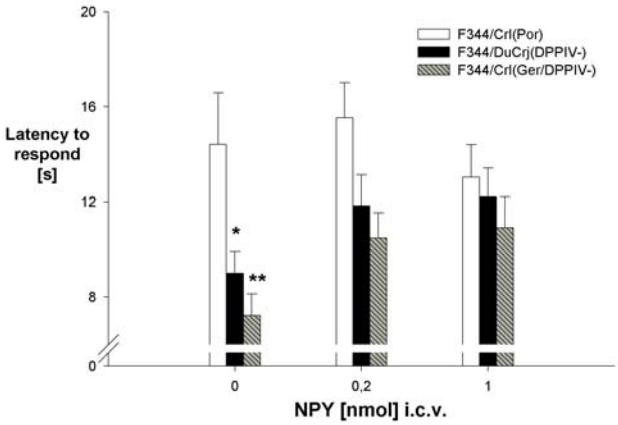
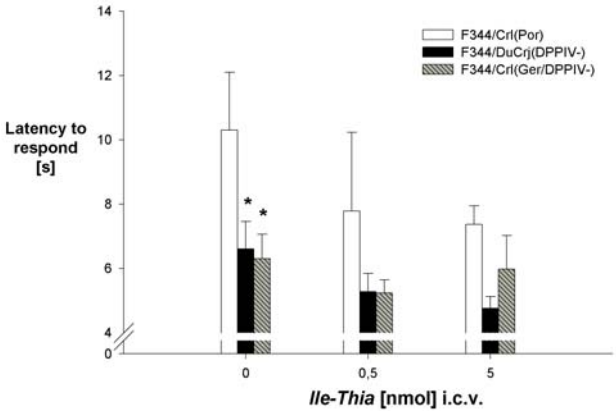


Fig. 2B: Nociception; pain sensitivity [s] was analyzed in the hot plate test; the rat was tested after receiving an i.c.v. injection of vehicle or *Ile-Thia* (0.5 nmol or 5.0 nmol); the latency to respond (*lick* or *raise a hindpaw*) was recorded (Hot plate II). Data represent means ± SEM. Asterisks (*$p < .05$) reflect significant differences versus F344/Crl(Por) – detected in the Fisher-PLSD-test.



7. Discussion

In a first step of our study, we screened different F344 rat substrains obtained from breeding colonies of Charles River Breeding Laboratories in Portage and in Raleigh (USA), in Atsugi (Japan), and in Sulzfeld (Germany) for their DPPIV-like activity (Karl et al., 2003b). These substrains were purchased at different time points in the years 1998 and 2001. We observed that F344/DuCrj(DPPIV-) rat substrains from Atsugi (Japan) constantly exhibit an almost complete lack of DPPIV-like enzymatic activity, while rats of the F344/Crl(Ger) substrain from the breeding colony in Sulzfeld (Germany) only occasionally showed a lack of DPPIV-like enzymatic activity [only in animals, which were obtained in 1998 from Crl: F344/Crl(Ger/98/DPPIV-)]. Recently, a genome wide screening of both DPPIV-deficient substrains (which were obtained in 1998) with anonymous microsatellite markers revealed that the genetic background between F344/DuCrj(DPPIV-) and F344/Crl(Ger/DPPIV-) is not identical (cooperation with Dr. D. Wedekind: Institute for Laboratory Animal Science and Central Animal Facility, Hannover Medical School, Germany - unpublished results). This finding suggests that segregation processes occurred in the F344/Crl(Ger/DPPIV-) colony, which were confirmed by the differences in the DPPIV-like activity between animals obtained from the German breeding colony of Crl (Sulzfeld) in 1998 and in 2001. Rats obtained from the Crl colony in Portage (USA) in 1998 [F344/Crl(Por/98)] and Raleigh (USA) in 2001 [F344/Crl(Ral/01)] show the expected wildtype-like DPPIV activity. Surprisingly, animals of the Portage colony obtained in 2001 show gender-dependent differences in DPPIV-like enzymatic activity. The differences in the DPPIV-like activity among the F344 rat substrains from the different breeding colonies of a world-wide operating vendor at different intervals and the results of the microsatellite analyses of F344/DuCrj(98/DPPIV-) and F344/Crl(Ger/98/DPPIV-) clearly indicates a persisting segregation for the *Dpp4* gene in some of these colonies (Portage, USA and Sulzfeld, Germany). Furthermore, the laboratory code of the substrains from the different breeding colonies in the USA and Germany does not differentiate between the various breeding locations.

These findings on variation in DPPIV-like enzymatic activity in the different F344 substrains indicate that scientists, who obtain F344 rats from this vendor (which have originated from above described colonies) or who work on DPPIV-dependent physiological processes in these rats should screen their animals in regard to their DPPIV-like activity and should be aware of the origin of their F344 rat substrain.

Furthermore, the extreme differences in the DPPIV-like activity found between several other rat strains (such as LEW/Ztm, DA/Ztm, BN/Ztm) suggest that a screening of DPPIV-like activity is an important experimental step in case of research in regard to DPPIV-dependent physiological processes (the differential DPPIV-like activity among various rat strains could be attributed to differences among coding regions of the *Dpp4* genes or to modifier genes of the respective genetic backgrounds).

For all our experiments we used animals received from the breeding colonies in Portage [F344/Crl(Por)], Atsugi [F344/DuCrj(DPPIV-)], and Sulzfeld [F344/Crl(Ger/DPPIV-)] in 1998 and screened every single test animal in regard to its DPPIV-like activity. Only the animals of the postulated DPPIV-deficient substrains exhibited the expected extreme reduction in the DPPIV-like activity (to nearly 0%). We assume that the deficiency in DPPIV-like activity in F344/DuCrj(DPPIV-) and F344/Crl(Ger/DPPIV-) could be attributed to a spontaneous mutation previously described in F344/DuCrj(DPPIV-) (Tsuji et al., 1992) resulting in a null allele. Very recently, we were able to confirm this point mutation identified by Tsuji and coworkers (1992) also in F344/Crl(Ger/DPPIV-) and additionally found three other point mutations in the *Dpp4* gene (in regard to the catalytic center of DPPIV) of both mutant substrains (cooperation with Dr. T. Hoffmann: Probiodrug AG, Halle, Germany - unpublished results).

Using an SSLP marker located within the sequence of rat *Dpp4* we were able to confirm the position of the *Dpp4* gene on rat chromosome 3 (RNO3). Furthermore, we demonstrated that the *Dpp4* gene is inherited in a semi-dominant fashion in the F344/DuCrj(DPPIV-) substrain. In these animals the DPPIV-like activity remains on an intermediate level in F344 rats heterozygous for the *Dpp4* gene. Thus, out- and intercrosses between homozygous DPPIV-deficient F344/DuCrj(DPPIV-) and homozygous wildtype-like F344/Ztm, F344/Crl(Por/98), or F344/Crl(Ral/01) animals could be a valuable tool to examine the impact of DPPIV-like activity on DPPIV-dependent physiological parameters.

We analysed the glucose tolerance in the F344 substrains obtained in 1998 to proof the physiological relevance of this enzymatic system. DPPIV is reported to play an essential role in the physiological control of blood glucose. This is reflected by an improved glucose tolerance in the DPPIV-deficient F344/DuCrj(DPPIV-) and F344/Crl(Ger/DPPIV-) rats. DPPIV-inhibition improves the glucose tolerance (Ahren et al., 2000; Pederson et al., 1998) and also enhances the insulin secretion (Pauly et al., 1999). Furthermore, it is effective to suppress the degradation of incretins like GLP-1 (Deacon et al., 1998; Holst and Deacon, 1998), which stimulates insulin gene expression, increases glucose-stimulated insulin

secretion (Ahren et al., 2000; Balkan et al., 1999), and inhibits glucagon secretion, all of which contribute to normalize elevated blood glucose levels (Holst and Deacon, 1998). Thus, active GLP-1 has a powerful influence on glucose tolerance. Inhibition of DPPIV results in increased levels of active GLP-1 and GIP (Kieffer et al., 1995) and an improved glucose tolerance. Therefore, the lack of DPPIV-like activity in the two DPPIV-deficient substrains seems to be responsible for the improved glucose tolerance by an incretin-mediated mechanism (Ahren et al., 2000).

Furthermore, we demonstrated that an extreme reduction in DPPIV-like activity is associated with a decreased NK cell mediated lysis of tumor cells. This finding on blunted NK cell mediated cytotoxicity against syngenic tumor cell targets suggests that DPPIV is involved in mediating specific aspects of NK cell function. It seems to be involved in the regulation of NK cell proliferation, whereas natural cytotoxicity seems to be regulated independently (Buhling et al., 1994; Shingu et al., 2003).

In conclusion, we present a gene linked SSLP marker, which allowed us to map *Dpp4* to rat chromosome 3 (RNO3). We found considerable differences in the DPPIV-like enzymatic activity between various rat strains and within different F344 substrains. The DPPIV deficiency of two F344 substrains [F344/DuCrj(DPPIV-) and F344/Crl(Ger/DPPIV-)] was found to be associated with an improved glucose tolerance and decreased NK cell function. Overall, the variations in DPPIV-like enzymatic activity reported here could act as confounding factors in biomedical research. The manipulation of plasma incretin concentrations by acute inhibition of DPPIV could be a therapeutic approach for improving the glucose tolerance and could prevent the transition to type 2 diabetes. Thus, our different F344 substrains may represent a useful tool for research focusing on glucose homeostasis (e.g. by developing a congene rat model with a $Lepr^{fa}$ mutation on a DPPIV-deficient F344 background).

Differences in the DPPIV-like activity probably affects not only the NK cell function and the glucose tolerance but also the degradation and half-life of regulatory peptides such as enterostatin, GLP-1, substance P and especially NPY, which are substrates for the DPPIV (see 1.2: Table 1). Therefore, in the second step of this study, the influence of DPPIV deficiency on behavioral and additional physiological topics (see 1.4: Table 2) was analyzed (Karl et al., 2003c). The systematic phenotyping of F344 substrains with or without endogenous DPPIV-like enzymatic activity demonstrated an important involvement of the ectopeptidase DPPIV in several behavioral and physiological processes, which are dependent on these regulatory peptides. The two DPPIV-deficient F344 substrains [F344/DuCrj(DPPIV-) and

F344/Crl(Ger/DPPIV-)] exhibited a significantly reduced body weight and water intake, a significant hyperalgesia in the non-habituated hot plate test, and a significant differential response to the habituation for this experiment. In the open field we observed significantly increased exploratory-like behavior in these rats, while the anxiety was significantly reduced in the F344/Crl(Ger/DPPIV-) rats in the social interaction test and in F344/DuCrj(DPPIV-) rats in the passive avoidance task. Interestingly, a reduced susceptibility to the sedative effect of ethanol was evident in the mutant F344 substrains.

The observed differential body weight in the different F344 substrains could be based on elevated endogenous levels of the DPPIV substrate enterostatin in young DPPIV-deficient rats (Erlanson-Albertsson and York, 1997). Enterostatin, which is stabilized by DPPIV inhibition with val-pro-asp-pro-arg (VPDPR) (Mentlein, 1999), produces a chronic decrease in body weight and body fat levels (Erlanson-Albertsson and York, 1997). The decreased water intake of DPPIV-deficient animals could be mediated via enhanced levels of active GLP-1, which functions as a potent inhibitor of basal and angiotensin II-induced water intake (Drucker, 1998). Studies with DPPIV knockout mice (Marguet et al., 2000) and F344/DuCrj(DPPIV-) rats (Kieffer et al., 1995; Nagakura et al., 2001) described already the inactivating effect of DPPIV on active GLP-1 levels *in vivo*.

The hyperalgesic phenotype of F344/DuCrj(DPPIV-) and F344/Crl(Ger/DPPIV-) rats in the hot plate test could be related to the involvement of DPPIV substrates such as substance P and NPY in pain sensitivity (Bannon et al., 2000; Hua et al., 1991; Luttinger et al., 1984; Post and Paulsson, 1985). The neurotransmitter NPY is known to increase the pain threshold in rats especially in the hot plate paradigm (Broqua et al., 1996; Merlo Pich et al., 1993) and overall thermal hyperalgesia (Tracey et al., 1995). Since a powerful stress response increases NPY release in the CNS (Colmers and Wahlestedt, 1993; Pralong et al., 1993), the endogenous NPY level of the wildtype-like substrain with its higher stress-associated behavioral response in tests of anxiety could be elevated compared to mutant animals. Thus, increased levels of NPY could be responsible for a decrease in pain sensitivity in the wildtype-like animals. In addition, the anxiogenic-like phenotype of these rats could increase the pain threshold by stress-induced hypoalgesia (Kelley, 1986; Millan, 1999).

The pattern of the findings in the anxiety-related paradigms strongly supports the concept of a reduced behavioral stress response in the mutant F344 substrains, which was associated with several indicators of reduced anxiety such as a high ambulation in the open field, an increased SI time, and an increased light-dark transversion time. These differences in the level of arousal and/or anxiety between the different F344 substrains could be based on a differential

degradation of NPY (De Meester et al., 2000). NPY administration decreases anxiety (Broqua et al., 1996; Kask et al., 2001b; Sajdyk et al., 1999; Thorsell et al., 2000) and has been reported to reduce stress responsiveness in transgenic NPY overexpressing rats (Thorsell et al., 2000). This anxiolytic-like effect of NPY is primarily mediated via the Y_1 receptor subtype (Heilig, 1995; Wahlestedt et al., 1993). The non-degraded NPY in the DPPIV-deficient rats is a more potent activator of the Y_1 receptor subtype than the degraded NPY_{3-36} in the wildtype-like animals (see 1.5.2: Fig. 1). Thus, differences in the NPY catabolism between the different F344 substrains, which are probably linked to DPPIV, seem to be responsible for the reduced anxiety of the DPPIV-deficient F344/DuCrj(DPPIV-) and F344/Crl(Ger/DPPIV-) substrains. The anxiolysis is probably due to elevated levels of non-cleaved native NPY in these substrains.

The DPPIV-deficient substrains exhibited a reduced susceptibility to the sedative effect of ethanol. But in regard to a possible involvement of the NPY system in this phenomenon, further investigations have to be conducted.

In conclusion, in this second experimental step, the DPPIV-deficient F344/DuCrj(DPPIV-) and F344/Crl(Ger/DPPIV-) rats exhibited a reduced behavioral stress response in tests like the hot plate, social interaction, and passive avoidance. These findings suggest an involvement of DPPIV-like activity in the regulation of anxiety-like behaviors, and these effects are possibly mediated by a differential degradation of NPY and/or alternative DPPIV substrates. Overall, this part of the study was based on a systematic behavioral and physiological phenotyping (Karl et al., 2003a). This includes a broad range of different behavioral tests, which are necessary for an extensive characterization and for detecting specific behavioral differences. This strategy for analyzing mutant rodents is becoming an increasingly important and useful tool in biomedical research.

To prove our hypothesis that the observed differences in behavior and physiology between the wildtype-like and mutant F344 rats could be based especially on differences in the metabolism of NPY, in the third experimental step of this study, we analyzed the effect of i.c.v. administration of different doses of NPY (0.0/0.2/1.0 nmol) in the wildtype-like [F344/Crl(Por)] and mutant [F344/DuCrj(DPPIV-) and F344/Crl(Ger/DPPIV-)] rats on behavioral tasks like feeding (food intake), anxiety, and nociception (Karl et al., 2003d; Karl et al., 2003e). Furthermore, we administered singularly different doses of the DPPIV-inhibitor *Ile-Thia* (0.0/0.5/5.0 nmol) to confirm that the observed differences in the behavioral and physiological phenotype of our different substrains are based on the DPPIV deficiency of F344/DuCrj(DPPIV-) and F344/Crl(Ger/DPPIV-) and not on other genetic or physiological

differences between the DPPIV-deficient and wildtype-like substrains (Karl et al., 2003e). The study shows for the first time a differential response to i.c.v. administration of NPY in rats that differ in their endogenous DPPIV-like enzymatic activity. Namely, the different doses of NPY induced a significant stimulatory effect on the feeding behavior, a significantly more pronounced sedative-like effect on elevated plus maze behavior, a significantly more potent anxiolytic-like effect on the rat social interaction test, and a significant more potent analgetic-like effect in the hot plate test in the DPPIV-deficient F344 rats. Furthermore, treatment with *Ile-Thia* resulted in a hyperalgesic phenotype in the wildtype-like F344/Crl(Por) substrain.

The stimulatory effect of NPY on the feeding behavior has already been described in literature (Brief et al., 1992; Jolicoeur et al., 1991; Levine and Morley, 1984; Merlo Pich et al., 1992; Schwartz et al., 2000; Stanley et al., 1986) and NPY, as a physiological appetite transducer (Kalra et al., 1999), is the only known peptide that can cause animals to eat until they are obese (Inui, 1999). The similarity in the feeding response to NPY administration between the different substrains is likely due to the affinity of cleaved NPY_{3-36} to Y_5 and Y_2 receptor subtypes, which are, like the Y_1 receptor subtype, involved in the feeding behavior of rodents (Bischoff and Michel, 1999; Inui, 1999; Inui, 2000; Kalra et al., 1999; Naveilhan et al., 1999; Sainsbury et al., 2002; Turnbull et al., 2002). Native NPY in the DPPIV-deficient and NPY_{3-36} in the wildtype-like rat substrains could both mediate potent orexigenic effects of similar power.

A dose-dependent sedative effect of NPY on locomotor activity, which is interpreted as sedation (Fuxe et al., 1983), has been reported by several studies (Broqua et al., 1995; Heilig and Murison, 1987; Heilig et al., 1989; Jolicoeur et al., 1995). This suppression of activity is supposed to be mediated by Y_1 receptors (Heilig et al., 1988; Kask et al., 1999; von Horsten et al., 1998a), which are also involved in the NPY-induced sensitization to sedation (Heilig et al., 1988; Kask et al., 1999; Naveilhan et al., 2001b; von Horsten et al., 1998a). In our study the NPY-treated mutant animals of F344/DuCrj(DPPIV-) and F344/Crl(Ger/DPPIV-) exhibited a dose-dependent reduction in motor activity. This effect in the DPPIV-deficient animals is based on the differences in the NPY catabolism between the F344 substrains with a DPPIV-dependent cleavage of NPY to the Y_1 receptor unspecific NPY_{3-36} only in control rats. The dose-dependent differential response of the three F344 substrains with respect to this parameter supports our hypothesis regarding the DPPIV-dependent differential NPY catabolism in wildtype-like and mutant animals.

I.c.v. administration of NPY is also deeply involved in anxiety-like behaviors of rodents (Broqua et al., 1995; Heilig et al., 1989; Kask et al., 2002; von Horsten et al., 1998a). The results of several studies suggest a role for the Y_1 receptor in mediating the anxiolytic-like action of NPY (Heilig et al., 1989; Kask and Harro, 2000; Kask et al., 2001a; Kask et al., 1996; Wahlestedt et al., 1993), although also the Y_2 (Kask et al., 2001a; Kask et al., 1998) and Y_5 (Sajdyk et al., 2002) receptor subtype could be involved.

NPY administration had an anxiolytic-like effect in all substrains in the elevated plus maze, which could be influenced also by the sedative-like effect of NPY. At least, a very narrow pharmacological window in the F344 genetic background between sedative-like and anxiolytic-like effects of NPY especially in the elevated plus maze has to be considered in order to avoid false positive results. Therefore, we also applied the social interaction test for recording the anxiolytic-like potency of NPY, in which the DPPIV-deficient rats exhibited an increased anxiolytic-like response (independent from the sedative-like effect) to the NPY administration compared to the wildtype-like animals. Motor activity was dose-dependently reduced by NPY treatment, but despite this sedative effect on ambulatory activity, social-like behaviors remained unaffected by NPY. Overall, these data show the suggested differences between the wildtype-like and mutant F344 substrains regarding their NPY catabolism because of the more potent Y_1 receptor-mediated anxiolytic-like and sedative-like effects of the neurotransmitter in the mutant, DPPIV-deficient animals. The differences in anxiety levels in the social interaction test between the two DPPIV-deficient substrains F344/DuCrj(DPPIV-) and F344/Crl(Ger/DPPIV-) are probably dependent on segregation-based differences in the genetic background between these substrains.

Centrally injected NPY exerted a dose-dependent hypoalgesic effect only in the mutant rats, whereas i.c.v. administration of the DPPIV inhibitor *Ile-Thia* had a hyperalgesic effect in the wildtype-like animals. The effect of NPY on nociception is discussed controversially, although there is evidence for the presence of NPY-immunoreactivity in some areas of the CNS, which are involved in pain modulation (Broqua et al., 1996; Jolicoeur et al., 1991). I.c.v. or intrathecal administration of NPY in the hot plate paradigm produced a dose-dependent elevation in the nociceptive threshold (Hua et al., 1991; Merlo Pich et al., 1990). In other studies NPY induced hyperalgesia (Broqua et al., 1996; von Horsten et al., 1998b) or did not have any effect on nociception (Heilig et al., 1993; Heilig et al., 1992; Heilig et al., 1989; Jolicoeur et al., 1991). However, Y_1 receptor knockout mice develop hyperalgesia to acute thermal pain (Naveilhan et al., 2001a). Therefore, it is concluded that the Y_1 receptor is

required for central physiological and pharmacological NPY-induced analgesia (Broqua et al., 1996; Wang et al., 2000).

The results of our study could point towards a Y_1 receptor-mediated analgetic-like effect of NPY. Wildtype-like F344/Crl(Por) rats with a normal DPPIV-like activity lost more rapidly the Y_1 receptor subtype-specific affinity of exogenously administered NPY by DPPIV-dependent cleavage processes than the DPPIV-deficient animals. In the mutant rats the exogenously administered native and non-cleaved NPY decreased their pain sensitivity, which is a strong hint for the antinociceptive effect of i.c.v. applied NPY. The hyperalgesic phenotype of the vehicle-treated DPPIV-deficient F344/DuCrj(DPPIV-) and F344/Crl(Ger/DPPIV-) animals was already discussed as an effect of the reduced anxiety levels and concomitant reduced stress-induced analgesia of these animals (Karl et al., 2003c). NPY-deficient mice appear to be hypoalgesic in the hot plate paradigm (Bannon et al., 2000). Also these mice exhibit an anxiogenic-like phenotype (such as F344/Crl(Por) rats). Their increased anxiety seems to increase their pain threshold probably by stress-induced analgesia, which is associated with or even triggered by a release of analgesia-inducing endogenous opioid peptides from central and peripheral sites (Kelley, 1986). This process could also occur in our wildtype-like rats. Furthermore, a potent stress-induced NPY release in the wildtype-like animals paired with the described stress-induced analgesia could manifest in the observed hypoalgesic phenotype. In addition, substance P and endomorphin-2, two other substrates of the DPPIV are involved in nociception and could be differentially degraded in the different substrains.

I.c.v. treatment with the DPPIV-inhibitor *Ile-Thia* exhibited no effects in the DPPIV-deficient animals but rather exerted an hyperalgesic effect in the control animals. This could be based on DPPIV inhibition-caused reduced anxiety levels in the wildtype-like animals, which could be linked to a decrease in endogenous NPY-release and a reduction in stress-induced analgesia.

In conclusion, this third experimental step demonstrates for the first time a differential potency and specificity of exogenously administered NPY between wildtype-like and DPPIV-deficient rat substrains (based on differential cleavage processes of NPY). Thereby, the study supports the concept that these animals provide a useful model to study the various behavioral and physiological effects associated with DPPIV-enzymatic activity. Furthermore, we could confirm the stimulatory effect of NPY on feeding behavior (food intake) and its sedative-like, anxiolytic-like, and probably also analgetic-like effect in rats.

In the present series of studies we found an overall wide range of differences between the wildtype-like and DPPIV-deficient F344 substrains. These differences seem to be based on a differential DPPIV-dependent cleavage of regulatory peptides such as enterostatin, GLP-1, and especially NPY. The results of the i.c.v. administration of NPY show that the mutant animals exhibited an increased responsiveness to the sedative-like, anxiolytic-like effect, and probably analgetic-like effect of i.c.v.-administered NPY, which is most likely mediated via increased and prolonged activation of NPY Y_1 receptor-dependent pathways and mechanisms. Thus, a differential cleavage of NPY in our different substrains is strongly suggested and can be considered at least as one of the main reasons for the observed differences in behavior and physiology between wildtype-like and DPPIV-deficient rats. Our F344 substrains F344/Crl(Por), F344/DuCrj(DPPIV-), and F344/Crl(Ger/DPPIV-) represent an excellent and valid animal model to study the "DPPIV-NPY-axis" and other DPPIV-dependent behavioral and physiological processes. Furthermore, these animals could serve as a physiological and molecular model for behavioral modulations. Since pharmacological inhibition of DPPIV-like activity has been demonstrated to potentiate the effects of NPY administration *in vivo* (Dimitrijevic et al., 2002), it seems possible that DPPIV inhibitors specifically targeting the CNS are useful modifiers of centrally mediated effects of NPY.

8. References

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9. Curriculum vitae

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Present Position:	PhD-student for behavioral biology in a graduate programme of the German Research Foundation (DFG) at the Medical School of Hannover
Nationality:	German
Sex:	male
20.9.1973:	born in Kirchhellen
1980 - 1984:	primary school
1984 - 1993:	secondary school and graduation from school (“Abitur”)
1993 - 1994:	civil service in a children´s home and on a ward of the Department for Psychiatry of the St. Antonius Hospital in Bottrop-Kirchhellen
1994 - 1999:	study of biology and German for teaching in a secondary school at the Westfaelische Wilhelms-University (WWU) of Muenster
1999:	graduation work: “Comparison of the mice strains AB/Gat, AB/Hal, and of the congene strain CS in their attack latency and in other physiological parameters” in the Department of Neurological and Behavioral Biology (Prof. Sachser) of the WWU Muenster
June 2000:	graduation from university
since 2001:	working as a PhD-student (for behavioral biology) at the Medical School of Hannover in the Department for Functional and Applied Anatomy (Prof. Pabst) under supervision of Prof. von Hoersten: “Characterization of neuropeptide Y-mediated behavioral effects in F344 rat substrains with a differential dipeptidyl-peptidase IV

(DPPIV; CD26) enzymatic activity”

Additional education

1996 – 1999:	working on a ward for handicapped women of the Stift Tilbeck in Havixbeck every second weekend;
Nov. 1998:	scientist practicum in the animal station of the Department of Psychology (WWU Muenster) – observation of monkeys concerning their learning behavior
1998 - 2000:	working as a tutor in the Department of Biology (WWU Muenster)
1998 - 2001:	zoo guide in the “Westfälischer Zoologischer Garten Münster” – organization of guided tours for the visitors

Collaborations

March 2002:	working as a visiting scientist at the Garvan Institute of Medical Research (Prof. Herzog) for 3 months - project: “Behavioral phenotyping of NPY Y1 knockout animals”
Feb. 2003:	second visit of the Garvan Institute of Medical Research (Prof. Herzog) for 2 months - project: “Characterization of NPY Y1 and Y2 germline knockout and Y1 conditional knockout animals in regard to aggression”

Conferences/Workshops

July 1999:	participation in a behavioral biology workshop in the Department of Animal Physiology (University Bayreuth) (Prof. von Holst)
Sept. 1999:	attendance at the 1. German Endocrine Brain Immune Network (GEBIN)-symposium in Essen (poster presentation)
Sept. 2001:	attendance at the 2. GEBIN-symposium in Regensburg
Aug. 2002:	attendance at the 1. European Conference on Behavioral Biology in Muenster
Okt. 2003:	attendance at the 3. GEBIN-symposium in Munich (oral presentation)

3.10.03

10. Publication list:

Original publications

Karl, T., Chwalisz, W.T., Wedekind, D., Hedrich, H.J., Hoffmann, T., Jacobs, R., Pabst, R., and von Horsten, S.: Localization, transmission, spontaneous mutations, and variation of function of the *Dpp4* (Dipeptidyl-peptidase IV ; CD26) gene in rats. Regul Pept 115. 2003. 81-90.

Karl, T., Hoffmann, T., Pabst, R., and von Horsten, S.: Extreme reduction of dipeptidyl-peptidase IV activity in F344 rat substrains results in major behavioral differences. Physiol Behav 80. 2003. 123-134.

Karl, T., Hoffmann, T., Pabst, R., and von Horsten, S.: Behavioral effects of neuropeptide Y in F344 rat substrains with a reduced dipeptidyl-peptidase IV activity. Pharmacol Biochem Behav 75. 2003. 869-79.

Reviews and contributions to books

Karl, T., Pabst, R., and von Horsten, S.: Behavioral phenotyping of mice in pharmacological and toxicological research. Exp Toxicol Pathol 55 (1). 2003. 69-83.

Von Horsten, S., Hoffmann, T., Alfalah, M., Wrann, D.C., Karl, T., Pabst, R., and Bedoui, S.: Formation, storage, release and degradation of NPY, PYY and PP. In: Michel, M.C. (ed.): Handbook of Experimental Pharmacology: Neuropeptide Y and related peptides. Springer Verlag Berlin. 2003. In press.

11. Erklärung

Hiermit erkläre ich, Tim Karl, 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.

3.10.2003

12. Danksagung

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