DPP4-deficient congenic rats display blunted stress, improved fear extinction and increased central NPY

Fabio Canneva, Yulia Golub, Joerg Distler, Julia Dobner, Sandra Meyer, Stephan von Hörsten

Department of Experimental Therapy, Präklinisches Experimentelles Tierzentrum, Universitätsklinikum Erlangen, 91054 Erlangen, Germany
Department of Child and Adolescent Mental Health, University Clinic of Erlangen, 91054 Erlangen, Germany

Received 28 July 2014; received in revised form 9 January 2015; accepted 9 January 2015

KEYWORDS
NPY; Anxiety; Stress; Fear; CD26; DPP4

Summary
Background: Inhibitors of dipeptidyl peptidase 4 (DPP4, CD26) are used for the treatment of type 2 diabetic patients and better glucose tolerance has been confirmed in functionally DPP4-deficient congenic rats (DPP4mut), along with immunological alterations and, interestingly, a stress-resilient phenotype. All these findings are in agreement with the "moonlighting" properties of DPP4, whose proteolytic action is responsible for the inactivation of a number of regulatory peptides including, but not limited to, neuropeptide Y (NPY).

Among all candidate substrates, DPP4 displays highest affinity for NPY, an endogenous anxiolytic neurotransmitter that is suggested as a candidate biomarker in post-traumatic stress disorder (PTSD) and depression.

Methods and results: Central and peripheral NPY levels were measured by ELISA in DPP4mut and DAwt rats revealing a significantly higher concentration of the peptide in the CSF of DPP4mut animals. This finding positively correlated with the blunted stress phenotype measured on an analgesia-meter. Additionally, when a classical fear-conditioning paradigm was investigated, short-term fear extinction was significantly potentiated in DPP4mut rats as compared to wt controls.

Conclusions: Our findings indicate a positive correlation between reduced stress-responsiveness and increased central NPY, in DPP4mut rats. Most interestingly, the behavioral phenotype

* Corresponding author at: Department of Experimental Therapy, Präklinisches Experimentelles Tierzentrum (PET2), Universitätsklinikum Erlangen, Palmsanlage 5, 91054 Erlangen, Germany. Tel.: +49 9131 8523530; fax: +49 9131 8523502.
E-mail address: Fabio.Canneva@uk-erlangen.de (F. Canneva).
extends to facilitation of fear extinction. These observations raise further interest in DPP4-modulating drugs for the potential effect on NPY metabolism, as a therapeutic tool for psychiatric conditions such as anxiety disorders and PTSD.

© 2015 Elsevier Ltd. All rights reserved.

1. Introduction

Inhibitors of dipeptidyl peptidase 4 (DPP4) are used as alternative therapeutics for the management of glucose homeostasis in patients affected by type 2 diabetes (Drucker, 2003; Gallwitz, 2007). In such clinical context, DPP4 inhibition enhances the activity of endogenous insulin-releasing gut hormones, especially glucagon-like protein 1 (GLP1), improving glucose homeostasis. Better glucose tolerance has been confirmed in functionally DPP4-deficient rats (DPP4mut), which express an inactive mutant form of the peptide (Karl et al., 2003a). DPP4mut animals are also characterized by reduced weight gain under high-caloric diet, immunological alterations, and importantly a stress-resilient phenotype (Frerker et al., 2009; Karl et al., 2003b,c; Stephan et al., 2011). These results are in agreement with the "moonlighting" properties of DPP4, whose proteolytic action is responsible for the inactivation of a number of regulatory proteins, spanning from the incretin system (e.g. GLP1) to several neuropeptides (e.g. NPY, substance P) and chemokines (e.g. macrophage chemotactic proteins) (Boonacker and Van Noorden, 2003).

Mirroring such a pleiotropic role, DPP4 is rather ubiquitously distributed throughout the body (Mentlein, 1999), with highest expression in kidney, lung liver and small intestine. Additionally, the extracellular domain can be shed, releasing a soluble form of the active enzyme particularly enriched in the glucose plasma (Mentlein, 2004). In the adult central nervous system, DPP4 expression is restricted to the circumventricular organs and leptomeningeal cells (Mitro and Lojda, 1988); additionally, brain capillaries and some ependymal cells are strongly immunopositive for DPP4 (Bernstein et al., 1987).

Among all candidate substrates, DPP4 displays highest affinity for NPY and peptide YY (Bjelke et al., 2006; Hartel et al., 1988). NPY is the most abundant neuropeptide in the central nervous system (CNS), and its role as an endogenous anxiolytic neurotransmitter has been thoroughly investigated (for a review see Heilig, 2004). Additionally, being highly expressed in the hypothalamic arcuate nucleus, NPY is critical in stimulating food intake and regulating energy stores (Beck, 2006; Kuo et al., 2007b). NPY activity is exerted through the interaction with several receptors, designated Y1 through Y6. Receptor specificity is highly influenced by the integrity of the molecule, so that full-length NPY preferentially binds to receptor Y1, mediating its anxiolytic effects (Fuhnderoff et al., 1990), whereas the truncated form released by DPP4 (NPY3-36) displays higher affinity for the receptor Y2, mediating opposite effects (King et al., 1999, 2000) through presynaptic inhibition of further NPY release (although alternative effects of pre- and postsynaptic activation of Y2 receptor have also been described, suggesting a more complex regulation of this pathway; for a review see Kask et al., 2002). Besides, NPY is a vasoconstrictor peptide, released by peripheral noradrenergic terminals innervating blood vessels and the heart (Lundberg and Tatemoto, 1982; Lundberg et al., 1982).

Finally, in some rodents, including the rat, NPY is also accumulated in platelets (Myers et al., 1988; Ogawa et al., 1989, also reviewed in Kuo et al., 2007a) from where it is released during platelet aggregation.

Because NPY can cross the blood—brain-barrier (BBB) (Kastin and Akerstrom, 1999), the presence of DPP4-like activity at sites of physiological barrier implies a regulatory role in the trafficking of the peptide across bodily fluids, from the periphery to the CNS.

Recent studies have pointed at NPY levels in plasma and CSF as candidate biomarkers for the clinical diagnosis and prognosis of post-traumatic stress disorder (PTSD) (Rasmusson et al., 2000; Sah et al., 2009; Yehuda et al., 2006), depression (Heilig et al., 2004) and metabolic syndrome (Abe et al., 2010; Rasmusson et al., 2010). In this context, aim of our work was to measure central and peripheral levels of NPY in DPP4mut rats, and to investigate stress-responsive and fear behaviors in this animal model.

2. Methods and materials

2.1. Animals

DA/Ztm (DAwt) and DA.F344-Dpp4+/SvH (DPP4mut) rats were housed and bred at our central animal facility as described previously (Frerker et al., 2009). All research and animal care procedures were approved by the local district governments (#33-42502-06/1173 and #54-2532.1-19/09), and performed according to international guidelines for use of laboratory animals. Animals were kept under a standard 12:12 light:dark cycle (lights on at 4.00, lights off at 16.00), in stable social groups of 3–4 rats. Food and water were available ad libitum. Sitagliptin (Selleckchem, Munich, Germany) was administered in the drinking water at a concentration suitable to provide 30 mg/kg/day, according to a published study (Chen et al., 2011).

2.2. Behavioral procedures

Independent sets of animals were used for each behavioral experiment.

2.2.1. Stress induced analgesia (SIA)

Stress induced analgesia (SIA) was measured using the hot-plate assay, as previously reported (Karl et al., 2003c). Briefly, animals were exposed to a preheated plate kept at the temperature of 52.5 °C, and latency to lick or raise hind paws was recorded. In order to avoid any tissue damage, all animals were removed from the plate within 30 s. Habituation to the testing procedure was achieved by
exposure of the animals to the cold plate for 30 s on three alternate days. A test session on the hot plate followed 48 h later.

2.2.2. Fear conditioning and extinction training

Fear conditioning and extinction training were adapted from previously published methods (Golub et al., 2011; Plendl and Wotjak, 2010). All procedures were performed during the active phase of the animals, at least 1 h after dark onset (17:00). Briefly, conditioning was performed in context A (dark, square chamber with electrified grid floor, cleaned with 70% ethanol); following 3 min of habituation to the testing environment, rats were exposed to a conditioning stimulus (CS, 80 dB, 5 kHz sine-wave tone) for 20 s, co-terminating with an unconditioned stimulus (UCS, 0.8 mA electrical foot-shock, 2 s long). Animals were returned to their home cages 60 s later.

Contextual fear was tested 24 h later, by measuring freezing behavior during a 180 s exposure to the original conditioning context A. Cued-fear was tested 8 days after fear conditioning. In order to measure the freezing response to the tone independently from the confounding influence of contextual memory, a new context B (transparent circular chamber, absorbent pad floor, cleaned with 1% acetic acid solution) was applied. After 3 min of habituation to the new environment, animals were presented with a 3 min-long CS, and freezing during this time was recorded.

Extinction training was carried out with half of the conditioned animals during five consecutive days, starting 48 h after fear conditioning. Training was performed in context B by means of repeated non-reinforced tone presentations. Each training session started after an initial 3 min period of habituation and consisted of 10 consecutive presentations of the CS (30 s) interspaced by variable inter-stimulus intervals (ISI, 60 ± 30 s). Rats were returned to their home cage 60 s after the last CS presentation. Twenty-four hours after completion of the extinction protocol, fear retention was measured in all animals as described above.

2.3. Collection of samples

Animals subjected to fear conditioning were sacrificed 30–45 min after re-exposure to the CS, in a completely new context (C), located in a different behavioral room, whereas rats tested on the hot-plate were anesthetized immediately after testing. Rats were injected i.p. with a mixture of Ketamine and Xylazine (Adamcak and Otten, 2000) and CSF was collected as previously described (Heffner et al., 1980; Pegg et al., 2010). Blood was drawn by cardiac puncture and EDTA-plasma was promptly prepared. The animals were then quickly perfused with ice-cold saline solution and the brains collected on ice and dissected into the anatomical regions of interest, following published guidelines (Heffner et al., 1980). All samples were immediately frozen in liquid nitrogen (tissue) or at −80 °C (CSF, plasma), and stored at −80 °C until further processed. Brain specimens for immunohistochemistry (IHC) were post-fixed in 4% para-formaldehyde (PFA) in physiological saline solution for 22 h and then equilibrated in 30% sucrose before being frozen at −80 °C.

2.4. NPY quantification

NPY concentration was measured by ELISA in the CSF and plasma samples using a commercially available kit (IBL America, Minneapolis, MN, USA). Briefly, all samples were gently thawed on ice, properly diluted and applied to the 96-well ELISA plate, following the manufacturer’s instructions. Each sample was loaded in duplicate, and the mean value used for further analysis.

2.5. DPP4 activity assay

DPP4 activity was measured in plasma samples using a published colorimetric assay (Dubois et al., 2008), based on the transformation of the chromogenic substrate glycyl-prolyl-para-nitroanilide (Gly-Pro-pNA). The assay was performed in a 96-well plate format, and the reactions run in 250 μl final volume. Briefly, 20 μl of undiluted plasma were mixed with 130 μl of Tris–HCl buffer (100 mM, pH 8.0) and incubated 15 min at 37 °C. Reactions were started by adding 100 μl of the substrate (1 mM in water). Enzymatic activity was determined kinetically during 10 min at 37 °C by measuring the velocity of pNA release, determined as an increase of absorbance at 405 nm in a microplate spectrophotometer (Benchmark Plus, BioRad, Munich, Germany). A positive control of the assay was run in parallel, using the purified DPP4 enzyme. Additionally, the specificity of the enzymatic activity measured was confirmed using the DPP4-selective inhibitor sitagliptin (1 μM).

2.6. Immunohistochemical detection of NPY and DPP4, and stereological quantification

Frozen, fixed brains were sliced into 40-μm-thick coronal sections on a cryostat (CM3050S, Leica Biosystems, Wetzlar, Germany). The sections were then stained using a free-floating immunohistochemistry (IHC) procedure. Briefly, PBS-T (PBS containing 0.2% TritonX-100) was used throughout all steps, and incubations were all performed at room temperature, unless otherwise specified. Tissue was permeabilized with PBS-T and then treated with 0.3% hydrogen peroxide for 20 min, rinsed and blocked for 30 min with 5% normal donkey serum (Jackson ImmunoResearch Europe Ltd., Suffolk, UK). Incubation with primary antibody (rabbit polyclonal anti-NPY, 1:5000, BioTrends, Cologne, Germany) diluted in REAL antibody diluent® (Dako, Hamburg, Germany) was performed overnight at 4 °C. The following day sections were rinsed and incubated for 1 h with secondary antibody (donkey-anti-rabbit IgG, 1:500; Santa Cruz Biotechnology, Heidelberg, Germany). Finally, sections were washed and incubated for 1 h with VECSTASTAIN® ABC reagent (Vector Laboratories, Peterborough, UK). The staining was developed with Ni-enhanced DAB reagent (Vector Laboratories, Peterborough, UK). After extensive washing, sections were blocked with streptavidin- and biotin-blocking agents (Vector Laboratories, Peterborough, UK), and then a second staining was performed on the same section, similarly to what described so far. In this case DPP4-specific primary (mouse monoclonal OX-61, 1:100, Becton Dickinson, Heidelberg, Germany) and donkey-anti-mouse secondary (1:500, Santa Cruz Biotechnology, Heidelberg,
Germany) antibodies were used, and staining was developed using HistoGreen reagent (Linaris, Dossenheim, Germany). Rinsed sections were then mounted on Superfrost Plus glass slides (Thermo Scientific, Germany), air-dried, dehydrated in ascending ethanol concentrations, cleared with xylol, and coverslipped with DPX mounting medium (Sigma, Munich, Germany). Images were acquired on a Keyence BZ9000E microscope, equipped with imaging software (Keyence, Neu-Isenburg, Germany). Stereological quantification of NPY positive (NPY+) neurons followed our previous reports (von Horsten et al., 2000) and was carried out under blind conditions using the StereoInvestigator software (MicroBrightField, Magdeburg, Germany). Total number of NPY+ positive neurons was estimated in the region of interest (ROI) (baso-lateral amygdala – BLA) using the optical fractionator method, and the Cavalieri’s principle applied to estimate the volume of ROI (Sauerhofer et al., 2012).

### 2.7. Quantification of NPY and NPY receptors mRNA expression

NPY and NPY-Rs mRNA expression levels were quantified in brain specimens, according to standard reverse transcriptase-coupled quantitative real-time PCR (qRT-PCR) procedure. Briefly, total RNA was extracted from tissue using RNA-Pure (Peqlab, Erlangen, Germany) and retrotranscribed into cDNA with First-Strand reagent (Invitrogen, Life Technologies, Darmstadt, Germany). qPCR reactions were then performed on an iQ5 thermo-cycler (Bio-Rad, Munich, Germany) using SYBR-Green reagent (BioRad, Munich, Germany) in a final volume of 25 µL. The relative amount of each transcript was calculated using the cycle threshold method, after normalization with a housekeeping gene (rpl13a). Primers used in this study are listed in Table 1.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>GenBank</th>
<th>5’ primer</th>
<th>3’ primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Npy</td>
<td>NM_012614.2</td>
<td>AGA GAT CCA GCC CTG AGA CA</td>
<td>AAC GAC AAC AAG GGA AAT GG</td>
</tr>
<tr>
<td>Npy-y1</td>
<td>NM_001113357.1</td>
<td>GGC GAA CAG ACG GAT TCT TT</td>
<td>TGG ATC AGA TCT TCA GCT GTC T</td>
</tr>
<tr>
<td>Npy-y2</td>
<td>NM_023968.1</td>
<td>CCA TCA TCT TGC TGG GGC T</td>
<td>CTC CCC CAT CAA GGT ATA GG</td>
</tr>
<tr>
<td>Npy-y5</td>
<td>NM_012869.1</td>
<td>GCT GCT CGG AAT GCA GCC</td>
<td>ACT GTA GTC TTC TGA TTG CG</td>
</tr>
<tr>
<td>c-fos</td>
<td>AY_780203.1</td>
<td>ACT CCA GTC CTC ACC TCT TC</td>
<td>ACA TGC TAC TAA CTA CCA GCT C</td>
</tr>
<tr>
<td>Rpl13a</td>
<td>NM_173340.2</td>
<td>GCT GCC GAA GAT GGC GGA GG</td>
<td>CAC CTT TCG GCC CAG CAG TAC C</td>
</tr>
</tbody>
</table>

NPY mRNA levels were measured across key areas of the brain rich in NPY-positive neurons (Fig. 1F), revealing no differences between DAwt (n = 13) and DPP4mut (n = 12) animals (4 month old). Similarly, stereological quantification of NPY+ neurons of the basolateral amygdala (BLA) did not reveal any difference between genotypes (Fig. 1G). Expression of NPY and NPY-receptors were further investigated by RT-qPCR in the amygdala of animals tested in the SIA paradigm (Fig. 2). Interestingly, an overall trend toward decreased levels of NPY receptors was found in specimens isolated from DPP4mut animals, reaching significance in the case of Y1 (unpaired t-test, p = 0.04) and Y5 (p = 0.036).

### 3.2. Stress-induced analgesia is reduced in DPP4mut rats under acute, but not habituated, conditions

Responsiveness to heat was used in order to investigate SIA (Amit and Galina, 1986) in 6–7 month old DAwt (n = 13) and DPP4mut (n = 12) male rats (Fig. 2). Experimentally naïve rats were tested on an analgesia-meter before and after habituation to the testing paradigm: while all animals responded to the habituation training with a significantly decreased latency to raise/lick a hind paw (F(1,24) = 68.04, p < 0.0001, two-way ANOVA for repeated measures), a significant difference between groups was only observed in the non-habituated version of the test (p = 0.0002, Sidak’s post-hoc test). Similar results were also obtained with female rats (Supplementary Fig. 3). This observation also indicates that supra-spinal regulation of reaction to pain, but not pain perception per se, is blunted in DPP4mut congenic animals. Additionally, no differences between genotypes were observed in the tail-flick assay, which measures spinal reflex to heat (Supplementary Fig. 1A).

Supplementary Figs. 1 and 3 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jspyneu.2015.01.007.

### 3.3. NPY concentration is increased in the CSF of DPP4mut rats

NPY was measured in adult (6 month old) male rats right after performing the non-habituated version of the hot-plate test. As predicted, latency to respond to heat was significantly reduced in DPP4mut rats (17.3 ± 0.3 s vs. 21.2 ± 1.9 s, p = 0.04, one-tailed t-test) indicating blunted stress responsiveness (Fig. 3A). Further supportive of this behavioral
Anxiolytic phenotype and central NPY in CD26-deficient rats

**Figure 1** Expression and distribution of NPY and DPP4, in the rat brain. NPY (Ni-DAB, black staining) and DPP4 (HistoGreen, green staining) immunoreactivity (ir) is shown in sample brain sections collected from a DAwt rat (A, overview image taken at 4× magnification). Higher magnifications (20×) show the localization of DPP4-ir at the meninges (B) and choroid plexus (C), indicated by arrowheads. Functional lack of DPP4 activity was confirmed in plasma samples from DPP4mut rats, as compared to DAwt (E, white and dark bars) and to rats treated with sitagliptin for three weeks (E, light gray bars). Specificity of the assay was further confirmed by inhibition of the measured peptidase activity with sitagliptin, in vitro. NPY expression was measured in the brain of DAwt and DPP4mut rats by means of RT-qPCR (F) and stereological quantification of NPY+ neurons in the basolateral amygdala (BLA, G); in either cases, no differences were detected between the two genotypes. Quantification of NPY mRNA levels in the amygdala of a different set of animals confirmed no significant differences between DAwt and DPP4mut rats (H). Nonetheless, an overall reduction of NPY-Rs was measured in DPP4mut rats, as compared to wt controls, which reached statistical significance in the case of Y1 and Y5 receptors. *p < 0.05; ****p < 0.0001. Scale bar in A represents 1 mm and 50 μm for 4× and 20× magnifications, respectively.
result, corticosterone levels were significantly reduced in DPP4mut rats as compared to wt (Supplementary Fig. 1B).

NPY levels were analyzed in the same animals by ELISA: NPY concentration was two-fold higher in the CSF of DPP4mut rats (0.90 ± 0.07 ng/ml), as compared to DAwt (0.43 ± 0.03 ng/ml; Fig. 3B). Conversely, no differences were detected in the plasma (37.5 ± 3.9 ng/ml and 36.1 ± 1.8 ng/ml).

Analysis of variance revealed a significant difference in the distribution of the data between the two groups for both the SIA (F(10,16) = 4.663, p = 0.0064) and central NPY (F(15,10) = 6.191, p = 0.0061) data. A Pearson test was therefore applied, in order to investigate the correlation between these two parameters (Fig. 3C). Our analysis revealed a modest, yet significant, negative correlation (r = −0.4262, p = 0.03, two-tailed), supporting the hypothesis of a causative link between central NPY and pain-responsive behavior, under these experimental conditions.

Sub-chronic, pharmacological inhibition of DPP4 activity further supported the behavioral results: DAwt animals treated with sitagliptin (30 mg/kg/day) for three consecutive weeks also displayed reduced SIA (Fig. 1E and 3D).

3.4. Fear conditioning and extinction in DPP4mut rats

A classical cued fear-conditioning paradigm was applied to investigate fear behavior in DPP4mut (n = 18) rats, as compared to DAwt (n = 21; Fig. 4A), at the age of 4 months. Two-way ANOVA for repeated measures ("time") was used throughout the study to analyze the impact of the factors "genotype" and "extinction"; Sidak’s multiple comparisons test was applied in order to calculate statistical significance between given experimental groups.

All rats responded similarly during the initial exposure to the conditioning environment and CS. No differences were detected between genotypes when fear memory was measured 24 and 48 h after conditioning: in particular, both DAwt and DPP4mut rats displayed significantly increased contextual (F(1,24) = 8.841, p = 0.0008), and tone-elicited (F(1,16) = 16.11, p = 0.001) freezing behavior, as compared to non-shocked controls (Fig. 4B and C).

Extinction learning was investigated by exposing half of the conditioned animals to repeated, non-reinforced, presentations of the CS in context B. High levels of freezing were measured also in the absence of CS, indicating a certain level of generalized fear to the context and, possibly, handling procedures. This generalized fear decreased during consecutive days of extinction training (from day 2 to 7: F(6,80) = 17.49, p < 0.0001; Supplementary Fig. 2A). Likewise, fear to the conditioned tone significantly decreased throughout the extinction training sessions (from day 3 to 7: F(6,64) = 11.94, p < 0.0001; Supplementary Fig. 2B). No significant differences between genotypes were displayed.

Supplementary Fig. 2 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.psyneuen.2015.01.007.

Conversely, within-session response to the repeated presentation of non-reinforced CS differed significantly between genotypes. In particular, DPP4mut rats displayed an overall diminished freezing behavior as compared to DAwt (F(1,16) = 6.679, p = 0.02) across time (Fig. 4F). This phenotype was stronger on the last day of extinction training, but was also observed on extinction days 3 and 4.

Finally, freezing behavior was measured 24 h after the last extinction session, in order to evaluate the extent of fear extinction achieved (Fig. 4D and E). Overall, animals that received extinction training exhibited significantly reduced freezing (F(1,29) = 27.91, p < 0.0001). A trend toward a genotype difference (F(1,29) = 3.712, p = 0.064) and a significant interaction with the factor "extinction training"
Figure 3  Stress-induced analgesia and NPY concentration in DPP4mut rats. Adult male rats were tested in the non-habituated version of the test, and latency to respond to heat was measured (A). NPY concentration was analyzed in the CSF (B) of the same animals. A negative correlation between the two values was revealed (C), indicating a putative role of NPY in mediating the blunted response to mildly stressful manipulations. Pharmacological inhibition of DPP4 activity in DAwt rats for three weeks also produced a significant decrease in the latency to respond to heat, as measured by the SIA test (D). Light and dark bars represent DAwt and DPP4mut animals, respectively. * p < 0.05; *** p < 0.001.

(F₁,₂⁹) = 8.163, p = 0.0078) were also revealed. Indeed, DPP4mut rats that underwent extinction training showed significantly reduced freezing as compared to DAwt (30.1% vs. 62.96%, p = 0.0027). Generalized fear to context B was also reduced in rats trained for fear extinction, independently of their genotype (F₁,₂⁹ = 29.86, p < 0.0001). c-fos mRNA expression was measured in brain specimens isolated from all experimental animals, as a biological correlate of CS-induced neuronal activation in DAwt and DPP4mut rats (Fig. 4G). A trend toward a genotype difference was revealed by two-way ANOVA (F₁,₂⁷ = 4.069, p = 0.0537), and an increased expression of c-fos was measured in DPP4mut rats naive to extinction training as compared to DAwt subjects (p = 0.05, Sidak’s post-hoc test).

4. Discussion

The “moonlighting” properties of DPP4 make this enzyme a relevant target in rather different clinical fields. Pharmacological inhibition of DPP4 activity results in prolonged half-life of several peptides, and such mechanism is currently in clinical practice for the treatment of diabetic patients. In the present work we show how congenic functional deficiency for DPP4 results in a stress-resilient phenotype, along with increased levels of NPY in the CSF. Like in our previous reports (Karl et al., 2003c), the phenotype of reduced SIA in DPP4mut rats was specifically replicated only under non-habituated conditions of the hot-plate-testing procedure (Frerker et al., 2009), thus ruling out the possibility of altered pain perception as a confounding factor in the behavioral test.

A number of publications have explored the anxiolytic properties of NPY. In particular, intra-cerebral application of the peptide is reported to improve the performance of rats in the elevated plus-maze (Heilig et al., 1989) and in the social interaction test of anxiety (10 pmol, intra-amygdala) (Sajdyk et al., 1999), as well as to effectively reverse a fear-potentiated acoustic startle paradigm (Broqua et al., 1995). Improved stress coping has already been reported for DPP4mut congenic rats, which display, among others, reduced stress-induced hyperthermia, plasmatic ACTH and corticosterone levels, as well as increased social interaction in a novel environment and increased exploration of the open arms in the elevated plus maze (Frerker et al., 2009).

Central expression of NPY was reported to increase in response to repeated exposure to a stressor (e.g. physical restraint), along with habituation to the stressor itself (Thorssell et al., 1999), thus suggesting a role for NPY in the habituation to stress eliciting stimuli.

Here we report that central NPY concentration is increased in animals lacking DPP4 activity, as compared to wt controls. Although NPY could be a substrate for the
Figure 4  Fear conditioning and extinction. A schematic representation of the experimental schedule is depicted in panel A. Conditioning to the context (B) and the tone (C) was successfully achieved in both DAwt and DPPmut rats. Contextual ("generalized", D) and cued (E) fear was measured again 8 days after conditioning, 24h after completion of the extinction protocol. Within-session extinction of fear is reported for the last day of extinction training (F); data from the initial habituation phase (30s bins) and of the first three CS presentations are shown for DAwt and DPPmut rats. c-fos mRNA expression was analyzed by RT-qPCR in the amygdala of animals sacrificed 30–45 min after exposure to the conditioning stimulus (G). Data were normalized to the respective observation interval and expressed as mean ± SEM. Light and dark bars represent DAwt and DPPmut animals, respectively. Non-shocked controls are indicated with gray-dashed bars. Results of the one- and two-way ANOVA for the factors "time", "genotype" and "extinction training" are described in Section 3. *p < 0.05; **p < 0.01; ***p < 0.001.

action of other dipeptidyl-peptidases, our results support the hypothesis that DPP4 is physiologically responsible for the inactivation and possibly further degradation of NPY. Recent observations from our working group demonstrate that the N-terminal cleavage operated by DPP4 is the first and major proteolytic event leading to further degradation of NPY, within the CNS (Wagner et al., unpublished data). Based on our experimental evidence we propose a
mechanism of NPY accumulation in the CSF of DPP4mut rats. In particular, our data indicate that neither the upregulation of gene expression, nor increased number of NPY-ergic neurons, at least in the BLA, are likely to be responsible for this upregulation. We suggest that inactivation of DPP4 activity at sites of physiological barrier between the periphery and the CNS could be responsible, in DPP4mut rats, for an increased shuttling, and prolonged half-life of NPY in the CSF. The reduced expression of Y1, Y2 and Y5 receptors measured in the amygdala of DPP4mut rats further supports our observation, possibly indicating a negative feedback mechanism responsive, in congenic deficient rats, to the increased availability of central NPY. The exact length of the peptide measured in our study is unfortunately unknown, since the highly standardized and sensitive kit used in our study, like similar ELISA and RIA assays used for the detection of NPY, is insensitive to the N-terminal truncation operated by DPP4.

Overall, we propose that the increased accumulation of NPY in the CSF of DPP4mut rats buffers the endogenous mechanisms responsive to environmental stress, resulting in an increased threshold to react to external cues, similarly to what is induced by habituation mechanisms. Although not specifically addressed in our investigation, clear evidence for a substance-P mediated effect on pain perception per se was not revealed in our experiments, as indicated by the similar latency to react to heat under habituated conditions (Fig. 2A) and in the tail-flick paradigm (Supplementary Fig. 1A).

The effect of acute central application of NPY in the context of fear behavior has been pharmacologically and behaviorally dissected (Fendt et al., 2009; Gutman et al., 2008), demonstrating how intra-amygdala and intra-ventricular infusion of NPY decreases fear expression and/or fear extinction, depending on the experimental conditions. Furthermore, fear extinction was found significantly impaired in Y1 ko mice (Verma et al., 2012), but not in Y2 ko animals, therefore suggesting a specific role for Y1 signaling in facilitating the fear extinction process.

These findings, along with the upregulation of NPY levels in the CSF of DPP4mut rats, motivated us to investigate fear behavior in DPP4mut rats. We applied a standard cued fear conditioning procedure (Plendl and Wotjak, 2010) followed by extinction training. Fear sensitization is an important process associated with the development of PTSD, and fear conditioning can be used to model some of the symptoms associated with this disorder (Siegmund and Wotjak, 2006). Moreover, fear extinction in laboratory animals is meant to model exposure therapy in humans, a cornerstone of behavioral intervention for anxiety disorders such PTSD and phobias (Bisson et al., 2007). Our results indicate that fear conditioning and expression is intact in DPP4mut rats, thus ruling out impairment of fear-memory, in this animal model. Additionally, all animals included in the study reacted similarly to the foot-shock used during the conditioning session, displaying a quick burst of activity (jump/avoidance) followed by protracted freezing.

Interestingly, DPP4mut rats showed significantly lower levels of cued fear 24 h following extinction training, suggesting a role for NPY in facilitating extinction learning. Moreover, c-fos expression was increased in the amygdala of DPP4mut rats that did not receive extinction training as compared to all other experimental groups. c-fos expression is typically associated with neuronal activity and memory formation/remodeling (Hall et al., 2001). Because all animals were sacrificed after re-exposure to the CS, our results may indicate specific reactivation and/or modification of the fear memory in DPP4mut rats, under such experimental conditions, associated with the faster/more efficient extinction of the fear behavior. Since a non-reinforced tone presentation can render several different processes, including extinction learning and stimulus habituation (Kamprath et al., 2006), further studies are necessary in order to disentangle the exact mechanisms of DPP4 and NPY involvement in modulating this molecular mechanism.

NPY is able to inhibit excitatory transmission in the BLA (Giesbrecht et al., 2010), and NPY agonists inhibit glutatione release in the hippocampus (Colmers et al., 1987; Qian et al., 1997). Acute infusion of 10 pmol of NPY in the BLA inhibits fear-expression in a model of fear-potentiated startle (Gutman et al., 2008), and i.c.v. injection of 10 μg NPY enhances within-session extinction as well as extinction retention, in the same experimental setting. Although far from the physiological range, these results indicate how an increased NPY-ergic tone can modulate fear behavior, most likely by inhibiting the excitatory output from those brain areas responsible for the expression of fear.

In this context, our data suggest that a modest, physiological increase of central NPY produces a biologically relevant effect. In particular, DPP4mut rats display a coherent behavioral shift toward increased stress tolerance. Such phenotype extends from baseline responsiveness to mild stressors (e.g. the non-habituated handling involved during testing for SIA) to modulation of a learned fear-conditioned response (i.e. fear extinction). The improved extinction acquisition and inhibition of fear expression in DPP4mut rats further suggests a fear-modulating role for NPY. Interestingly, fear expression was found reduced shortly after an intensive fear conditioning protocol, consisting of 100 presentations of a tone-shock pairing over 10 days of training (Pickens et al., 2009), and, although not measured, it is reasonable to expect that increased (central) NPY expression/release would be associated with this phenomenon. Under such experimental setting, the authors also reported that i.c.v. injection of NPY was able to inhibit fear expression also one month later, when the effect of the ‘tolerance-like’ phenomenon had vanished in the vehicle treated animals. These findings, along with great part of the published literature, suggest a pivotal function for NPY in determining the subjective perception of environmental inputs and integration into a coherent stress-responsive behavior. Adaptation to chronic stress was previously found associated with increased expression of central NPY (Thorsell et al., 1999), and here we report a rat model displaying blunted response to stressful manipulations where central NPY is constitutionally elevated. Lack of differences in peripheral NPY concentrations in DPP4mut rats, under our experimental conditions, could be due to a number of reasons. Firstly, platelets-enriched plasma was used for all quantifications in our study, and since platelets are an important source of NPY, in the rat (Kuo et al., 2007a), possible differences in circulating plasma may have been masked in our analysis. Preliminary investigations carried out in platelets-deprived plasma seemed, nonetheless, to rule out such scenario (not shown). Secondly, NPY is rather abundant in the plasma,
as compared to the CSF, and a number of proteases capable to degrade the peptide are present in the blood stream (Wagner et al., unpublished data); it is therefore conceivable that, at least in the rat, homeostatic mechanisms are responsible for a rapid turnover of newly synthesized circulating NPY, thus resulting in no apparent differences in our study. Further investigations targeting a selective sampling at specific time-points before and after exposure to different stressors (e.g. restraint, repeated presentation of inescapable foot-shock) may reveal differences in the adaptive release of the peptide, in the periphery.

Involvement of the NPY-ergic system in the modulation and adaptation to a stressful, or so perceived, environment represents an interesting venue to explore, specifically in the context of clinical conditions such as anxiety, post-traumatic stress disorder (PTSD) and depression. Decreased NPY concentration was measured in the CSF of post-combat veterans affected from PTSD as compared to healthy subjects (Sah et al., 2009), as well as in patients with treatment-refractory unipolar major depression (Hellig et al., 2004). Conversely, increased NPY levels were detected in combat-exposed veterans not affected by PTSD as compared to PTSD+ patients (Yehuda et al., 2006), and the same study reported that recovery from previous PTSD was also associated with increased plasma NPY, as compared to exposed veterans that did not develop PTSD. Exposure to chronic stress or traumatic experiences results in lower plasmaic NPY in both rats (Corder et al., 1992) and humans (Morgan et al., 2003). On the other hand, acute stress induces a rapid increase of circulating NPY (Morgan et al., 2002), along with cortisol, norepinephrine (NE) and signs of subjective distress. In a previous study Morgan and colleagues had shown that increase of plasmaic NPY following acute exposure to psychological stress (i.e. captivity experience-like interrogation) was higher in Special Forces (SF) soldiers, as compared to non-SF soldiers (Morgan et al., 2000). Additionally, decreased NPY was measured in non-SF subjects 24h after interrogation as compared to pre-stress baseline values, and this phenomenon was abolished in SF soldiers. NPY inhibits the release of NE from the sympathetic nerves, via presynaptic Y2 receptors, while potentiating the effects of NE postsynaptically, via Y1 receptors (Colmers and Bleakman, 1994), and catecholamine and cortisol levels were found altered in some studies on individuals with PTSD (for a review see Pitman et al., 2012).

DPP4 expression and activity were not investigated in the studies mentioned above, thus leaving unanswered the question whether such clinical conditions are associated with alterations in the functionality of this enzyme. Conversely, a few publications have described polymorphisms of the NPY gene sequence associated with altered expression of the peptide and stress-responsive phenotype, in humans (Pickens et al., 2009; Zhou et al., 2008). To our knowledge, no clinical or preclinical investigation has yet attempted to modulate NPY activity/overflow by inhibition of NPY-degrading enzymes. Our study indicates the existence of a physiological relationship between DPP4 activity in the periphery and central accumulation of NPY, and how abolishment of DPP4 function produces an anxiolytic phenotype. More pre-clinical and clinical observations are necessary in order to extend our knowledge on the role of NPY as a functional peptide involved in inter-individual variation of resiliency to stress. Our data underpin the further exploration of pharmacological inhibition of DPP4 activity as a tool to modulate NPY inactivation/degradation, in the context of diseases, such as PTSD, where fear sensitization represents a clinically relevant process associated with a large part of the symptoms lamented by the patients, such as hyper-arousal, irritability and increased startle response. To this regard, recent work by Serova et al. (2013, 2014) describes the positive effect of intranasal application of synthetic NPY on reducing the development of PTSD-like symptoms in a rat model of exposure to prolonged stress. NPY modulation, through inhibition of DPP4 activity, may therefore represent an interesting option for the enhancement of classical exposure therapy (Bradley et al., 2005; Choy et al., 2007) by strengthening the endogenous system naturally in charge of assessing and modulating stress responses. Such therapeutic venue will require careful evaluation, since several and possibly serious side effects can be associated with the inhibition of such pleiotropic enzyme (Azim et al., 2014; Forssmann et al., 2008; Stephan et al., 2013). For this reason, preclinical models are highly valuable for the assessment of effectiveness, specificity and safety of systemic vs. topical/targeted DPP4 inhibition.

Role of the funding sources

The present work was partially supported by the ELAN Fund (ET-11.09.06.1 to F.C.). The funding source had no role in designing the study, collecting and analyzing the data.

Conflicts of interest

The authors have no conflicts of interest or financial disclosures to declare.

Acknowledgments

The present work was partially supported by the ELAN fund (ET-11.09.06.1 to F.C.). The authors would like to thank Ms. G. Minakaki and Ms. A.C. Plank for helpful discussion and revision of the manuscript.

References


