1) TITLE PAGE

Title: Pathology and glia type specific changes of the DPP4 activity in the spinal cord contributes to the development and maintenance of hyperalgesia and shapes opioid signalling in chronic pain states.

Contribution of glial cells to the development and maintenance of hyperalgesia in inflammatory and neuropathic pain states through dipeptidyl peptidase 4 activity in rats

Authors: Kornél Király[1†], Márk Kozsurek[2†], Erika Lukácsi[2], Benjamin Barta[2], Alán Alpár[2] Tamás Balázsa[2], Csaba Fekete[3], Szabon Judit[3], Zsuzsanna Helyes[4,5], Kata Bölcskei[4], Valéria Tékus[4] , Zsuzsanna E. Tóth[2], Károly Pap[6], Gábor Gerber[2], Zita Puskár[2*]

† Kornél Király and Márk Kozsurek contributed equally to the paper.

Affiliations:
[1] Department of Pharmacology and Pharmacotherapy, Semmelweis University, H-1089 Budapest, Hungary
[2] Department of Anatomy, Histology and Embryology, Semmelweis University, H-1094 Budapest, Hungary
[3] "Lendület" Laboratory of Integrative Neurobiology, Institute of Experimental Medicine of the Hungarian Academy of Sciences, H-1083 Budapest, Hungary
[4] Department of Pharmacology and Pharmacotherapy, Medical School & Szentágothai Research Centre, University of Pécs, H-7624 Pécs, Hungary
[5] MTA-PTE NAP B Chronic Pain Research Group, University of Pécs, H-7624 Pécs, Hungary
[6] Department of Traumatology, Semmelweis University, H-1113 Budapest, Hungary & Department of Orthopaedics and Traumatology, Uzsoki Hospital, H-1145 Budapest, Hungary

E-mails:
kiraly.kornel@med.semmelweis-univ.hu, kozsurek.mark@med.semmelweis-univ.hu, lukacsi.erika@med.semmelweis-univ.hu, bartabenji@gmail.com, alpar.alan@med.semmelweis-univ.hu, tbalazsa@gmail.com, feketecs@koki.hu, juditszabon@gmail.com, zsuzsanna.helyes@aok.pte.hu, kata.bolcskei@aok.pte.hu, valeria.teku@aok.pte.hu, toth.zsuzsanna.emese@med.semmelweis-univ.hu, drpapster@gmail.com, gerber.gabor@med.semmelweis-univ.hu, puskar.zta@med.semmelweis-univ.hu

Running title: Role of DPP4 in spinal pain sensation.

Number of words: Abstract: **249**
Text: **5853**
Introduction: **569**
Materials and Methods: **3049**
Results: **967**
Discussion: **1266**
Acknowledgements: **85**
Bibliography: **1660**
Figure legends: **755**
Total word count: **8434**

Number of figures: 6
Number of tables: 1

*** Corresponding author**: Zita Puskár
Szentágothai János Laboratory
Department of Anatomy, Histology and Embryology
Semmelweis University
H-1094 Budapest, Tuzolto utca 58., Hungary
puskar.zita@med.semmelweis-univ.hu

Main points: Inhibition of glial DPP4 may provide a novel approach to pain management by induction or modulation of endogenous opioidergic systems.

Key words: nociception, spinal cord, astrocyte, microglia, opioids

2) ABSTRACT

Altered pain sensations such as hyperalgesia and allodynia are characteristic features of various chronic pain states, and remain difficult to treat. We have shown previously that spinal application of dipeptidyl peptidase 4 (DPP4) enzyme inhibitors results in a strong antihyperalgesic effect during inflammatory pain states. In this study we observed a low level of mRNA for DPP4 in the rat spinal dorsal horn in physiological conditions, which did not change significantly either in carrageenan-induced inflammatory or partial nerve ligation-generated neuropathic states. Although DPP4 protein was detected in neurons, astrocytes and microglia in naïve animals its expression significantly increased in astrocytes during inflammation and in microglia in neuropathic conditions. Intrathecal application of two DPP4 inhibitors the tripeptide isoleucin-prolin-isoleucin (IPI) and the antidiabetic drug vildagliptin resulted in robust opioid-dependent antihyperalgesic effect during inflammation and an opioid-independent effect in the Seltzer model. The opioid-mediated antihyperalgesic effect of IPI was exclusively related to mu-opioid receptors, while vildagliptin affected mainly delta-receptor activity, although mu- and kappa-receptors were also involved.

Our results suggest a pathology and glia type specific changes of the DPP4 activity in the spinal cord which contributes to the development and maintenance of hyperalgesia and shapes opioid signalling.

3) TEXT

Introduction

Inflammation, nerve injury or ongoing diseases induce peripheral sensitization of primary sensory afferents and central sensitization of spinal neurons, resulting in hyperalgesia (increased response to noxious stimuli), allodynia (painful response to normally innocuous stimuli) and spontaneous pain (Sandkuhler 2009). Direct and specific interactions between primary afferents and spinal lamina I projection neurons are necessary for development of hyperalgesia (Khasabov et al. 2002; Nichols et al. 1999) and this involves Ca^{2+}-dependent long term potentiation (LTP) in projection neurons via activation of NMDA and neurokinin-1 receptors (NK1R) following continuous release of glutamate and substance P (SP) from peptidergic C primary afferents (Sandkuhler and Gruber-Schoffnegger 2012).

C fiber-related mu-opioid receptor (MOR) activation results in acute and reversible synaptic depression through inhibition of voltage-gated calcium channels and can reverse various forms of LTP, however prolonged application of MOR agonists and opioid withdrawal also leads to LTP and hyperalgesia (Heinke et al. 2011). Morphine hyperalgesia is mediated by microglia (Clark and Malcangio 2012; Ferrini and De Koninck 2013; Ji et al. 2013) and involves functional MORs (**Laurie-Anne Roeckel et al 2017, Scientific Reports)** Pronociceptive cytokines, such as interleukin-1β (IL-Iβ) and tumour necrosis factor (TNF), released from the microglia can affect the spinal synaptic transmission and contribution of microglia to LTP has also been demonstrated (Clark et al. 2015; Gruber-Schoffnegger et al. 2013; Zhong et al. 2010).

DPP4 is an integral membrane glycoprotein expressed on many cell types, but appears also in soluble form in body fluids including cerebrospinal fluid (Boonacker and Van Noorden 2003). As a serine protease, DPP4 cleaves dipeptides from oligopeptides and proteins containing proline/alanine in the penultimate position. DPP4 processes neuropeptides, hormones, cytokines and chemokines leading to their biological activation or inactivation. Potential substrates include bradykinin, glucagon-like peptide-1 (GLP-1) and -2, SP, neuropeptide Y (NPY), vasoactive intestinal polypeptide (VIP) and TNF-α (Mentlein 1999; Sakurada et al. 1999; Turner et al. 1987). DPP4 is also known as cell surface antigen CD26 on T-lymphocytes (Gorrell et al. 1991; Yu et al. 2011) and receptor for Coronaviruses (Lu et al. 2013). Although only one type of DPP4 mRNA is detected (Hong et al. 1989), molecular heterogeneity of the enzyme can result from posttranslational modifications (Kahne et al. 1996).

DPP4 mRNA was detected in the brain of adult animals and its level did not change after cerebral ischemia. In contrast, DPP4 immunoreactivity was not found in the same regions in physiological state but appeared in neurons and glial cells during cerebral ischaemia (Rohnert et al. 2012). We demonstrated dramatic reduction of mechanical hyperalgesia following spinal application of DPP4 inhibitors in subacute inflammation and this action was naloxone reversible suggesting an opioid receptor-mediated effect. None of the inhibitors affected allodynia in inflammatory conditions or changed the nociceptive threshold in acute nociceptive tail-flick

test (Kiraly et al. 2009). Antihyperalgesic and anti-inflammatory effects of DPP4 inhibitors were also showed in chronic inflammatory models in mice (Ujhelyi et al. 2014).

Machinery of the endogenous opioid system has been intensely investigated and clarified in recent decades. Although inducing/regulating the endogenous opioid machinery would provide a powerful tool to control pain propagation this possibility has remained largely unexploited. Here, we identify DPP4 in the spinal dorsal horn, show that its expression changes during pathological conditions, and demonstrate that it shapes opioid signalling in a receptor- and treatment-specific manner. Although synaptic DPP4 may have a key role in neuronal mechanisms of pain propagation, we identify glial cells as inducible DPP4-batteries, in this way playing a role in hyperalgesia and opioid signalling.

Materials and Methods

Animals

Nociceptive threshold measurements were carried out on male Wistar rats weighing 170–230 g and received from the breeding colony of the Semmelweis University that were used for carrageenan induced hyperalgesia model, or on animals weighing 100–160 g at the start of the partial sciatic nerve ligation experiments (Seltzer model) and obtained from Charles River Laboratories via Innovo Ltd. (Gödöllő, Hungary) or Toxi-Coop Ltd. (Budapest, Hungary). These later groups were bred and kept at the Laboratory Animal Centre of the University of Pécs. Rats were housed under similar conditions both at Semmelweis University and University of Pécs including temperature-controlled rooms with 12 h light / 12 h dark cycles and standard rodent chow and tap water supplied *ad libitum.*

Experiments were performed in accordance with the European Communities Council Directive 86/609/ECC and were approved by the Committees on Animal Experiments of the Semmelweis University, Budapest (XIV-I-001/2265-4/2012) and the Medical School of the University of Pécs (BA02/2000-9/2011) Hungary.

Drugs

Diprotin A (isoleucin-prolin-isoleucin, IPI; Sigma-Aldrich, I9759) stock solution was made up in 25% (w/v) hydroxypropyl-beta-cyclodextrin (HPβCD, Sigma-Aldrich, H107) and dilutions were made with sterile saline and administered intrathecally (i.t.) in 30 nmol/rat dose. vildagliptin (VIL) was received from Prof. Ingrid De Meester of the Laboratory of Medical Biochemistry, University of Antwerp, Wilrijk, Belgium and was administered i.t. in 3 nmol/rat dose. Naltrexone hydrochloride (NTX) was a generous gift from DuPont Pharmaceuticals (Geneva, Switzerland), and was injected subcutaneously (s.c.) in 0.5mg/g b.w. dissolved in saline.

The MOR antagonist D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2 (CTAP; Sigma-Aldrich, C6352; 200 pmol/rat), delta-opioid receptor (DOR) -antagonist H-Tyr-Tic(CH_2NH)-Phe-Phe-OH (TIPP[Ψ]; Sigma-Aldrich, T7075; 1 nmol/rat) and kappa-opioid receptor (KOR) -antagonist 5'-guanidinonaltrindole (gNTI; Sigma-Aldrich, G3416; 10 nmol/rat) were dissolved in distilled water.

I.t. injections were delivered in 5µl volume by a 250µl Hamilton syringe set into a Hamilton dispenser. The 23-Ga needle was introduced at the L5-6 intervertertebral space (Kiraly et al. 2011; Kiraly et al. 2009).

Behavioural experiments

Carrageenan-induced subacute inflammation and hyperalgesia. Hyperalgesia was induced by intraplantar injection of 100µl l% λ-carrageenan dissolved in saline (Sigma-Aldrich, 22049) into the right hindpaw. Nociceptive threshold to pressure was determined by the Randall-Selitto method (Kiraly et al. 2011; Kiraly et al. 2009; Randall and Selitto 1957) using a type 37215 Analgesy-Meter (Ugo Basile, Comerio, Italy). Rats were lightly restrained and an evenly increasing force was applied onto their paws inserted between the cone-shaped clamps of the apparatus. At the moment of paw withdrawal, the actual force was recorded as the nociceptive threshold. The baseline nociceptive threshold was initially determined on both hindpaws (at -5 min), then carrageenan was injected into the right hindpaw (at 0 min). The nociceptive threshold was measured again at 180 min and i.t. injection of vehicle, DPP4 inhibitors alone (IPI or VIL) or in combination with subtype specific opioid receptor antagonists (CTAP, TIPP[Ψ] or gNTI), was performed. Nociceptive threshold readings were repeated at 185, 195, 210 and 240 minutes. Five rats were involved in each vehicle experiment, while for drug and drug combinations, 7–10 animals were used.

Time-matching data sets on different ipsilateral curves were compared with two-way repeated measures ANOVA followed by Bonferroni *post hoc* test. Percentage maximum possible antihyperalgesic effects were calculated according to the following equation: MPE (%) = 100 x (ipsilateral threshold 30 min after i.t. drug application – hyperalgesic baseline) / (contralateral threshold at the same time – hyperalgesic baseline), where hyperalgesic baseline was defined as the nociceptive threshold of the inflamed hindpaw 180 min after intraplantar carrageenan injection, then comparisons were made with one-way ANOVA followed by Dunnett's *post hoc* test (Kiraly et al. 2011; Kiraly et al. 2009).

Partial sciatic nerve ligation-induced chronic neuropathic pain model (Seltzer model). Baseline nociceptive thresholds were determined on two consecutive days using three different methods: dynamic plantar aestesiometry (DPA, mechanical allodynia), noxious cold stimulation (cold allodynia) and Randall-Selitto test (mechanical hyperalgesia). Under deep pentobarbital anesthesia (50 mg/kg i.p. Euthasol, Produlab Pharma, Raamsdonksveer, Netherlands), the sciatic nerves of rats (n=100) were tightly ligated high in the thigh unilaterally

using a braided silk suture (Mersilk 6-0, Ethicone) so that approximately 1/3–1/2 of the diameter of the nerve was trapped in the ligature (Seltzer et al. 1990). The wound was closed afterwards with 4-0 silk sutures and the animals were allowed to recover for one week.

On the 7th postoperative day, nociceptive threshold measurements were repeated for each animal at short intervals and percentage hyperalgesia/allodynia values were calculated for the nerve-injured paws with the following formula: hyperalgesia/allodynia (%) = 100 x (preoperative – postoperative values) / (preoperative values). Only animals that developed a minimum of 20% decrease of threshold with each method were included in treatment groups (n=71). Rats were arranged into groups having similar degree of hyperalgesia/allodynia and received (1) i.t. vehicle or (2) DPP4 inhibitor or (3) DPP4 inhibitor 15 min after s.c. NTX pretreatment. For i.t. vehicle and DPP4 inhibitor experiments, 8-10 animals were used in each group, while groups undergoing i.t. DPP4 inhibitor application following s.c. NTX pretreatment consisted of 5-8 rats. Nociceptive measurements from each animal were carried out 20-30 min after i.t. injection starting with DPA at 20 min, followed by Randall-Selitto test at 25 min and finishing with noxious cold stimulation at 30 min. During dynamic plantar aesthesiometry rats were placed into an observation chamber positioned on a metal mesh surface. The touch stimulator unit was placed under the animal's paw and increasing upward force (10 g/s) was exerted until the rat removed its paw. Withdrawal thresholds were measured 3 times in turns for each hindpaw and the mean values were used for statistical analysis. If no withdrawal occurred, the preset maximum (50 g) was used in the evaluation. Randall-Selitto test was performed as detailed above. To measure noxious cold sensitivity, hindpaws of lightly restrained rats were immersed into a 0°C water bath and the latency to paw withdrawal was recorded. The cut-off time was set to 180 seconds.

Withdrawal thresholds recorded before nerve ligation, then on the 7th postoperative day before and after drug applications were compared with two-way repeated measures ANOVA followed by Bonferroni *post hoc* test. Percent maximum possible effects were calculated according to the following formula: MPE (%) = 100 x (withdrawal threshold after drug application – withdrawal threshold before nerve ligation) / (withdrawal threshold before drug application – withdrawal threshold before nerve ligation), then comparisons were made with one-way ANOVA followed by Dunnett's *post hoc* test.

RNA isolation and real-time PCR analyses

Carrageenan was injected into both hindpaws of 6 rats and partial ligation of both sciatic nerves was performed in 9 animals. Survival time was 3 hours and 7 days, respectively. Development of inflammatory or neuropathic hyperalgesia was confirmed by the Randall-Selitto test, and then rats were sacrificed by decapitation, with a further 6 animals used as controls. L4-L6 spinal segments were removed and frozen on dry ice. RNA was isolated using the RNeasy Lipid Tissue Mini Kit (QIAGEN) from spinal cord samples according to the manufacturer's instructions. The purity and concentration of the RNA were analyzed using a SmartSpec Plus

spectrophotometer (Bio-Rad, UK). Reverse transcription was performed with 1 μg of RNA to convert the total RNA to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems by Life Technologies). Concentration of the generated cDNA was determined using the Qubit 2.0 Fluorometer with the Qubit ssDNA Assay Kit (Life Technologies). Expression of DPP4 mRNA was measured by real-time quantitative TaqMan RT-PCR reaction with a ViiA 7 Real-Time PCR System (Life Technologies), using commercially available TaqMan probe (Rn00562910_n1) on 10-ng cDNA template in duplicates. Glyceraldehyde-3-phosphate dehydrogenase (Rn99999916_s1) was used as a housekeeping gene, and its expression did not vary between the experimental groups.

For statistical analyses, qPCR data were expressed as relative quantification values (RQ; mean ± SEM) and compared between groups by one-way ANOVA.

In situ hybridisation

The 1632-2051 bp long fragment of the rat DPP4 cDNA (gene bank accession #NM_012789) was purchased from Blue Heron Biotechnology Inc. (Bothell, WA, USA), subcloned into pBC KS+ **(**Addgene, Cambridge, MA, USA) vector, and verified by sequencing. *In situ* hybridisation (ISH) was performed as described earlier (Toth et al. 2008). Riboprobes in sense and antisense directions were prepared by *in vitro* transcription (MAXIscriptKit, Life Technologies, Carlsbad, CA, USA) and labelled using [^{35}S]UTP-(Per-Form Hungaria Kft, Budapest, Hungary). Carrageenan was injected into both hindpaws of 3 rats and partial ligation of both sciatic nerves was performed in 3 animals. Survival time was 3 hours and 7 days, respectively. Development of inflammatory or neuropathic hyperalgesia was confirmed by the Randall-Selitto test, and then rats were sacrificed by decapitation, with a further 3 animals used as controls. The spinal dorsal horn of L4-L6 segments were removed and frozen on dry ice. Serial coronal sections were cut in a cryostat and mounted onto positively charged Superfrost Plus slides (Life Technologies). Slides were hybridized overnight in humid chambers at 55°C with 10^6 cpm/slide of the radioactively labelled probes, washed and dehydrated. Slides were dipped into NTB nuclear track emulsion (Carestream Health Deutschland GmbH, Stuttgart, Germany) for 4 weeks. Emulsion-coated slides were developed using Kodak Dektol developer and Fixer (Sigma-Aldrich Kft, Budapest, Hungary). Sections were counter-stained with 0.5% Giemsa solution (Sigma), air dried and coverslipped using Depex mounting medium. Dark-field images of three samples per animal were captured by a BX51 Olympus microscope (Olympus Corporation, Hamburg, Germany) attached to a QICAM (Qimaging, Surrey, BC, Canada) camera. The grain density indicating the level of the DPP4 mRNA expression was calculated as the area percent occupied by the silver grains within a given region of interest (ROI; 100x100 pixel^2) using the Image J 1.32j program. In each section, 3 ROIs from the background (area outside of the tissue) and 5 ROIs from the dorsal horn were measured and averaged. Then the background was subtracted from the value that was obtained from the tissue. The sense

and antisense signals were compared using Student's t-tests with SigmaStat 3.5program (Systat Software, Inc. San Jose, CA, USA). One way ANOVA was used for comparing the antisense *in situ* hybridization signals among the groups.

Western blotting

Five rats with unilateral carrageenan-induced hindpaw inflammation, 9 rats undergoing unilateral partial nerve ligation one week earlier with further 4 naïve rats were tested with the Randall-Selitto method as it was described above. After confirming the obvious decrease of nociceptive thresholds, animals were sacrificed by decapitation, and the spinal dorsal horn of L4-L6 segments were removed and snap-frozen on dry ice. Samples were homogenized in TNE buffer containing 0.5% Triton X-100 (Sigma), 5 mM NaF, 100 μM Na_3VO_4 and a cocktail of protease inhibitors (CompleteTM, Roche) and briefly sonicated. Cell debris and nuclei were pelleted by centrifugation (800g, 30 min at 4°C). Protein concentrations were determined by Bradford's colorimetric method (Bradford 1976). Samples were diluted to a final protein concentration of 2 μg/μl, denatured in 5x Laemmli buffer, and analysed by SDS-PAGE on a 10% resolving gel. After transferring onto Immobilon-FL polyvinylidene difluoride membranes (Millipore), membrane-bound protein samples were blocked in 3% BSA and 0.5% Tween-20 diluted in TBS for 1.5h, and subsequently exposed to the goat DPP4 primary antibody **(Table 1.)** overnight at 4 °C. Signal detection was achieved by using a HRP-conjugated donkey anti-goat secondary antibody (Jackson; 1:10,000). Image acquisition and analysis were performed on a Bio-Rad XRS+ imaging platform.

Immunofluorescent labelling

Antibodies

Polyclonal goat DPP4 antibody was raised against the synthetic peptide C-PPHFDKSKKYP representing the internal region of DPP4 according to NP_001926.2 and labelled one band at approx. 110 kDa in rat lung lysate in Western blot experiments (for details see the supplier's datasheet). In our Western blot experiments rat lung and pancreas lysates were used as positive controls **(Fig. 1C)**. Monoclonal mouse DPP4 antibody was produced against the full length rat CD26 protein. To test the specificity of the two DPP4 antibodies double immunofluorescent staining was carried out (Lorincz and Nusser 2008). Both antibodies labelled the same profiles in neurons, astrocytes and microglia **(Fig. 2)**.

Mouse anti-neuron-specific nuclear protein (NeuN) was used for labelling neuronal somata (Todd et al. 1998) together with NeuroTrace 435/455 blue-fluorescent Nissl stain (Pap et al. 2015) (Thermo Fischer Scientific - Invitrogen, Cat#: N-21479; 1:200). Dendrites were identified with monoclonal mouse antibody produced in mice against microtubule-associated protein 2 (MAP2) (Attems et al. 2012; Binder et al. 1986). Antibody stained one single lane at 280 kDa in Western blot experiment using rat brain extract. Ionized calcium-binding adaptor molecule-1 (IBA1) was used as specific microglia/macrophage marker and anti-IBA1 antibody was isolated from

the serum of rabbits immunized with a synthetic peptide corresponding to C-terminus of IBA1. According to the provider antibody stains one single lane in Western blot experiments at around 17 kDa. Monoclonal mouse antibody against glial fibrillary acidic protein (GFAP) was used for specific labelling of astrocytes. Guinea pig antibody against vesicular glutamate transporter 2 (VGLUT2) was used to identify excitatory axon terminals. The antibody is raised against a 18 amino acid long sequence of the rat protein and Western blot analysis on rat brain lysate showed 52kDa lane as it is seen on the provider's datasheet related to Lot#: NG1866937. This VGLUT2 antibody is heavily used and well characterized (Ganley et al. 2015). Inhibitory boutons were labelled with polyclonal antibodies against vesicular GABA transporter (VGAT) raised in rabbits immunized by the synthetic peptide AEPPVEGDIHYQR (amino acids 75-87 in rat VGAT) coupled to key-hole limpet hemocyanin via an added N- terminal cysteine. Based on the supplier's data sheet this antibody is knock-out verified. Synaptophysin (SYN) was used as a synaptic marker and monoclonal mouse antibody was raised against synthetic peptide corresponding to a region near to the C-terminal end of the full peptide. SYN antibody validation by immunohistochemistry and Western blot is found in the Human Protein Atlas (http://www.proteinatlas.org). Calcitonin gene-related peptide (CGRP) antibody raised in guinea pig and used for labelling peptidergic unmyelinated primary afferents recognizes identical structures to those detected by well characterized rabbit and goat antibodies against rat α-CGRP (Kozsurek et al. 2007). For detailed specifications of antibodies see **Table 1.**

Secondary antibodies were all raised in donkey: Alexa Fluor (AF) -488 labelled anti-goat, AF-555 conjugated anti-mouse, AF-555 labelled anti-rabbit (all from Thermo Fischer Scientific-Invitrogen-Molecular Probes; 1:500) and Rhodamine Red X-anti guinea pig, Cyanine 5-anti mouse, Cyanine 5-anti rabbit (all from Jackson ImmunoResearch; 1:100).

General immunofluorescent staining protocol

Transcardial perfusion of deeply anesthetized (75 mg ketamine and 7.5 mg xylazine; i.m.) rats (5 naive, 5 unilateral carrageenan treated, and 5 unilateral nerve ligated as described above) was initiated with 4% (para)formaldehyde and completed with 4% (para)formaldehyde containing 10% sucrose. L4-L5 spinal segments were removed and immersed overnight into 20% sucrose dissolved in PBS. Segments were frozen with liquid nitrogen and then 50 μm thick sections were cut on a Vibratome. Endogenous peroxidase activity was blocked for 30 minutes with 1% hydrogen peroxide diluted in phosphate buffer (PB), then sections were transferred into phosphate buffered saline (PBS) with 5% normal horse serum (NHS). After the blocking procedure they were incubated overnight in the DPP4 antibodies then reacted with Alexa Fluor 488 labelled species specific secondary antibodies. Sections were incubated for 72 hours in mixtures of the other primary antibodies and were reacted overnight with fluorescently labelled species specific secondary antibodies. All the primary and secondary antibodies were dissolved in PBS. In some sets of experiments, sections were immersed into Neurotrace fluorescent Nissl dye (Invitrogen-Molecular Probes; 1:200 in 0.1M phosphate buffer) for half an hour before

mounting. After rinsing, sections were mounted in Vectashield (Vector Laboratories) and scanned on a confocal laser scanning system (Zeiss, LSM780).

Densitometry of DPP4 immunostaining

L4 and L5 spinal cord segments taken from 5 control, 5 carrageenan-treated and 5 nerve-ligated rats were used for quantitative analysis. Three to six spinal cord sections were taken from each segment on the basis of the gray matter shape and 5 to 8 confocal optical sections were scanned from each section. DPP4 staining was not viewed prior to selecting any sections, ROIs or cell profiles. The quantitative analysis was carried out by an independent observer, who was blind to the experimental conditions and was not involved in the scanning either.

To determine DPP4 immunoreactivity and its alteration under different circumstances fields containing the whole dorsal horn were scanned through a 20x lens of the confocal microscope to produce z-stacks with z separation of 1 μm. The scanning parameters were selected and optimized in sections from control animals and were used further in sections of treated rats. Single optical sections containing black and white images were selected from each z-series and analysed using the Image J program (Rasband WS, Image J, NIH, Bethesda, Maryland). The medial two third of the dorsal horn containing the first four laminae (the area receiving inputs from the sciatic nerve) was drawn in each section and used as region of interest (ROI). The threshold was adjusted and the density of the immunostaining was calculated as the area percentage occupied by the immunostained dots within a given ROI. Data were compared among groups by one-way ANOVA.

To analyse the density of the DPP4 immunoreactivity in individual cell types, non-overlapping fields of 135 μm x 135 μm within the medial two third of the spinal dorsal horn were scanned through a 63x oil immersion lens to generate z-stacks with a z-separation of 0.5 μm. The same optimized parameters were used for all types of sections. IBA1-, GFAP- and DPP4-immunolabellings (for glial cells) or DPP4 immunolabelling with fluorescent Nissl staining (neuronal cell bodies) were imaged in different colour channels sequentially. Outlines of microglia and astrocytes were determined automatically by using the AutoThreshold plugin of ImageJ in IBA1 and GFAP image channels, respectively. To exclude non-specific labelling of glial cells and nuclei by the fluorescent Nissl dye, contours of randomly selected neurons (8 neuron/field) were drawn manually. Then integrated density values from DPP4 image channel were measured in previously delineated glial or neuronal profiles to detect changes in protein expression due to different treatments. The density values were subjected to statistical analysis.

Statistical analysis

Statistical methods used are detailed at each experiment individually. Analyses were made with SigmaStat 3.5program (Systat Software, Inc. San Jose, CA, USA) and curves/bar graphs were created with the GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA, USA). In general, data were represented as mean±SEM when the population was normally distributed or as median with 25% and 75%, otherwise. In both cases, $p<0.05$ was considered as statistically significant.

Results

DPP4 transcripts in the rat spinal dorsal horn in physiological, inflammatory and neuropathic states

Taqman qPCR detected DPP4 mRNA in the dorsal horn of L5 spinal segments taken from control, inflamed and neuropathic rats. Neither carrageenan treatment nor neuropathic condition caused significant alteration in the DPP4 mRNA levels (control *vs.* carrageenan-induced inflammation *vs.* neuropathy (RQ): 1.0±0.2 *vs.* 0.7±0.1 *vs.* 1.3±0.3, one-way ANOVA P=0.301; **Fig. 1. A**).

DPP4 mRNA showed a low expression by *in situ* hybridization in the spinal dorsal horn of L4-L6 segments. While the grain density observed in sections hybridized using the sense probe was equal to the background, a significant signal was detected with the antisense probe (antisense: N=27, median=110.4, 25% at 80.7 and 75% at 135.5; sense: N=19, median=0, 25% at 0, 75% at 21.7; Mann-Whitney Rank Sum Test p<0,001). DPP4 mRNA was distributed evenly within the dorsal horn, and no significant difference among the experimental groups has been detected (control: N=9, 1.2±0.2; inflamed: N=9, 1.1±0.1; neuropathic: N=9, 1.0±0.1; one-way ANOVA, p=0,21; **Fig. 1. B**).

DPP4 protein expression in physiological condition and its changes during inflammation and neuropathy in the spinal dorsal horn

Western-blot analysis demonstrated an elevated protein level in the inflamed spinal cord being significantly different from those found in naïve and neuropathic spinal cords (control: N=4, 0.16±0.05; inflamed: N=5, 0.54±0.06; neuropathic: N=9, 0.31±0.06; one-way ANOVA, Holm-Sidak method, p=0,023; **Fig. 1. C, E**).

DPP4 immunoreactivity in the spinal cord appeared in naïve, inflamed and also in neuropathic animals. Immunostaining for the enzyme was weaker in the superficial laminae than in the deeper laminae in control and neuropathic conditions but increased significantly in the medial two third of the dorsal horn during inflammation (**Fig. 1 D**). Densitometry of the DPP4 immunolabelling showed a significant increase in inflamed spinal cord in those areas corresponding to the inflamed hind paw compared to naïve and neuropathic conditions (control: N=7, 3.42±0.38; inflamed: N=10, 5.66±0.80; neuropathic: N=8, 3.18±0.44; one-way ANOVA p=0,016; **Fig. 1. F**).

DPP4 immunoreactivity and its changes in individual cell types during pathology

Punctate-like immunostaining was detected in neuronal cell bodies (**Fig. 2. A**) and also in axon terminals in naive animals. Puncta representing DPP4 were embedded in synaptophysin stained elements suggesting close relationship between synaptic and DPP4 activity in many cases **(Fig. 3.)**. DPP4-immunopositive dots that appeared on MAP2 labelled dendrites were always associated with synaptophysin positivity **(Fig. 3. A)** suggesting that dendrites did not express the enzyme but received synapses from DPP4-containing boutons. The enzyme appeared both in vesicular glutamate transporter 2 (VGLUT2) immunolabelled excitatory **(Fig. 3. C)** and in vesicular GABA transporter (VGAT) positive inhibitory axon terminals **(Fig. 3. D)**, as well as in CGRP stained primary afferent boutons **(Fig. 3. B)**. DPP4 labelling also occurred in GFAP-positive astrocytes (**Fig. 2. B)** and IBA1-stained microglia cells **(Fig. 2. C)**. In naive rats, the density of the enzyme staining was the highest in astrocytes and differed significantly from that in other cell types. DPP4 density was also significantly higher in microglia than in neurons (astrocyte: N=109, median=18652.86, 25% at 8185.077, 75% at 31208.903; microglia: N=83, median=3196.484, 25% at 2338.702, 75% at 5189.725; neuron: N=96 median=343.95, 25% at 247.461, 75% at 420.691; Kruskal-Wallis one-way ANOVA on ranks, Dunn's method at $P<0.001$). During inflammation, DPP4 expression increased significantly in astocytes but not in microglia and neurons. Significant increase in the DPP4 immunoreactivity appeared only in microglia and significant decrease in neurons in the Seltzer model (astrocyte control: N=109, median=18652.86, 25% at 8185.077, 75% at 31208.903, infl.: N= 112, median=33379.275, 25% at 7651.471, 75% at 43072.64, Seltzer: N= 80, median=15583.715, 25% at 8802.536, 75% at 22288.04, Kruskal-Wallis one-way ANOVA on ranks, Dunn's method at $P<0.001$; microglia control: N=83, median=3196.484, 25% at 2338.702, 75% at 5189.725, infl.: N= 86, median=2996,707, 25% at 2446.597, 75% at 4359.576, Seltzer: N=67, median=4926.802, 25% at 3312.321, 75% at 6493.72, Kruskal-Wallis one-way ANOVA on ranks, Dunn's method $P<0.001$; neuron control: N=96, median=343.95, 25% at 247.461, 75% at 420.691, infl.: N=64, median=312.079, 25% at 203.529, 75% at 416.453, Seltzer: N=64 median=278.455, 25% at 213.089, 75% at 362.673, Kruskal-Wallis one-way ANOVA on ranks, Dunn's method at $P<0.001$, in all cases N means the number of the analysed optical sections of neuronal or glial elements). It should be noted that DPP4 immunolabelling existed not only in the membranes of the different cell types but also in intracellular compartments **(Fig. 4 .A, B, C)**.

Opioid receptor types involved in the antihyperalgesic effect of DPP4 inhibitors in inflammation

To challange the involved opioid receptor types, selective opioid receptor antagonists were applied spinally together with two different DPP4 inhibitors in carrageenan-induced subacute inflammation. I.t. application of 30 nmol/rat IPI and 3 nmol/rat vildagliptin eliminated 93.8±1.2% and 88.3±1.6% of mechanical hyperalgesia measured by the Randall-Selitto test in intraplantar carrageenan-induced inflammation. Co-administration of the mu-selective inhibitor CTAP reduced the antihyperalgesic effect of IPI to -5.0±4.5%, while

following co-application of kappa-receptor antagonist gNTI and the delta-opioid antagonist TIPP[Ψ] the antihyperalgesic effect of IPI remained at 92.2±2.4% and 90.1±3.0%, respectively. Following co-administration of mu- and kappa-antagonists, antihyperalgesic effect of vildagliptin was 51.0±4.4% and 45.8±4.2%, respectively, while the delta-antagonist TIPP[Ψ] completely blocked the antihyperalgesic effect of vildagliptin by reducing its antihyperalgesic effect to -1.4±2.1% **(Fig. 5.)**.

Effects of DPP4 inhibitors in neuropathy

Different modalities of hyperalgesia and allodynia appear not only in inflammatory conditions but also in neuropathic pain states. None of the tested DPP4 inhibitors had significant effect on mechanical and cold allodynia, while both i.t. IPI and Vildagliptin had an obvious mechanical antihyperalgesic effect measured with the Randall-Selitto test one week after partial sciatic nerve ligation with MPE values of 37.9±12.4% and 41.8±10.4%, respectively. In contrast to inflammatory states, NTX did not affect the antihyperalgesic action of the DPP4 inhibitors signifficantly in neuropathic conditions suggesting completely different actions of the enzyme on hyperalgesia in the two pain states. **(Fig. 6.)**.

Discussion

DPP4 expression in the spinal dorsal horn

A high level of DPP4 expression in the developing brain and spinal cord was described, which dramatically decreased in adults, remaining in leptomeningeal cells and capillary endothelial cells of the choroid plexus (Bernstein et al. 1987; Haninec and Grim 1990; Mitro and Lojda 1988). DPP4 mRNA was detected in cortical areas in adult naïve animals and its level did not change after cerebral ischemia. In contrast, DPP4 immunoreactivity was not found in the same regions in physiological state but its expression appeared in microglia, neurons and astrocytes in different time points during cerebral ischaemia (Rohnert et al. 2012). We are first to show that transcripts as well as the protein of DPP4 are detectable in the mammalian spinal dorsal horn. Similarly to ischemic injuries in the brain, our q-PCR and *in situ* hybridization analysis showed that DPP4 mRNA level remained similarly low in inflammation or neuropathy compared to physiological state. At the same time, a significant shift was experienced at the protein level in the investigated spinal cord, reflecting a five-fold increase in DPP4 protein expression and suggesting the posttranscriptional control of DPP4 expression during quickly developing inflammation and ischemia in neurons and glia. Our understanding of DPP4 molecular regulation is far from being complete. Studies on lymphocytes have demonstrated that while retinoic acid and interferon administration result in DPP4 transcription, IL12 upregulates DPP4 translation only and TNFα merely decrease

cell surface expression (Rohrborn et al. 2015; Zhong et al. 2015). Further studies are necessary to discover the molecular machinery that drives changes in DPP4 expression in the CNS during pathological states.

High resolution confocal laser scanning imaging revealed that neuronal DPP4 was typically confined to presynaptic, and also to somatic domains, with significantly decreased densities in neuropathy. In contrast, astrocytes were amply decorated with DPP4-immunoreactive profiles, with significantly increasing density in inflammatory but not neuropathic states. Finally, a third-party involvement in spinal neuropathic mechanisms was likely reflected by the increase of DPP4 expression in microglia which showed an obvious protein expression level in control animals, too.

Detectable levels of the DPP4 protein in healthy dorsal spinal cord, in contrast to brain tissue, could be due to the quick on-demand regulatory role of the moonlight protein in nociceptive processes where SP and NPY offer ample and typical substrates for DPP4. The presynaptic location of DPP4 in neurons suggests a possible role for this protein in synaptic physiology. On the other hand, glial expression of DPP4 which is significantly upregulated in a pathology and cell type specific manner opens further aspects to detect alternative roles of this protein in chronic pain states.

Antihyperalgesic effect of the DPP4 inhibitors

Previously, we have demonstrated that DPP4 inhibitors do not change the nociceptive threshold in acute nociceptive condition (Kiraly et al. 2009). In contrast to this, robust antihyperalgesic effects of different DPP4 inhibitors such as IPI, vildagliptin and sitagliptin in carrageenan-induced and CFA-provoked chronic inflammatory models have been reported previously (Kiraly et al. 2011; Ujhelyi et al. 2014). The antihyperalgesic action of IPI and vildagliptin appeared opioid-mediated since the general opioid receptor antagonist NTX reversed their effects (Kiraly et al., 2009; Kiraly et al., 2011). In this study, we examined the opioid receptors involved by using selective antagonists against MOR, DOR, and KOR and measuring mechanical hyperalgesia in carrageenan-induced acute inflammation. Surprisingly, the antihyperalgesic effect of the IPI exclusively related to MOR, while vildagliptin affected mainly DOR but had also effect on MOR and KOR. It has been demonstrated previously that IPI does not activate MORs directly (Ronai et al. 1999). Both IPI and vildagliptin are inhibitors of the DPP4 but IPI with a penultimate proline is also a substrate of the enzyme and its competitive inhibition is a kinetic artefact (Rahfeld et al. 1991). Although both inhibitors target the active site of the enzyme, the extent of inhibition depends on the residual interaction between drug and active site residues. X-ray crystallography analysing the co-crystal structure of different inhibitors with DPP4 demonstrated that the inhibitors, but not the substrates could bind well beyond the S2 subsite to increase their inhibitory activity. (Nabeno et al. 2013). Taking all these together supports that different residual interactions of the two inhibitors can affect the DPP4 activity in different ways.

Endogenous opioids, especially enkephalins, dynorphins and endorphins, are released from spinal and supraspinal sites during acute inflammation, but are degraded very quickly by extremely high enzymatic activity (Chen and Marvizon 2009). A common feature of these opioids is that they can activate each opioid receptor with different potencies. Since both IPI and vildagliptin are very selective DPP4 inhibitors, it is very unlikely that it has inhibitory effect on opioid degrading enzymes. On the other hand, it has been demonstrated that glial cells express opioid receptors and can synthesize endogenous opioids (Fan et al. 2015; Ruzicka and Akil 1997; Ruzicka et al. 1995). These processes are at least partly regulated by inflammatory mediators including IL-1β (Ruzicka and Akil 1997). The interaction between the two systems is mutual, since the endogeneous opioids also have effect on the production of inflammatory mediators released by glial cells (Finley et al. 2008).

GLP1 receptor activation results in β-endorphin release from microglia and blocks inflammatory nociception and mechanical allodynia in the spinal nerve ligation model (Fan et al. 2015; Gong et al. 2014). In this study, we have found an increase in the expression of DPP4 both in microglia and astrocytes that can facilitate the degradation of many peptides including GLP1. Systemic increase of GLP-1 peptide has been detected during inflammation (Kahles et al. 2014) and IPI/vildagliptin blocking DPP4 activity may increase further the GLP1 level in the spinal cord and induce β-endorphin release from microglia.

In this study, we have found a substantial increase in the expression of DPP4 in astrocytes during inflammation. It has been also demonstrated that astrocytes can synthesize both proenkephalins and dynorphins (Batter et al. 1991; Wahlert et al. 2013) that makes them good candidates for the source of these peptides. It has been shown that purinergic and toll-like receptor (TLR) activation results in dynorphin-A and -B releases from this glia type (Wahlert et al. 2013) and proenkephalin release was also detected in cultured astrocytes (Batter et al. 1991). However, the conditions in which astrocytes release proenkephalin *in vivo* have not been determined.

Recent *in vitro* studies in monocytes demonstrated that DPP4 inhibitors suppressed TLR4 mediated upregulation of proinflammatory cytokines including IL-1β, IL-6 (Ta et al. 2011), NLRP3 inflammasome which is a key molecule to process and release of IL-1β and IL-18 (Dai et al. 2014), and extracellular-regulated kinase (ERK) activation that has also critical role in expression of inflammatory cytokines (Ta et al. 2010). These data suggest an important interaction between TLR4 and DPP4 activity which may exist also in glial cells and regulates the synthesis and release of inflammatory mediators and endogenous opioids.

IPI and vildagliptin both produced a significant decrease in mechanical hyperalgesia in the Seltzer model but this effect was not opioid dependant. In addition to the mechanical hyperalgesia, mechanical and cold allodynia also appeared in this model. None of the inhibitors affected either types of allodynia. Our results indicate the contribution of the DPP4 enzyme to the development and maintenance of mechanical hyperalgesia in this model of neuropathy, but the underlying mechanisms are not known. The increased expression of the enzyme was most obvious in microglia suggesting their role in this process. The contribution of microglia to the development of allodynia in neuropathic condition is intensively studied (Beggs et al. 2012; Ferrini and De

Koninck 2013; Tsuda 2016), but its role in the induction and sustaining of hyperalgesia and its relationship to the DPP4 requires further studies.

Therapeutic potential of the DPP4 inhibitors

Our study has demonstrated that DPP4 exists in the spinal cord and, similarly to that in higher brain area, its expression significantly increases during pathological conditions. Inhibitors of the enzyme do not affect acute nociceptive processing but can selectively block glial mechanisms that contribute to the development and maintenance of hyperalgesia both in inflammatory and neuropathic conditions. This raises the possibility that DPP4 inhibitors targeting the central nervous system could be an important antihyperalgesic and anti-inflammatory component of new analgesics for the treatment of severe and persistent pain without serious side effects.

4) ACKNOWLEDGEMENTS

We thank Professor A. J. Todd for helpful discussion and advice, Ms. Katalin Keserű Góglné, Dóra Pintér Ömböliné, Andrea Németh, Judit Szalay for the professional technical assistance. We also say thanks to Csaba Dávid for his technical advices to image analysis.

The work was supported by National Research, Development and Innovation (NKFI) Fund: PD-OTKA 101248, OTKA 115422 and the National Brain Research Programme B: KTIA_NAP_13-2014-0022, Hungary, Kornél Király was supported by “János Bolyai Fellowship” from the Hungarian Academy of Sciences.

5) REFERENCES

Attems J, Alpar A, Spence L, McParland S, Heikenwalder M, Uhlen M, Tanila H, Hokfelt TG, Harkany T. 2012. Clusters of secretagogin-expressing neurons in the aged human olfactory tract lack terminal differentiation. Proc Natl Acad Sci U S A 109:6259-64.

Batter DK, Vilijn MH, Kessler J. 1991. Cultured astrocytes release proenkephalin. Brain Res 563:28-32.

Beggs S, Trang T, Salter MW. 2012. P2X4R+ microglia drive neuropathic pain. Nat Neurosci 15:1068-73.

Bernstein HG, Schon E, Ansorge S, Rose I, Dorn A. 1987. Immunolocalization of dipeptidyl aminopeptidase (DAP IV) in the developing human brain. Int J Dev Neurosci 5:237-42.

Binder LI, Frankfurter A, Rebhun LI. 1986. Differential Localization of Map-2 and Tau in Mammalian Neurons Insitu. Annals of the New York Academy of Sciences 466:145-166.

Boonacker E, Van Noorden CJ. 2003. The multifunctional or moonlighting protein CD26/DPPIV. Eur J Cell Biol 82:53-73.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-54.

Chen W, Marvizon JC. 2009. Acute inflammation induces segmental, bilateral, supraspinally mediated opioid release in the rat spinal cord, as measured by mu-opioid receptor internalization. Neuroscience 161:157-72.

Clark AK, Gruber-Schoffnegger D, Drdla-Schutting R, Gerhold KJ, Malcangio M, Sandkuhler J. 2015. Selective activation of microglia facilitates synaptic strength. J Neurosci 35:4552-70.

Clark AK, Malcangio M. 2012. Microglial signalling mechanisms: Cathepsin S and Fractalkine. Exp Neurol 234:283-92.

Dai Y, Dai D, Wang X, Ding Z, Mehta JL. 2014. DPP-4 inhibitors repress NLRP3 inflammasome and interleukin-1beta via GLP-1 receptor in macrophages through protein kinase C pathway. Cardiovasc Drugs Ther 28:425-32.

Fan H, Gong N, Li TF, Ma AN, Wu XY, Wang MW, Wang YX. 2015. The non-peptide GLP-1 receptor agonist WB4-24 blocks inflammatory nociception by stimulating beta-endorphin release from spinal microglia. Br J Pharmacol 172:64-79.

Ferrini F, De Koninck Y. 2013. Microglia control neuronal network excitability via BDNF signalling. Neural Plast 2013:429815.

Finley MJ, Happel CM, Kaminsky DE, Rogers TJ. 2008. Opioid and nociceptin receptors regulate cytokine and cytokine receptor expression. Cell Immunol 252:146-54.

Ganley RP, Iwagaki N, del Rio P, Baseer N, Dickie AC, Boyle KA, Polgar E, Watanabe M, Abraira VE, Zimmerman A and others. 2015. Inhibitory Interneurons That Express GFP in the PrP-GFP Mouse Spinal Cord Are Morphologically Heterogeneous, Innervated by Several Classes of Primary Afferent and Include Lamina I Projection Neurons among Their Postsynaptic Targets. J Neurosci 35:7626-42.

Gong N, Xiao Q, Zhu B, Zhang CY, Wang YC, Fan H, Ma AN, Wang YX. 2014. Activation of spinal glucagon-like peptide-1 receptors specifically suppresses pain hypersensitivity. J Neurosci 34:5322-34.

Gorrell MD, Wickson J, McCaughan GW. 1991. Expression of the rat CD26 antigen (dipeptidyl peptidase IV) on subpopulations of rat lymphocytes. Cell Immunol 134:205-15.

Gruber-Schoffnegger D, Drdla-Schutting R, Honigsperger C, Wunderbaldinger G, Gassner M, Sandkuhler J. 2013. Induction of thermal hyperalgesia and synaptic long-term potentiation in the spinal cord lamina I by TNF-alpha and IL-1beta is mediated by glial cells. J Neurosci 33:6540-51.

Haninec P, Grim M. 1990. Localization of dipeptidylpeptidase IV and alkaline phosphatase in developing spinal cord meninges and peripheral nerve coverings of the rat. Int J Dev Neurosci 8:175-85.

Heinke B, Gingl E, Sandkuhler J. 2011. Multiple targets of mu-opioid receptor-mediated presynaptic inhibition at primary afferent Adelta- and C-fibers. J Neurosci 31:1313-22.

Hong WJ, Petell JK, Swank D, Sanford J, Hixson DC, Doyle D. 1989. Expression of dipeptidyl peptidase IV in rat tissues is mainly regulated at the mRNA levels. Exp Cell Res 182:256-66.

Ji RR, Berta T, Nedergaard M. 2013. Glia and pain: is chronic pain a gliopathy? Pain 154 Suppl 1:S10-28.

Kahles F, Meyer C, Mollmann J, Diebold S, Findeisen HM, Lebherz C, Trautwein C, Koch A, Tacke F, Marx N and others. 2014. GLP-1 secretion is increased by inflammatory stimuli in an IL-6-dependent manner, leading to hyperinsulinemia and blood glucose lowering. Diabetes 63:3221-9.

Kahne T, Kroning H, Thiel U, Ulmer AJ, Flad HD, Ansorge S. 1996. Alterations in structure and cellular localization of molecular forms of DP IV/CD26 during T cell activation. Cell Immunol 170:63-70.

Khasabov SG, Rogers SD, Ghilardi JR, Peters CM, Mantyh PW, Simone DA. 2002. Spinal neurons that possess the substance P receptor are required for the development of central sensitization. J Neurosci 22:9086-98.

Kiraly K, Lambeir AM, Szalai J, Szentirmay A, Luyten W, Barna I, Puskar Z, Kozsurek M, Ronai AZ. 2011. The dipeptidyl peptidase IV (CD26, EC 3.4.14.5) inhibitor vildagliptin is a potent antihyperalgesic in rats by promoting endomorphin-2 generation in the spinal cord. Eur J Pharmacol 650:195-9.

Kiraly K, Szalay B, Szalai J, Barna I, Gyires K, Verbeken M, Ronai AZ. 2009. Intrathecally injected Ile-Pro-Ile, an inhibitor of membrane ectoenzyme dipeptidyl peptidase IV, is antihyperalgesic in rats by switching the enzyme from hydrolase to synthase functional mode to generate endomorphin 2. Eur J Pharmacol 620:21-6.

Kozsurek M, Lukacsi E, Fekete C, Wittmann G, Rethelyi M, Puskar Z. 2007. Cocaine- and amphetamine-regulated transcript peptide (CART) is present in peptidergic C primary afferents and axons of excitatory interneurons with a possible role in nociception in the superficial laminae of the rat spinal cord. Eur J Neurosci 26:1624-31.

Lorincz A, Nusser Z. 2008. Specificity of immunoreactions: the importance of testing specificity in each method. J Neurosci 28:9083-6.

Lu G, Hu Y, Wang Q, Qi J, Gao F, Li Y, Zhang Y, Zhang W, Yuan Y, Bao J and others. 2013. Molecular basis of binding between novel human coronavirus MERS-CoV and its receptor CD26. Nature 500:227-31.

Mentlein R. 1999. Dipeptidyl-peptidase IV (CD26)--role in the inactivation of regulatory peptides. Regul Pept 85:9-24.

Mitro A, Lojda Z. 1988. Histochemistry of proteases in ependyma, choroid plexus and leptomeninges. Histochemistry 88:645-6.

Nabeno M, Akahoshi F, Kishida H, Miyaguchi I, Tanaka Y, Ishii S, Kadowaki T. 2013. A comparative study of the binding modes of recently launched dipeptidyl peptidase IV inhibitors in the active site. Biochem Biophys Res Commun 434:191-6.

Nichols ML, Allen BJ, Rogers SD, Ghilardi JR, Honore P, Luger NM, Finke MP, Li J, Lappi DA, Simone DA and others. 1999. Transmission of chronic nociception by spinal neurons expressing the substance P receptor. Science 286:1558-61.

Pap K, Berta A, Szoke G, Dunay M, Nemeth T, Hornok K, Marosfoi L, Rethelyi M, Kozsurek M, Puskar Z. 2015. Nerve stretch injury induced pain pattern and changes in sensory ganglia in a clinically relevant model of limb-lengthening in rabbits. Physiol Res 64:571-81.

Rahfeld J, Schierhorn M, Hartrodt B, Neubert K, Heins J. 1991. Are diprotin A (Ile-Pro-Ile) and diprotin B (Val-Pro-Leu) inhibitors or substrates of dipeptidyl peptidase IV? Biochim Biophys Acta 1076:314-6.

Randall LO, Selitto JJ. 1957. A method for measurement of analgesic activity on inflamed tissue. Arch Int Pharmacodyn Ther 111:409-19.

Rohnert P, Schmidt W, Emmerlich P, Goihl A, Wrenger S, Bank U, Nordhoff K, Tager M, Ansorge S, Reinhold D and others. 2012. Dipeptidyl peptidase IV, aminopeptidase N and DPIV/APN-like proteases in cerebral ischemia. J Neuroinflammation 9:44.

Rohrborn D, Wronkowitz N, Eckel J. 2015. DPP4 in Diabetes. Front Immunol 6:386.

Ronai AZ, Timar J, Mako E, Erdo F, Gyarmati Z, Toth G, Orosz G, Furst S, Szekely JI. 1999. Diprotin A, an inhibitor of dipeptidyl aminopeptidase IV(EC 3.4.14.5) produces naloxone-reversible analgesia in rats. Life Sci 64:145-52.

Ruzicka BB, Akil H. 1997. The interleukin-1beta-mediated regulation of proenkephalin and opioid receptor messenger RNA in primary astrocyte-enriched cultures. Neuroscience 79:517-24.

Ruzicka BB, Fox CA, Thompson RC, Meng F, Watson SJ, Akil H. 1995. Primary astroglial cultures derived from several rat brain regions differentially express mu, delta and kappa opioid receptor mRNA. Brain Res Mol Brain Res 34:209-20.

Sakurada C, Watanabe C, Sakurada S, Tan-No K, Sakurada T. 1999. Major metabolites of substance P degraded by spinal synaptic membranes antagonize the behavioral response to substance P in rats. J Pharm Sci 88:1127-32.

Sandkuhler J. 2009. Models and mechanisms of hyperalgesia and allodynia. Physiol Rev 89:707-58.

Sandkuhler J, Gruber-Schoffnegger D. 2012. Hyperalgesia by synaptic long-term potentiation (LTP): an update. Curr Opin Pharmacol 12:18-27.

Seltzer Z, Dubner R, Shir Y. 1990. A novel behavioral model of neuropathic pain disorders produced in rats by partial sciatic nerve injury. Pain 43:205-18.

Ta NN, Li Y, Schuyler CA, Lopes-Virella MF, Huang Y. 2010. DPP-4 (CD26) inhibitor alogliptin inhibits TLR4-mediated ERK activation and ERK-dependent MMP-1 expression by U937 histiocytes. Atherosclerosis 213:429-35.

Ta NN, Schuyler CA, Li Y, Lopes-Virella MF, Huang Y. 2011. DPP-4 (CD26) inhibitor alogliptin inhibits atherosclerosis in diabetic apolipoprotein E-deficient mice. J Cardiovasc Pharmacol 58:157-66.

Todd AJ, Spike RC, Polgar E. 1998. A quantitative study of neurons which express neurokinin-1 or somatostatin sst2a receptor in rat spinal dorsal horn. Neuroscience 85:459-73.

Toth ZE, Zelena D, Mergl Z, Kirilly E, Varnai P, Mezey E, Makara GB, Palkovits M. 2008. Chronic repeated restraint stress increases prolactin-releasing peptide/tyrosine-hydroxylase ratio with gender-related differences in the rat brain. J Neurochem 104:653-66.

Tsuda M. 2016. Microglia in the spinal cord and neuropathic pain. J Diabetes Investig 7:17-26.

Turner AJ, Hryszko J, Hooper NM, Dowdall MJ. 1987. Purification and characterization of a peptidyl dipeptidase resembling angiotensin converting enzyme from the electric organ of Torpedo marmorata. J Neurochem 48:910-6.

Ujhelyi J, Ujhelyi Z, Szalai A, Laszlo JF, Cayasso M, Vecsernyes M, Porszasz R. 2014. Analgesic and anti-inflammatory effectiveness of sitagliptin and vildagliptin in mice. Regul Pept 194-195:23-9.

Wahlert A, Funkelstein L, Fitzsimmons B, Yaksh T, Hook V. 2013. Spinal astrocytes produce and secrete dynorphin neuropeptides. Neuropeptides 47:109-15.

Yu DM, Slaitini L, Gysbers V, Riekhoff AG, Kahne T, Knott HM, De Meester I, Abbott CA, McCaughan GW, Gorrell MD. 2011. Soluble CD26 / dipeptidyl peptidase IV enhances human lymphocyte proliferation in vitro independent of dipeptidyl peptidase enzyme activity and adenosine deaminase binding. Scand J Immunol 73:102-11.

Zhong J, Maiseyeu A, Davis SN, Rajagopalan S. 2015. DPP4 in cardiometabolic disease: recent insights from the laboratory and clinical trials of DPP4 inhibition. Circ Res 116:1491-504.

Zhong Y, Zhou LJ, Ren WJ, Xin WJ, Li YY, Zhang T, Liu XG. 2010. The direction of synaptic plasticity mediated by C-fibers in spinal dorsal horn is decided by Src-family kinases in microglia: the role of tumor necrosis factor-alpha. Brain Behav Immun 24:874-80.

7) TABLES

Antibody	Species	Dilution	Source	Catalog No.
CGRP	guinea pig	1:5000	Bachem-Peninsula Laboratories	T-5027
DPP4	goat	1:500	Fischer Scientific-Novus Biological	NB100-61658
DPP4	mouse	1:500	Abcam	ab119346
GFAP	mouse	1:100	Leica Biosystems-Novocastra	NCL-GFAP-GA5
IBA1	rabbit	1:500	Wako Pure Chemical Industries Ltd	019-19741
MAP2	mouse	1:500	Sigma-Aldrich	M9942
NeuN	mouse	1:1000	Merck-Millipore-Chemicon	MAB377
VGAT	rabbit	1:1000	Synaptic Systems	131 002
VGLUT2	guinea pig	1:5000	Merck-Millipore-Chemicon	AB2251
Synaptophysin	mouse	1:1000	Leica Biosystems-Novocastra	NCL-SYNAP-299

Table 1. Specifications of primary antibodies used for immunohistochemistry

8) FIGURE LEGENDS

Figure 1. DPP4 mRNA and protein expression in the dorsal horn of control, carrageenan treated and neuropathic rats. DPP4 mRNA expression in the dorsal horn of the spinal cord assessed by qPCR (A) and *in situ* hybridization (B) did not show significant difference among the three experimental groups (mean±SEM, n=6-9, one-way ANOVA, p=0.30 and p=0.21 for qPCR and ISH, respectively). In Western-blot experiments (C) goat DPP4 antibody labelled one lane at 110kDa in spinal cord, lung and pancreas lysates. Western-blot detection of DPP4 in the spinal dorsal horn samples taken from naive, inflamed and neuropathic animals (D) and representative confocal images of transverse spinal dorsal horn sections obtained from rats of the three experimental groups and stained by mouse DPP4 antibody (E). Significantly increased DPP4 protein levels were detected in carrageenan-induced inflammation measured both by Western-blotting (F) and quantitative immunohistochemistry (G). (Values are given as mean±SEM, n=7-10, one-way ANOVA followed by Holm-Sidac *post hoc* test: p=0.023 for Western blot experiments and one-way ANOVA with Student-Neuman-Keuls *post hoc* test: p=0.016 for densitometry).

Figure 2. DPP4 immunoreactivity of different cell types in the spinal dorsal horn. DPP4-immunoreactive puncta appeared in Nissl stained neurons (row A), GFAP labelled astrocytes (row B) and IBA1 positive microglial cells (row C). Co-staining with mouse monoclonal antibody against the full length rat CD26 protein (DPP4mo, column 2) and polyclonal goat DPP4 antibody that was raised against the synthetic peptide C-PPHFDKSKKYP representing the internal region of DPP4 (DPP4gt, column 3) labelled the same puncta in all three cell types demonstrating the specificity of the two antibodies (arrows). All the images are single optical sections. Scalebar: 5 μm.

Figure 3. DPP4 immunoreactivity in various types of axon terminals. DPP4 immunolabelling on MAP2-stained dendritic surfaces was associated with synaptophysisn (SYN) indicating that not the dendrites but the axon terminals express the receptor (row A). DPP4 is expressed by the majority of peptidergic (CGRP-containing) C primary afferent terminals (row B) and coexpression of DPP4 with SYN and VGLUT2 (row C) or VGAT (row D) suggests that DPP4 is present both in excitatory and inhibitory nerve endings. All the images are single optical sections. Scalebar: 5 μm.

Figure 4. DPP4 immunoreactivity in glial cells in control, inflamed and neuropathic animals. Representative confocal images of the spinal dorsal horn obtained from control (A), inflamed (B) and neuropathic (C) animals demonstrate that DPP4 is expressed predominantly by glial cells (arrows: microglia, arrowheads: astrocytes). All

the images are single optical sections. Scalebar: 10 μm. Integrated density values (D) demonstrate that the majority of DPP4-immunopositivity is related to GFAP-labelled astrocytes. A proportion of DPP4 labelling belongs to microgila and very few DPP4 is expressed by neuronal cell bodies. Inflammation significantly increased the DPP4 expression on astocytes but not on the other cell types. In contrast to inflammation DPP4 density was significantly higher on microglia and lower in neurons during neuropathy (Kruskal-Wallis One way ANOVA on ranks, Dunn's Method, p<0,001).

Figure 5. Antihyperalgesic effect of DPP4 inhibitor IPI and vildagliptin in carrageenan-induced inflammation. (A) Antihyperalgesic effect of IPI has been completely abolished by co-administartion of CTAP, but (B) neither TIPP[Ψ] nor (C) gNTI altered IPI-evoked antihyperalgesia. (D) Antihyperalgesic effect of vildagliptin was significantly reduced by CTAP and (F) gNTI, but was completely eliminated when (E) TIPP[Ψ] was co-injected. Inhibitory effects of opioid antagonists on IPI and vildagliptin related antihyperalgesia are summarized on bar graphs (G) and (H) constructed from data recorded 210 min after i.t. drug application. Comparisons were made with two-way ANOVA, Bonferoni *post hoc* test; +: p<0.05; +++: p<0.001 (A-F) and one-way ANOVA followed by Dunnett's *post hoc* test. ***: p<0.001 (G and H). Asterisks always indicate significant differences between the time-matching points of *DPP4 inhibitor* and *DPP4 inhibitor+opioid antagonist* curves. Data on each curves and bars are given as mean and SEM.

Figure 6. Antinociceptive effects of the DPP4 inhibitor IPI and vildagliptin in chronic neuropathic condition induced by partial sciatic nerve ligation. DPP4 inhibitors were ineffective in dynamic plantar aesthesiometer (A and D) and noxious cold sensitivity (B and E) tests. In Randall-Selitto test, both IPI and vildagliptin had antihyperalgesic effect which was not antagonized by NTX (C and F). Maximal possible effects (MPE%) of DPP4 inhibitors alone or in combination with subtype specific opioid antagonists is given on bar graphs (G-I). Comparisons were made with two-way ANOVA, Bonferoni *post hoc* test; +: p<0.05; ++: p<0.01; +++: p<0.001 (A-F) and one-way ANOVA followed by Dunnett's *post hoc* test; *: p<0.1 **: p<0.01 (G-I). Data on each curves and bars are given as mean and SEM.