

## Accepted Manuscript

Title: Similarity and dissimilarity in antinociceptive effects of dipeptidyl-peptidase 4 inhibitors, Diprotin A and vildagliptin in rat inflammatory pain models following spinal administration

Authors: Mihály Balogh, Bence Kálmán Varga, Dávid Árpád Karádi, Pál Riba, Zita Puskár, Márk Kozsurek, Mahmoud Al-Khrasani, Kornél Király

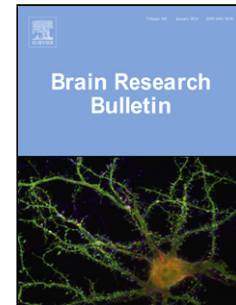
PII: S0361-9230(18)30977-8  
DOI: <https://doi.org/10.1016/j.brainresbull.2019.02.001>  
Reference: BRB 9620

To appear in: *Brain Research Bulletin*

Received date: 17 December 2018  
Accepted date: 5 February 2019

Please cite this article as: Balogh M, Varga BK, Karádi D, Riba P, Puskár Z, Kozsurek M, Al-Khrasani M, Király K, Similarity and dissimilarity in antinociceptive effects of dipeptidyl-peptidase 4 inhibitors, Diprotin A and vildagliptin in rat inflammatory pain models following spinal administration, *Brain Research Bulletin* (2019), <https://doi.org/10.1016/j.brainresbull.2019.02.001>

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.



## Similarity and dissimilarity in antinociceptive effects of dipeptidyl-peptidase 4 inhibitors, Diprotin A and vildagliptin in rat inflammatory pain models following spinal administration

Mihály Balogh<sup>1\*</sup>, Bence Kálmán Varga<sup>1\*</sup>, Dávid Árpád Karádi<sup>1</sup>, Pál Riba<sup>1</sup>, Zita Puskár<sup>2</sup>, Márk Kozsurek<sup>2</sup>, Mahmoud Al-Khrasani<sup>1</sup>, Kornél Király<sup>1‡</sup>

<sup>1</sup>: Department of Pharmacology and Pharmacotherapy, Faculty of Medicine, Semmelweis University, Nagyvárad tér 4, P.O. Box 370, H-1445 Budapest, Hungary

<sup>2</sup>: Department of Anatomy, Histology and Embryology, Faculty of Medicine, Semmelweis University, Tűzoltó u. 58, P.O.Box 2, H-1428 Budapest, Hungary

\*: Mihály Balogh and Bence Kálmán Varga equally contributed to the present work

‡: Address correspondence to this author: Kornél Király at Department of Pharmacology and Pharmacotherapy, Faculty of Medicine, Semmelweis University, Nagyvárad tér 4, P.O. Box 370, H-1445 Budapest, Hungary; Tel.: +36 1 2104416; fax: +36 1 2104412, e-mail: kiraly.kornel@med.semmelweis-univ.hu

### Highlights

- It. diprotin A produced  $\mu$  opioid mediated analgesia in subchronic inflammatory pain
- It. diprotin A produced  $\mu > \delta$  opioid mediated analgesia in acute inflammatory pain
- It. vildagliptin showed  $\delta$  opioid dominant analgesia in subchronic inflammatory pain
- Both compounds showed significant Y1 involvement in subchronic inflammatory model

### Abstract:

Dipeptidyl-peptidase 4 (DPP4) enzyme is involved in the degradation of many biologically active peptides including opioids. Its role in pain transmission is poorly elucidated. Recently we reported on the spinal antihyperalgesic effects of DPP4 inhibitors, Ile-Pro-Ile (Diprotin A) and vildagliptin in carrageenan-evoked acute inflammatory pain in rats. The present study investigated the effects of intrathecal (it.) diprotin A and vildagliptin in Complete Freund's Adjuvant- (CFA) and formalin induced pain in rats. The former assay can model the subchronic inflammatory pain condition and the later one reflects both acute tonic and inflammatory pain conditions. The involvement of opioid receptor (OR) subtypes, Y1-, and GLP1 receptors were also investigated.

In CFA pain model it. diprotin A or vildagliptin dose-dependently inhibit hyperalgesia in ipsilateral while have no effect in contralateral paws. The peak effect was achieved 30 min following drug administration which was used for further analysis. Both compounds showed naltrexone reversible antihyperalgesia. Co-administration of OR-subtype-selective antagonists with diprotin A and vildagliptin revealed involvement of  $\mu$  and  $\delta > \mu$  opioid receptors, respectively. Co-administered Y1 but not GLP1 receptor antagonists reversed the antihyperalgesic action of both DPP4 inhibitors. In touch-hypersensitivity both compounds were ineffective. In formalin test only diprotin A showed  $\mu$  and  $\delta$  OR-mediated antinociception and only in the 2<sup>nd</sup> phase. This effect was Y1 or GLP-1 receptor antagonist insensitive. Conclusion, diprotin A and vildagliptin display antinociception of different mechanisms of action in subchronic inflammatory pain. Furthermore, the spinal pain relay points of inflammatory pain of acute or subchronic conditions were more effectively affected by diprotin A than vildagliptin which needs future elucidation.

Keywords: Dipeptidyl peptidase-4, spinal administration, inflammatory pain, opioid, NPY, GLP-1.

## 1. Introduction:

Spinal cord dorsal horn is the first level pain processing area in the central nervous system. Peripheral injuries (inflammation, neuronal damage) can change pain processing and may lead to elevated pain signalization known as hyperalgesia [12]. The better understanding of the spinal nociceptive signalization and signal processing and the mechanisms of hyperalgesia

would provide new ways to develop novel analgesic compounds. Our group reported for the first time that dipeptidyl-peptidase 4 (DPP4) enzyme inhibitors can influence nociception in spinal cord under certain conditions [10, 17-19] and now we are to further study the role of DPP4 enzyme in the spinal nociceptive transmission.

DPP4 enzyme is recently under thorough investigation because of its multiple actions in a lot of physiological and pathophysiological processes. Many biologically active peptides can be found among its substrates (for review see [26]): peptide hormones, neurotransmitters and chemokines). Since DPP4 influences transmitters that are involved in the control processes of the immune system, metabolism and neurological functions, it is a logical target in drug development. On the other hand, it is known that DPP4 has functions that may be independent from its enzymatic activity. Protein-protein interactions are known on immune cells, the binding of DPP4 to adenosine-desaminase (ADA), CAVEOLIN-1 and CD45 and several other extracellular molecules (fibronectin, collagen) causes increased activities and the effect is mainly proinflammatory (for review see [20]).

Theoretically DPP4 could influence nociceptive transmissions: there are at least six peptides among its substrates known to influence pain processes: endomorphin 1 and 2 (EM1 and EM2), substance P (SP), neuropeptide Y (NPY), pituitary adenylate cyclase-activating peptide (PACAP) and glucagon like peptide 1 (GLP1) [11, 26-28, 31, 36, 40]. SP and PACAP peptides are generally known as proalgesic, the others are considered analgesic peptides. EM1, EM2, GLP1 and PACAP are fully inactivated by DPP4, truncation of NPY causes partial inactivation – it loses the activity on Y1 receptors [7], SP remains active on NK1 receptors but it is possibly the initial step of its inactivation. Despite to its assumed role there are only few reports about the proven effects of DPP4 or DPP4 inhibitors in pain processing. Recently we reported the expression of DPP4 in spinal cord dorsal horn neurons, astrocytes and microglia. The expression of the DPP4 protein was increased by peripheral inflammation in astrocytes and also after neural injury (partial ligation of sciatic nerve) in microglia [17]. Also, vincristine induced neuropathic pain caused an increase in DPP4 like enzymatic activity in the spinal cord [38]. These reports suggest that in pathologically painful situations the role of DPP4 becomes more pronounced, therefore the effects of DPP4 inhibitors could also increase. On the other hand, in vivo DPP4 KO mice were reported to be more sensitive to pain because of their higher SP level [10]. Furthermore, DPP4 deficient Fisher rats showed lower latencies on hot plate test without habituation. Interestingly, after habituation there was no difference compared to controls [15].

The DPP4 inhibitor diprotin A (Ile-Pro-Ile, IPI) was found to have an opioid mediated analgesic effect in high doses on tail flick test after intracerebroventricular (icv.) injection [33]. Chronic administration of Diprotin A prevented vincristin induced allodynia through the inhibition of increased spinal EM2 degradation [38]. In recent years, we found that both Diprotin A and the non-peptide DPP4 inhibitor vildagliptin were effective in different pain models after intrathecal (it.) administration. In the acute thermal nociceptive tail flick test both compounds were ineffective but in carrageenan induced subacute inflammatory hyperalgesia both compounds were able to fully restore nociceptive thresholds in Randall-Selitto (RS) test in 30 and 3 nmol/rat doses, respectively [18, 19]. The effect was opioid receptor mediated in both cases. In mononeuropathic pain model (partial sciatic nerve ligation) both compounds have partial effect: partially- and non-opioid actions on RS test but no effect on touch sensitivity and cold allodynia. We further analyzed the opioid action on carrageenan model and found that the effects of diprotin A and vildagliptin were different. The action of diprotin A was exclusively mediated by  $\mu$  receptors whereas all three opioid receptor subtypes were involved in the effect of vildagliptin with a  $\delta$  opioid dominance [17].

In our current research we focused on the analgesic effects of DPP4 inhibitors in two other inflammatory models. The subchronic Complete Freund's Adjuvant (CFA) induced inflammation which is an accepted model of human inflammatory pain and the acute and more intensive formalin test which is a model of both acute chemical pain (1<sup>st</sup> phase) and acute inflammatory pain (2<sup>nd</sup> phase). Formalin causes hyperalgesia of spontaneous pain related behavior. Pain evoked by formalin has been reported to be more intensive than that evoked by carrageenan or CFA [22]. In both models, both DPP4 inhibitor compounds were analyzed for opioid receptor subtype-involvement to determine whether the difference between them is special to the previously used carrageenan model. We also tested the impact of Y1 receptors of NPY and GLP1 receptors in the applied pain models. DPP4 inhibition would primary increase the Y1 mediated action [26]. Intrathecal GLP1 or its analogs were reported to produce similar opioid mediated effect in nociceptive tests [6, 8].

## 2. Materials and methods:

### 2.1. Animals:

170-240g Male Wistar rats were used. They were purchased from the central animal breeding facility of Semmelweis University, Budapest. They were kept in 12h light cycle  $21\pm 3^{\circ}\text{C}$  temperature and 60% humidity, fed with commercial rodent chow and tap water ad-libitum. Experiments were performed in accordance to guidelines of the local animal care committee (PEI/001/276-4/2013) and the Ethical Board of Semmelweis University based on the Declaration of the European Communities Council Directives (2010/63/EU).

### 2.2. Materials:

Stock solution of diprotin A (Sigma-Aldrich, Hungary) and BIBO3304 (Tocris, Hungary) were dissolved in 20% beta-cyclodextrine and diluted 10 fold with bidistilled water. Vildagliptin (Selleckchem, California, USA; Sigma-Aldrich, Hungary), CTAP ( $\mu$  antagonist, Sigma-Aldrich, Hungary), guanidino-naltrindole (gNTI,  $\kappa$  antagonist, Tocris, Hungary), TICP $\psi$  ( $\delta$  antagonist, courtesy gift from Géza Tóth, Biological Research Center, Szeged, Hungary [34]) and exendin(9-39) (Bachem, Germany) were dissolved in bidistilled water as stock. The final solutions were diluted with sterile saline. Naltrexone hydrochloride (NTX, nonselective opioid antagonist) was a kind gift from DuPont Pharmaceuticals, USA and it was dissolved in sterile saline. Complete Freund's Adjuvant was purchased from Calbiochem, Hungary. Formalin and isoflurane were purchased from Sigma-Aldrich, Hungary. Cyclodextrin ((2-hydroxypropyl)- $\beta$ -cyclodextrin, HP $\beta$ CD) was purchased from Cyclolab Ltd, Hungary.

### 2.3. Treatment of animals:

Intrathecal (it.) injections were given in a 5  $\mu\text{l}$  volume, delivered by a 250  $\mu\text{l}$  Hamilton syringe set into a Hamilton dispenser. The 23-Ga needle, with a depth controller at 6 mm from the tip, was inserted at the L5-6 intervertebral space [29]. In experiments with selective antagonist they were co-injected with the DPP4 inhibitor in the following doses: CTAP 200 pmol/rat, TICP $\psi$  1 nmol/rat and gNTI 10 nmol/rat, BIBO3304 4 and 8 nmol/rat, exendin(9-39) 2.96 nmol/rat. Subcutaneous (sc.) NTX injections were given in 1 ml/kg volume, 1.46  $\mu\text{mol/kg}$  dose.

The drugs were randomly given during an experiment, and the tester was blinded for the drugs and doses applied. During the longer series of experiments the treated groups were randomized among the experimental days.

#### 2.4. Complete Freund's Adjuvant induced subchronic inflammatory model

Randall-Selitto paw pressure test (RS test, Model 37215 Ugo Basile, Italy; setting: 10g/unit) was measured 3 times (-3, -5 and 0 days) before CFA treatment and the mean was accepted as baseline threshold. Dynamic plantar aesthesiometer (DPA, Model 37450 Ugo Basile, Italy; setting: 10g/sec, max. force 50g) was performed twice (-3 and -5 days) before CFA administration. Each time each paw was measured 3 times and the mean of the 3 measurements was calculated for each paws. The mean value of the 2 measurements was used as baseline. On day 0 150  $\mu$ l CFA suspension was injected into the right hindpaw of the rats under light isoflurane anesthesia. The experiments were performed on day 4 after CFA treatment. Baseline latencies were measured on DPA and RS tests. When NTX was used in the experiment, NTX or saline was given 10 min before the it. drug administration. The effects of the compounds were measured 30 min after it. injection, except in the first experiment when only RS test was used and the effect was measured 15, 30 and 60 min after the it. injection (determination of peak effect).

#### 2.5. Formalin induced acute inflammatory model:

5 minutes after it. injections of test compounds, 50  $\mu$ l 2.5% formalin solution was injected into the right paw of the rat. The typical pain reactions (flinching, biting and elevating) were counted in 5-minute periods from 0 to 60 minutes as described previously [1].

#### 2.6. Statistical analysis:

For all statistical analysis a professional statistical software was used (GraphPad Prism 6.0.; GraphPad Software Inc., San Diego, CA). In CFA model threshold-timepoint (baseline-hyperalgesia-treatment) curves, in formalin test the reaction count-time curves were compared with two-way repeated measure ANOVA and Bonferroni post hoc test. For formalin test we also compared the cumulated reaction count of the different groups in the 2<sup>nd</sup> phase (10-60 min), applying one-way ANOVA followed by Bonferroni post hoc test.

Results were considered statistically significant when  $P < 0.05$ .

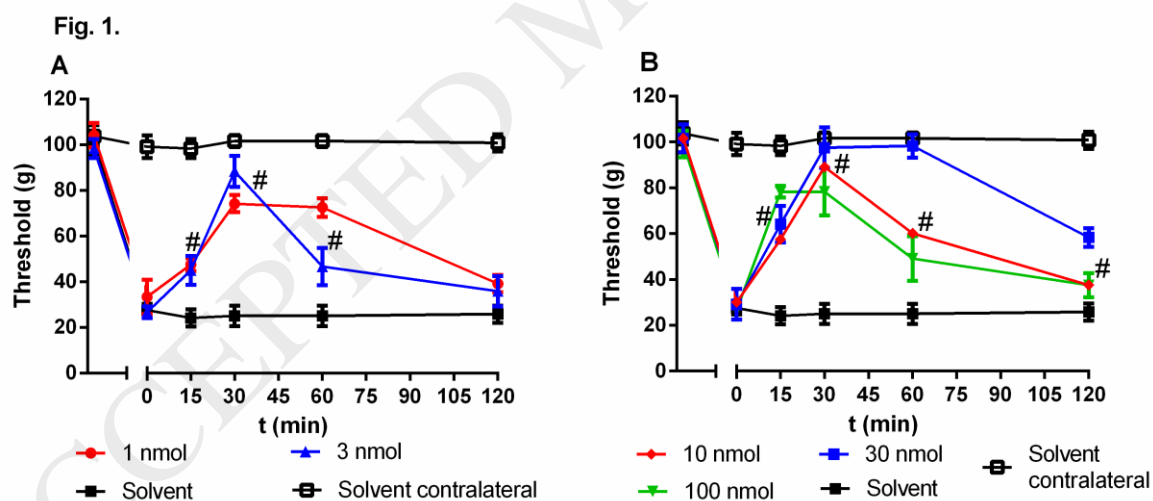
### 3. Results:

#### 3.1 CFA induced subchronic inflammatory model:

Intraplantar CFA treatment produced pressure and touch hyperalgesia in rat ipsilateral (CFA treated) paws measured by Randall Selitto and DPA, respectively on the 4<sup>th</sup> day following CFA injections in accordance with results obtained by other research groups [21, 23].

##### 3. 1. 1. Diprotin A produces antinociceptive effect after intrathecal administration in CFA-induced inflammatory pain in Randall-Selitto test

CFA injections resulted in a significant decrease in ipsilateral paw pressure threshold (PPT) (from  $100 \pm 1,22$  g to  $28,89 \pm 0,92$  g) and no change was observed in the contralateral PPT ( $101,1 \pm 0,98$  g). Diprotin A was injected it. in doses of 1, 3, 10, 30 and 100 nmol/rat. The analgesic action of tested doses was determined 15, 30 and 60 min after it injections. All tested doses increased PPT of the ipsilateral paw with a peak effect at 30 min (Fig. 1.). Diprotin A produced maximal analgesia in dose of 30 nmol in accordance with our previous work [17, 19]. This dose was therefore used in the subsequent experiments. All tested doses failed to affect the PPTs of non-inflamed paws (contralateral paws).

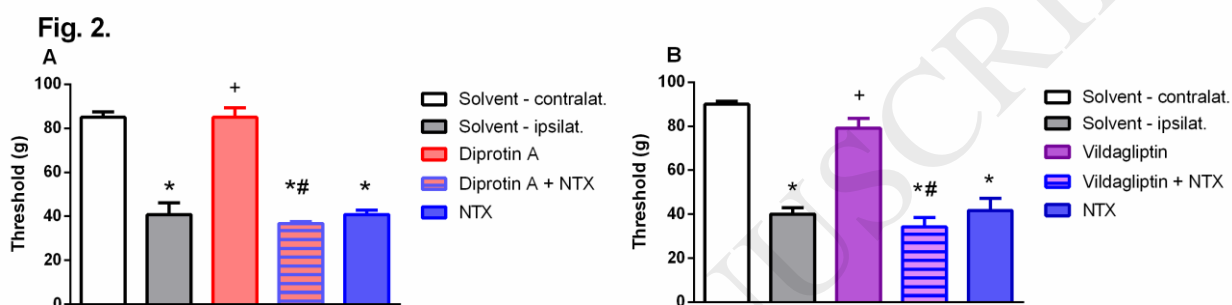


**Figure 1.** The antihyperalgesic effect of it. 1, 3, (A) 10, 30 and 100 (B) nmol/rat Diprotin A on CFA induced pressure hyperalgesia measured in Randall-Selitto test. Thresholds of contralateral paws of other groups are not shown (identical to solvent treated group).  $n = 6$ /group, data are represented as mean  $\pm$  SEM. #: significant difference vs. solvent ipsilateral



### 3. 1. 2. Vildagliptin produces antinociceptive effect after intrathecal administration in CFA-induced pain in Randall-Selitto test

In a pilot experiment, intrathecal vildagliptin was tested in 3 nmol/rat dose that was used in our previous work [17, 18] and 10 nmol/rat dose (data not shown). Both doses produced antinociception but 10 nmol caused complete antihyperalgesic effect in most of the experiments, therefore 10 nmol/rat and some experiments also 20nmol/rat doses were chosen for further analysis. 10 nmol/rat abolished the hyperalgesia in inflamed paws (Fig. 2b and 4). Vildagliptin failed to affect the PPT of contralateral paws.

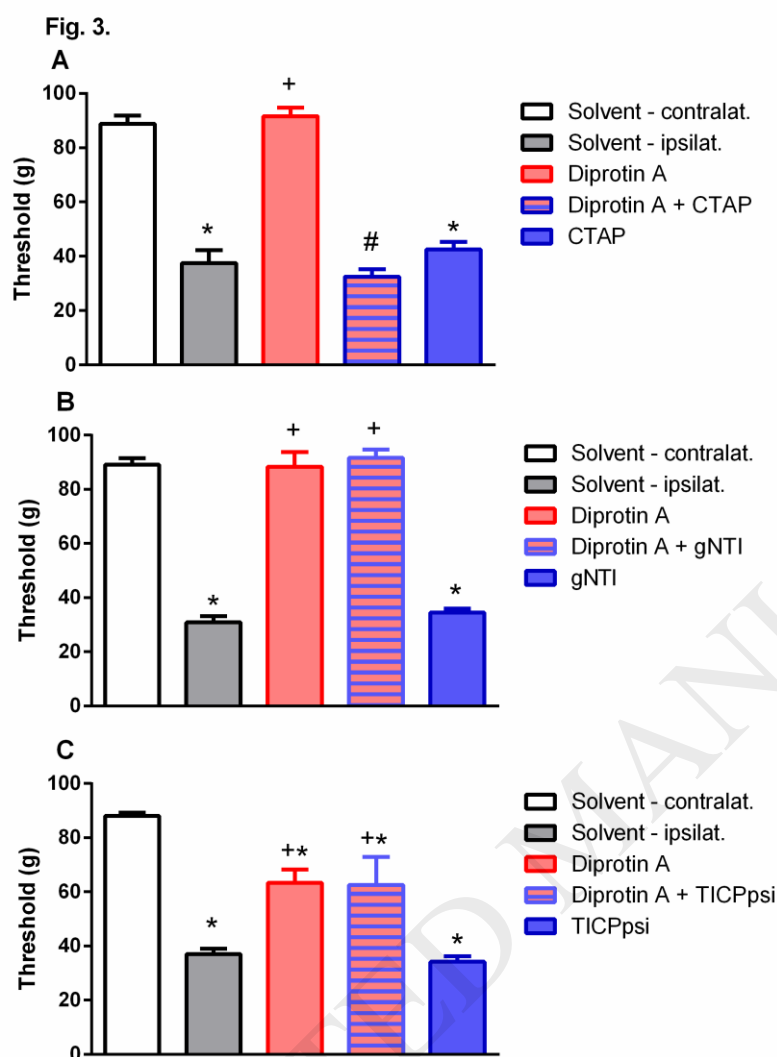


**Figure 2.** The effect of the subcutaneously given nonselective opioid receptor antagonist naltrexone (NTX) on the antihyperalgesic effect of it. diprotin A (panel A) and vildagliptin (panel B)  $n = 6/\text{group}$ , data are represented as mean  $\pm$  SEM. contralat.: values of contralateral paws ipsilat.: values of ipsilateral (inflamed) paws  
\*: significant difference vs. solvent-contralat.; + significant difference vs. solvent ipsilat.; # significant difference vs. diprotin A / vildagliptin (panel A/ panel B)

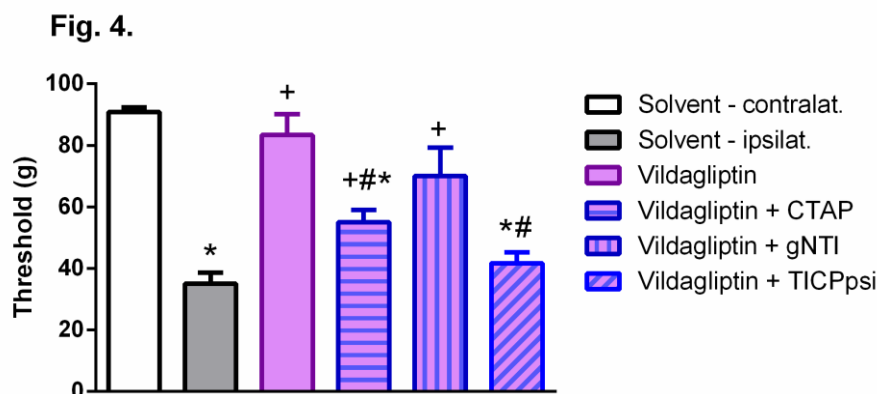
### 3. 1. 3. The antagonist actions of non-selective and subtype selective opioid antagonists on antinociceptive effects of intrathecal diprotin A or vildagliptin

Subcutaneously administered NTX (1,46  $\mu\text{mol}$ ), a non-selective opioid antagonist abolished the antinociceptive effects of it. diprotin A and vildagliptin. (Fig. 2.). Co-administered CTAP, a highly selective  $\mu$ -opioid receptor antagonist abolished the antihyperalgesic effect diprotin A (Fig. 3., panel A). Neither it.  $\kappa$  nor  $\delta$  opioid receptor antagonist affected the antinociceptive action of intrathecal diprotin A (Fig. 3., panels B and C). The  $\delta$ -selective antagonist, TICP $\psi$  fully, whereas CTAP only partially affected the antinociceptive action of it. vildagliptin. On the other hand, gNTI showed no significant impact on the antinociceptive effect of it. vildagliptin, but still a trend was recognizable (Fig. 4). Neither sc. NTX nor other it. tested

opioid antagonists caused any change in the PPT of ipsi- or contralateral paws when given alone.



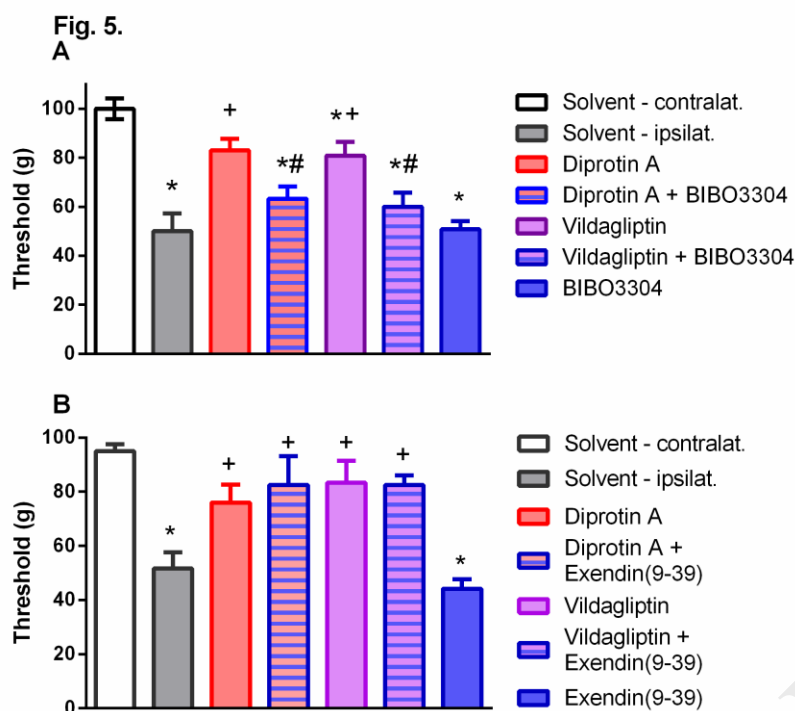
**Figure 3.** Effect of co-injected  $\mu$ -,  $\delta$ -, and  $\kappa$  subtype selective opioid receptor antagonists (CTAP, gNTI and TICPpsi, represented in panels A, B and C, respectively) on the antihyperalgesic effect of it. 30 nmol Diprotin A.  $n= 4-9$ /group, data are represented as mean  $\pm$  SEM. contralat.: values of contralateral paws; ipsilat.: values of ipsilateral (inflamed) paws  
\*: significant difference vs. solvent-contralat.; +: significant difference vs. solvent ipsilat.;  
#: significant difference vs. diprotin A



**Figure 4.** Effect of co-injected subtype selective opioid receptor antagonists on the antihyperalgesic effect of 10 nmol/rat *it.* vildagliptin.  $n = 6$ /group, data are represented as mean  $\pm$  SEM. contralat.: values of contralateral paws; ipsilat.: values of ipsilateral (inflamed) paws; \*: significant difference vs. solvent-contralat.; +: significant difference vs. solvent ipsilat.; #: significant difference vs. diprotin A

### 3. 1. 4. The antagonist effects of Y1- and GLP-1 receptor antagonists on the antinociceptive effects of intrathecal diprotin A or vildagliptin

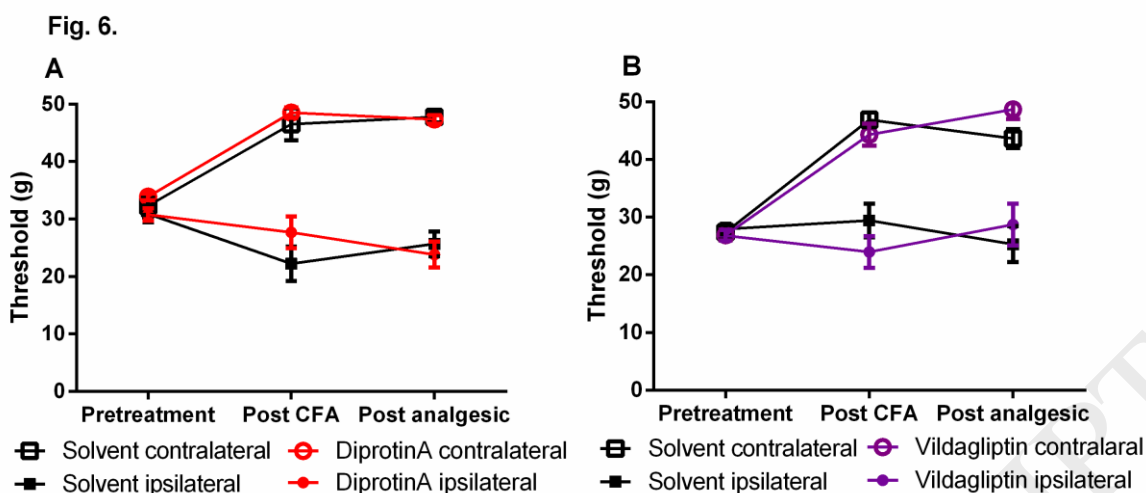
The Y1 receptor antagonist BIBO3304 (4 nmol/rat) partially inhibited the antinociceptive effect of diprotin A (data not shown). In addition, BIBO3304 in a dose of (8 nmol/rat) did produce similar partial reverse on antihyperalgesic action of both DPP4 inhibitors. (Fig. 5., panel A) The GLP-1 receptor antagonist, exendin(9-39) influenced neither the action of Diprotin A nor vildagliptin in high (2.96 nmol/rat) dose (Fig. 5., panel B).



**Figure 5.** Effects of co-injected Y1 receptor antagonist BIBO3304 (panel A) and GLP1 receptor antagonist exendin(9-39) (panel B) on the antihyperalgesic effect of it. administered 30 nmol/rat diprotin A and 20 nmol/rat vildagliptin.  $n = 5-6$ /group, data are represented as mean  $\pm$  SEM. contralat.: values of contralateral paws; ipsilat.: values of ipsilateral (inflamed) paws; \*: significant difference vs. solvent-contralat.; +: significant difference vs. solvent ipsilat.; #: significant difference vs. diprotin A

### 3.1. 5. Diprotin A or vildagliptin failed to alleviate CFA evoked light touch sensitivity

Since the results obtained in the above mentioned test (Randall-Selitto) demonstrated that i.t. diprotin A and vildagliptin inhibited mechanical hyperalgesia, the effects of test compounds were also assessed on light touch hyperalgesia measured by DPA. I.pl. injection of CFA evoked significant hyperalgesia in the right (ipsilateral, CFA treated) paws compared to contralateral paws. In this pain model both diprotin A and vildagliptin failed to produce analgesic action. Furthermore, the opioid antagonists also failed to cause alteration in ipsi- or contralateral paws of rats (Fig. 6.)



**Figure 6.** 30 nmol/rat Diprotin A (panel A) and 10 nmol/rat vildagliptin (panel B) showed no effect on increased touch sensitivity measured on Dynamic Plantar Aesthesiometer (DPA).  $n=6$ /group, data are represented as mean  $\pm$  SEM

### 3.2 Formalin induced acute inflammatory pain model

#### 3. 2. 1. Diprotin A but not vildagliptin produces antinociception after intrathecal administration in rat formalin test

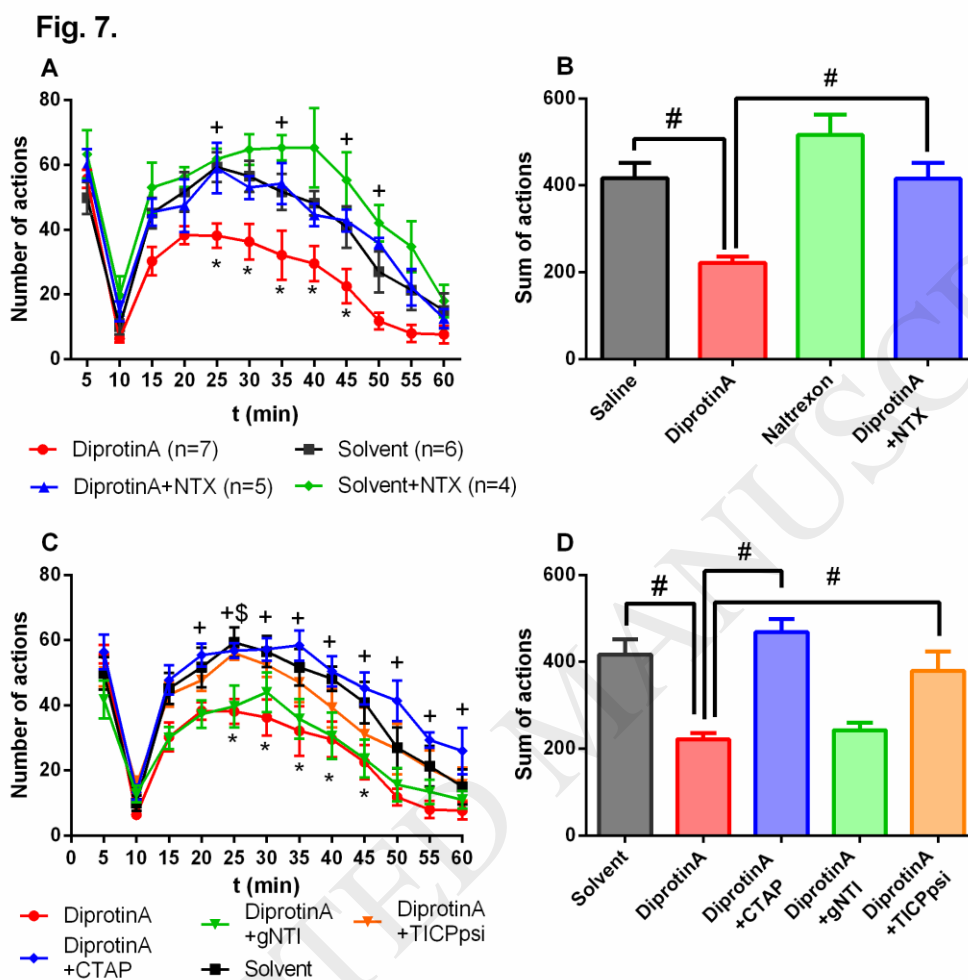
Intrathecal injection of diprotin A (30 nmol/rat) significantly reduced the pain reaction counts in the 2<sup>nd</sup> phase of the test, but not in the 1<sup>st</sup> phase (Fig. 7.).

On the other hand, vildagliptin in a dose of 10 or 20 nmol/rat failed to inhibit the pain behaviors in both phases (data not shown).

#### 3. 2. 2. The antagonist effects of non-selective and subtype selective opioid antagonists on antinociceptive effect of intrathecal diprotin A in rat formalin test

The effect of diprotin A was antagonized by sc. NTX (Fig. 7. panels A and B). Co-injected CTAP with diprotin A completely, while TICP $\psi$  partially inhibited its antinociceptive effect. The selective  $\kappa$  antagonist gNTI did not antagonize diprotin A (Fig. 7. panel C and D). The partial antagonism by TICP $\psi$  was statistically significant in one time period and the cumulated reaction count was also significantly higher than in the diprotin A group (Fig. 7., panels C and D).

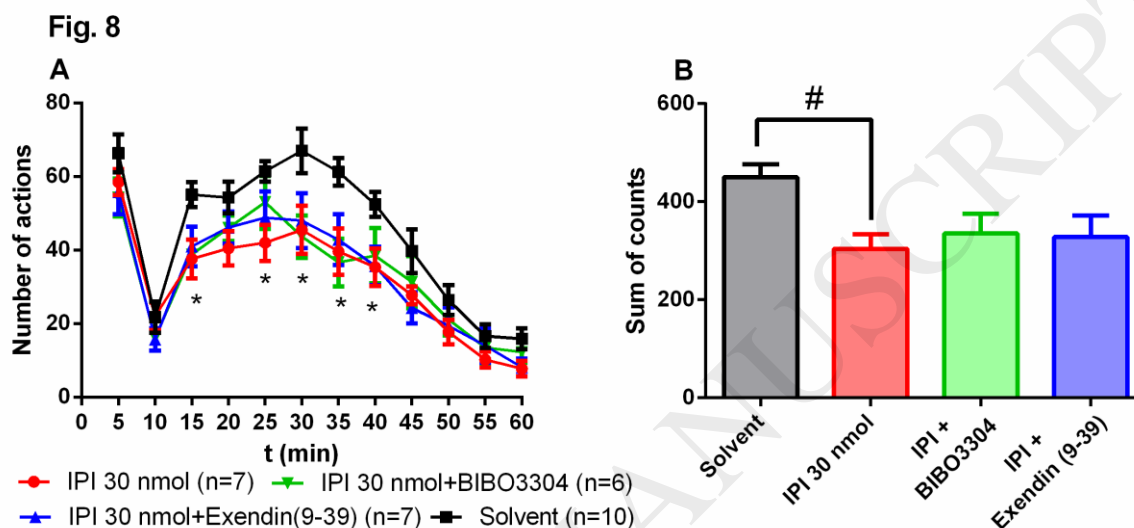
Naltrexone given alone slightly but not significantly elevated the reaction count (Fig. 7. panel A and B). In order to reduce the number of experimental animals subtype selective antagonists were not tested alone. The ineffectiveness of the nonselective opioid antagonist naltrexone predicts no effect of the selective antagonists when given alone.



**Figure 7.** Analgesic effect of it. 30 nmol/rat diprotin A on formalin test. Panel A: represents the effect of nonselective opioid antagonist naltrexone (NTX) against Diprotin A induced analgesic action. Panel B: sum of counts in the 2<sup>nd</sup> phase of the test, presented in panel A. Panel C: the effects of different subtype selective opioid antagonists ( $\mu$  - CTAP,  $\delta$  - gNTI, and  $\kappa$  - TICPpsi) against Diprotin A-induced analgesic action. Panel D: sum of counts in the 2<sup>nd</sup> phase of the test, presented in panel C. n= 5-7/group, data are represented as mean  $\pm$  SEM. \*: significant difference vs. solvent.; +: diprotin A vs. diprotin A+NTX / CTAP; \$: diprotin A vs. diprotin A+TICPpsi; # significant difference between signed columns

### 3. 2. 3. Y1 receptor and GLP-1 antagonists failed to affect the antinociceptive effect of intrathecal diprotin A in rat formalin test

In this test neither BIBO3304 nor exendin(9-39) influenced the effect of diprotin A indicating neither NPY nor GLP1 has any role in the analgesic effect of diprotin A under the present circumstances (Fig. 8).



**Figure 8.** The effect of co-injected Y1 receptor antagonist BIBO3304 and GLP1 receptor antagonist exendin(9-39) on the analgesic action of it. 30 nmol/rat diprotin A in formalin test. Panel A represents the counts of pain reactions in 5 minute intervals, panel B. represents the sum of actions in the 2<sup>nd</sup> phase of the test. n= 6-9/group, data are represented as mean  $\pm$  SEM. \*: significant difference vs. solvent; # significant difference between signed columns

#### 4. Discussion

Chronic pain management is still a huge clinical challenge. While opioids are undoubtedly effective in the management of moderate to severe pain, their use is hampered by the appearance of their adverse effects. One of the successful strategies in pharmacology is the utilization of the endogenous system to gain new therapeutic effects. Following this strategy could be more sophisticated than using exogenous opioid agonists. Among the possibilities to enhance an endogenous transmitter system is the inhibition of the transmitter termination by the enzymes. DPP4 has been considered as the main metabolizing enzyme of endomorphins but not of other endogenous opioids [26, 36]. In the current work we investigated the effects of two DPP4 inhibitors, namely diprotin A and vildagliptin in two inflammatory pain models that have not been reported so far. Our choice has fallen on CFA and formalin induced pain models. The former is accepted as model of human chronic inflammatory pain [9]. The later assay (formalin test) consists of a combination of acute nociceptive chemical (1<sup>st</sup> phase) and acute inflammatory (2<sup>nd</sup> phase) pain.

On CFA induced inflammatory pain model we tested the animals on two modalities: pressure pain (Randall-Selitto test, RS) and tactile sensitivity (DPA method). The drop of the thresholds was detectable with both methods in accordance with previous studies [21, 23].

In RS test, the antinociceptive effect was detectable only on the inflamed paw, indicating antihyperalgesic effects of test enzyme inhibitors. We applied antihyperalgesic terminology, because the thresholds never exceeded the values of prehyperalgesic condition following administration of the enzyme inhibitors. The antihyperalgesic effects of both DPP4 inhibitors in RS test were naltrexone reversible, indicating the contribution of endogenous opioid system. Earlier we could prove that DPP4 inhibitors could activate the endogenous opioid system and cause opioid mediated antihyperalgesic effect in subacute inflammatory pain. [17-19, 32]. Of note, the applied pain model was different. Naltrexone is a non-selective opioid antagonist to verify which opioid receptor subtypes are responsible for the measured antihyperalgesia. We used different opioid antagonists with high selectivity for each opioid receptor subtype. The opioid subtype analysis suggests that the effect of diprotin A was  $\mu$  opioid receptor mediated. On the other hand the effect of vildagliptin is mediated by both  $\mu$ - and  $\delta$ -opioid receptor subtypes. In addition, no significant  $\kappa$  opioid receptor contribution was observed. Previous studies by our and other research groups have established the upregulation of  $\mu$  opioid receptors in DRG, peripheral and central nerve terminals of primary afferent neurons [16, 30]. This condition reflects the effectiveness of opioids in inflammatory pain.



Based on previous studies and literature, we can hypothesize that at spinal level DPP4 inhibitors prevent the degradation of endogenous opioid peptides that consequently results in an increase of endogenous opioids, which in turn can result in antinociception. Furthermore, accepting that diprotin A prevents the degradation of endomorphins, then the measured antihyperalgesic action could be coined from interaction of endomorphins with spinal  $\mu$  opioid receptors. The result of pain inhibition is largely attributed to both DPP4 enzyme [17] and  $\mu$  opioid receptor upregulation [16] at the level of spinal cord as consequence of inflammation [21]. Nevertheless, the endogenous opioids are not directly influenced by DPP4 (with the exception of endomorphins), therefore we checked two further transmitters as intermediate mediators to verify the antihyperalgesic effect of DPP4 inhibitors. The possible role of NPY and GLP1 were also analyzed. NPY has two types of receptors in spinal cord: the pre- and postsynaptically localized Y2 and Y1, respectively [2-4]. Both could mediate antinociception, though in case of inflammatory pain Y1 receptor is better accepted as an analgesic mediator of intrathecally administered NPY [13, 25, 35]. Despite the above mentioned mechanism the role of NPY is still not fully understood [5]. Based on literature data, truncation of NPY by DPP4 changes its affinity to Y1 but not to Y2 [27]. Consequently, if NPY is a mediator of DPP4 inhibitor caused analgesia it would act through the enhancement of Y1 receptor activity. There is no literature data proving that spinal NPY would act through opioid system but in the case of supraspinal NPY administration there are publications about naltrexone sensitive analgesic effect [24, 37, 39]. Therefore, it could be also possible at the spinal level.

GLP1 is less accepted as a spinal mediator but Fan et al. found that spinally administered exogenous GLP-1 caused  $\mu$  receptor mediated analgesic effect on CFA model and also on formalin test – similarly to our results in case of diprotin A – through releasing  $\beta$ -endorphin from spinal microglia cells [6]. So GLP1 could also be a possible alternative answer to how DPP4 inhibitors may act.

Based on our experiments we could fully exclude the involvement of GLP1 from the spinal action of DPP4 blockers. Exogenous GLP1 could have very similar action to our enzyme inhibitors but endogenously it cannot be activated by DPP4 inhibition. However, NPY through Y1 seems to have a role here but Y1 is not the exclusive mediator of the analgesic effect of the tested DPP4 inhibitors. BIBO3304 in 4 nmol dose partially blocked the effect of both diprotin A and vildagliptin. The elevation to 8 nmol did not increase the inhibitory action: it caused a similar partial effect as the lower dose. That suggests a partial involvement in both pathways in this model. From the results above we can conclude that both DPP4

inhibitors can activate the endogenous opioid system in the spinal cord and can alleviate hyperalgesia. This opioid activation is partially mediated by NPY via Y1 receptors, but not by GLP1. Similarly to the carrageenan model the opioid receptor profile of vildagliptin and diprotin A suggest that the two compounds activate the opioid system differently.

The DPP4 inhibitors, diprotin A and vildagliptin failed to alter the lowered tactile thresholds indicating that no antiallodynic effect was found with DPA method in contrast to the almost complete antihyperalgesia detected on Randall-Selitto test as mentioned above. To clear up this contradiction we tested the effects of the highly selective  $\mu$  receptor agonist DAMGO on mechanical hyperalgesia and light touch sensitivity (see supplementary figs). DAMGO in doses of 3 and 10 pmol produced significant antinociceptive effect only in mechanical hyperalgesia but at higher dose (30 pmol/animal) abolished both the mechanical hyperalgesia and light touch sensitivity. Of note, DAMGO in a dose of 30 pmol produced greater antihyperalgesic effect in RS than in DPA test. So it seems that RS test is more sensitive to detect moderate antihyperalgesic action of the tested compounds.

In the formalin test neither diprotin A nor vildagliptin affected the number of pain reactions in the phase I. This result is in accordance with our previous study utilizing acute nociceptive tests, whereby we showed that diprotin A and vildagliptin are ineffective [18, 19]. On the other hand, diprotin A but not vildagliptin caused significant effect in doses that were proved to be effective in the present study (CFA model) and previous work (carrageenan model) in phase II, which in contrast to phase I is accepted as an inflammatory reaction. Nevertheless, vildagliptin in doses of 10 or 20 nmol was ineffective. Our above mentioned hypothesis on endogenous opioids and antinociception following intrathecal administration of diprotin A may also stand for the measured antinociception in formalin test. The exception is the appearance of  $\delta$ -opioid receptor mediated antinociceptive action. To elucidate this effect further future studies are needed. We have also paid attention to a reasonable question that might be raised on the antinociception of diprotin A and its dosing time regarding phase I. To verify this issue we also tested the antinociceptive effect of diprotin A administered 20 min prior to formalin injection. In this setting diprotin A also failed to produce antinociception in phase I, indicating that the ineffectiveness persisted even when the time of dosing was matched to phase I experiments (data not shown). Furthermore, Y1 and GLP1 receptor antagonists failed to affect the antinociceptive effect of diprotin A. This result rules out the contribution of Y1 and GLP1 receptors to the measured antinociceptive effect of diprotin A under the present experimental conditions. The ineffectiveness of vildagliptin could be

explained as follows: the pathway it activates has lower maximal effect – cannot block the more intensive pain in this model – or it needs longer activation, so the short time window of formalin test is not long enough for its activation. In addition, diprotin A has a moderate effect in formalin test compared to RS test, meaning that diprotin A is not able to fully suppress the nociceptive reactions as a directly acting opioid agonist [1].

We can speculate that the only neuronal conductive pathway affected by diprotin A was enough to suppress pain reactions. The present results reveal that vildagliptin is inferior to diprotin A. However, these results further support the assumption that utilization of the endogenous opioid system may offer a new pain treatment approach.

The most intriguing question is the difference in the antinociceptive effects of the two DPP4 inhibitors in the different inflammatory pain models (CFA model vs formalin test). To fully elucidate these questions more future parallel studies are needed. Our hypothesis is based on two possible mechanisms. First, both compounds share the same inhibitory binding site on DPP4, yet the enzyme activity is altered differently. Second, one of the inhibitors might have another active binding site. The first possibility proposes that the target of both DPP4 inhibitors is the DPP4 molecule. In this case, both drugs could bind to the same active site of the enzyme but their inhibitory mechanisms are different. Vildagliptin, as other gliptins, binds to the enzyme as a competitive inhibitor. In the other hand, diprotin A is a slowly metabolized substrate of DPP4 [14], so it is possible that the cleavage of natural substrates by DPP4 is influenced differently. Moreover, we did not find any difference in case of GLP-1 or NPY in the present study: GLP-1 was not involved in any case, whereas NPY is similarly involved in both cases through Y1 receptors in CFA model. In case of diprotin A the involvement of EM2 appears to be more prominent, though the existence of endomorphins as endogenous peptides is still a debated question. It could also be possible that the binding of these inhibitors might result in changes of the enzyme structure which in turn makes them to drive alternate protein-protein interactions of DPP4. The second assumption could be tested on DPP4 deficient rats. If on those animals diprotin A or vildagliptin had antihyperalgesic / analgesic action then our compounds would have another binding site which might be (also) responsible for the antihyperalgesic action. One of our future plans is to test our hypothesis in DPP4 deficient rats.

### **Conclusions:**

Utilization of the endogenous opioid system can lead to strong analgesic action in inflammatory conditions. Both diprotin A and vildagliptin show antinociceptive actions in subchronic pain model, yet their mechanisms of action differ. In addition, diprotin A but not

vildagliptin alleviates intense acute inflammatory pain in rats. The present study further supports the assumption that the utilization of the endogenous opioid system may offer a new pain treatment approach.

**Acknowledgements:**

Funding: This work was supported by the Ministry of Human Capacities, Hungary (New National Excellence Program: ÚNKP-18-3-III awarded to Mihály Balogh) and Kornél Király was supported by “János Bolyai Fellowship” from the Hungarian Academy of Sciences: BO 00124/14 and by Faculty of Medicine, Semmelweis University and also by Semmelweis Technology and Innovation Fund 2017.

Declarations of interest: none

**References:**

- [1] M. Balogh, Z.S. Zadori, B. Lazar, D. Karadi, S. Laszlo, S.A. Mousa, S. Hosztafi, F. Zador, P. Riba, M. Schafer, S. Furst, M. Al-Khrasani, The Peripheral Versus Central Antinociception of a Novel Opioid Agonist: Acute Inflammatory Pain in Rats, *Neurochem Res*, 43 (2018) 1250-1257.10.1007/s11064-018-2542-7
- [2] P. Brumovsky, C. Hofstetter, L. Olson, G. Ohning, M. Villar, T. Hokfelt, The neuropeptide tyrosine Y1R is expressed in interneurons and projection neurons in the dorsal horn and area X of the rat spinal cord, *Neuroscience*, 138 (2006) 1361-1376.10.1016/j.neuroscience.2005.11.069
- [3] P. Brumovsky, D. Stanic, S. Shuster, H. Herzog, M. Villar, T. Hokfelt, Neuropeptide Y2 receptor protein is present in peptidergic and nonpeptidergic primary sensory neurons of the mouse, *J Comp Neurol*, 489 (2005) 328-348.10.1002/cne.20639
- [4] P.R. Brumovsky, T.J. Shi, H. Matsuda, J. Kopp, M.J. Villar, T. Hokfelt, NPY Y1 receptors are present in axonal processes of DRG neurons, *Exp Neurol*, 174 (2002) 1-10.10.1006/exnr.2001.7845
- [5] M. Diaz-delCastillo, D.P.D. Woldbye, A.M. Heegaard, Neuropeptide Y and its involvement in chronic pain, *Neuroscience*, (2017).10.1016/j.neuroscience.2017.08.050
- [6] H. Fan, N. Gong, T.F. Li, A.N. Ma, X.Y. Wu, M.W. Wang, Y.X. Wang, The non-peptide GLP-1 receptor agonist WB4-24 blocks inflammatory nociception by stimulating beta-endorphin release from spinal microglia, *Br J Pharmacol*, 172 (2015) 64-79.10.1111/bph.12895
- [7] C. Gerald, M.W. Walker, L. Criscione, E.L. Gustafson, C. Batzli-Hartmann, K.E. Smith, P. Vaysse, M.M. Durkin, T.M. Laz, D.L. Linemeyer, A.O. Schaffhauser, S. Whitebread, K.G. Hofbauer, R.I. Taber, T.A. Branchek, R.L. Weinshank, A receptor subtype involved in neuropeptide-Y-induced food intake, *Nature*, 382 (1996) 168-171.10.1038/382168a0
- [8] N. Gong, Q. Xiao, B. Zhu, C.Y. Zhang, Y.C. Wang, H. Fan, A.N. Ma, Y.X. Wang, Activation of spinal glucagon-like peptide-1 receptors specifically suppresses pain hypersensitivity, *J Neurosci*, 34 (2014) 5322-5334.10.1523/JNEUROSCI.4703-13.2014
- [9] N.S. Gregory, A.L. Harris, C.R. Robinson, P.M. Dougherty, P.N. Fuchs, K.A. Sluka, An overview of animal models of pain: disease models and outcome measures, *J Pain*, 14 (2013) 1255-1269.10.1016/j.jpain.2013.06.008

- [10] R. Guieu, E. Fenouillet, C. Devaux, Z. Fajloun, L. Carrega, J.M. Sabatier, N. Sauze, D. Marguet, CD26 modulates nociception in mice via its dipeptidyl-peptidase IV activity, *Behav Brain Res*, 166 (2006) 230-235.10.1016/j.bbr.2005.08.003
- [11] E. Heymann, R. Mentlein, Liver dipeptidyl aminopeptidase IV hydrolyzes substance P, *FEBS Lett*, 91 (1978) 360-364
- [12] H. Ikeda, J. Stark, H. Fischer, M. Wagner, R. Drdla, T. Jager, J. Sandkuhler, Synaptic amplifier of inflammatory pain in the spinal dorsal horn, *Science*, 312 (2006) 1659-1662.10.1126/science.1127233
- [13] A.B. Intondi, M.N. Dahlgren, M.A. Eilers, B.K. Taylor, Intrathecal neuropeptide Y reduces behavioral and molecular markers of inflammatory or neuropathic pain, *Pain*, 137 (2008) 352-365.10.1016/j.pain.2007.09.016
- [14] J. Jiang, Ghosh S. , Dipeptidyl Peptidase-4 (DPP-4), in, Protein Data Bank PDB-101, 2016,
- [15] T. Karl, T. Hoffmann, R. Pabst, S. von Horsten, Extreme reduction of dipeptidyl peptidase IV activity in F344 rat substrains is associated with various behavioral differences, *Physiol Behav*, 80 (2003) 123-134
- [16] B.I. Khalefa, S.A. Mousa, M. Shaqura, E. Lacko, S. Hosztafi, P. Riba, M. Schafer, P. Ferdinandy, S. Furst, M. Al-Khrasani, Peripheral antinociceptive efficacy and potency of a novel opioid compound 14-O-MeM6SU in comparison to known peptide and non-peptide opioid agonists in a rat model of inflammatory pain, *Eur J Pharmacol*, 713 (2013) 54-57.10.1016/j.ejphar.2013.04.043
- [17] K. Kiraly, M. Kozsurek, E. Lukacsi, B. Barta, A. Alpar, T. Balazsa, C. Fekete, J. Szabon, Z. Helyes, K. Bolcskei, V. Tekus, Z.E. Toth, K. Pap, G. Gerber, Z. Puskar, Glial cell type-specific changes in spinal dipeptidyl peptidase 4 expression and effects of its inhibitors in inflammatory and neuropathic pain, *Sci Rep*, 8 (2018) 3490.10.1038/s41598-018-21799-8
- [18] K. Kiraly, A.M. Lambeir, J. Szalai, A. Szentirmay, W. Luyten, I. Barna, Z. Puskar, M. Kozsurek, A.Z. Ronai, 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 (2011) 195-199.10.1016/j.ejphar.2010.09.060
- [19] K. Kiraly, B. Szalay, J. Szalai, I. Barna, K. Gyires, M. Verbeken, A.Z. Ronai, 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 (2009) 21-26.10.1016/j.ejphar.2009.08.018

- [20] C. Klemann, L. Wagner, M. Stephan, S. von Horsten, Cut to the chase: a review of CD26/dipeptidyl peptidase-4's (DPP4) entanglement in the immune system, *Clin Exp Immunol*, 185 (2016) 1-21.10.1111/cei.12781
- [21] E. Lacko, P. Riba, Z. Giricz, A. Varadi, L. Cornic, M. Balogh, K. Kiraly, K. Cseko, S.A. Mousa, S. Hosztafi, M. Schafer, Z.S. Zadori, Z. Helyes, P. Ferdinandy, S. Furst, M. Al-Khrasani, New Morphine Analogs Produce Peripheral Antinociception within a Certain Dose Range of Their Systemic Administration, *J Pharmacol Exp Ther*, 359 (2016) 171-181.10.1124/jpet.116.233551
- [22] D. Le Bars, M. Gozariu, S.W. Cadden, Animal models of nociception, *Pharmacol Rev*, 53 (2001) 597-652
- [23] B.S. Lee, I.G. Jun, S.H. Kim, J.Y. Park, Interaction of morphine and selective serotonin receptor inhibitors in rats experiencing inflammatory pain, *J Korean Med Sci*, 27 (2012) 430-436.10.3346/jkms.2012.27.4.430
- [24] Y. Li, J.J. Li, L.C. Yu, Anti-nociceptive effect of neuropeptide Y in the nucleus accumbens of rats: an involvement of opioid receptors in the effect, *Brain Res*, 940 (2002) 69-78
- [25] T.B. Mahinda, B.K. Taylor, Intrathecal neuropeptide Y inhibits behavioral and cardiovascular responses to noxious inflammatory stimuli in awake rats, *Physiol Behav*, 80 (2004) 703-711.10.1016/j.physbeh.2003.12.007
- [26] R. Mentlein, Dipeptidyl-peptidase IV (CD26)--role in the inactivation of regulatory peptides, *Regul Pept*, 85 (1999) 9-24
- [27] R. Mentlein, P. Dahms, D. Grandt, R. Kruger, Proteolytic processing of neuropeptide Y and peptide YY by dipeptidyl peptidase IV, *Regul Pept*, 49 (1993) 133-144
- [28] R. Mentlein, B. Gallwitz, W.E. Schmidt, Dipeptidyl-peptidase IV hydrolyses gastric inhibitory polypeptide, glucagon-like peptide-1(7-36)amide, peptide histidine methionine and is responsible for their degradation in human serum, *Eur J Biochem*, 214 (1993) 829-835
- [29] C. Mestre, T. Pelissier, J. Fialip, G. Wilcox, A. Eschalier, A method to perform direct transcutaneous intrathecal injection in rats, *J Pharmacol Toxicol Methods*, 32 (1994) 197-200
- [30] S.A. Mousa, M. Shaqura, U. Brendl, M. Al-Khrasani, S. Furst, M. Schafer, Involvement of the peripheral sensory and sympathetic nervous system in the vascular endothelial expression of ICAM-1 and the recruitment of opioid-containing immune cells to inhibit inflammatory pain, *Brain Behav Immun*, 24 (2010) 1310-1323.10.1016/j.bbi.2010.06.008
- [31] I. Nausch, E. Heymann, Substance P in human plasma is degraded by dipeptidyl peptidase IV, not by cholinesterase, *J Neurochem*, 44 (1985) 1354-1357

- [32] A.Z. Ronai, K. Kiraly, A. Szebeni, E. Szemenyei, Z. Prohaszka, Z. Darula, G. Toth, I. Till, B. Szalay, E. Kato, I. Barna, Immunoreactive endomorphin 2 is generated extracellularly in rat isolated L4,5 dorsal root ganglia by DPP-IV, *Regul Pept*, 157 (2009) 1-2.10.1016/j.regpep.2009.06.006
- [33] A.Z. Ronai, J. Timar, E. Mako, F. Erdo, Z. Gyarmati, G. Toth, G. Orosz, S. Furst, J.I. Szekely, Diprotin A, an inhibitor of dipeptidyl aminopeptidase IV(EC 3.4.14.5) produces naloxone-reversible analgesia in rats, *Life Sci*, 64 (1999) 145-152
- [34] I. Szatmari, G. Toth, I. Kertesz, P.W. Schiller, A. Borsodi, Synthesis and binding characteristics of [3H] H-Tyr-Ticpsi[CH<sub>2</sub>-NH] Cha-Phe-OH, a highly specific and stable delta-opioid antagonist, *Peptides*, 20 (1999) 1079-1083
- [35] O.B. Taiwo, B.K. Taylor, Antihyperalgesic effects of intrathecal neuropeptide Y during inflammation are mediated by Y1 receptors, *Pain*, 96 (2002) 353-363
- [36] C. Tomboly, A. Peter, G. Toth, In vitro quantitative study of the degradation of endomorphins, *Peptides*, 23 (2002) 1573-1580
- [37] J.Z. Wang, Microinjection of neuropeptide Y into periaqueductal grey produces antinociception in rats with mononeuropathy, *Sheng Li Xue Bao*, 56 (2004) 79-82
- [38] Y. Yang, Y.G. Zhang, G.A. Lin, H.Q. Xie, H.T. Pan, B.Q. Huang, J.D. Liu, H. Liu, N. Zhang, L. Li, J.H. Chen, Spinal changes of a newly isolated neuropeptide endomorphin-2 concomitant with vincristine-induced allodynia, *PLoS One*, 9 (2014) e89583.10.1371/journal.pone.0089583
- [39] Y. Zhang, T. Lundeberg, L. Yu, Involvement of neuropeptide Y and Y1 receptor in antinociception in nucleus raphe magnus of rats, *Regul Pept*, 95 (2000) 109-113
- [40] L. Zhu, C. Tamvakopoulos, D. Xie, J. Dragovic, X. Shen, J.E. Fenyk-Melody, K. Schmidt, A. Bagchi, P.R. Griffin, N.A. Thornberry, R. Sinha Roy, The role of dipeptidyl peptidase IV in the cleavage of glucagon family peptides: in vivo metabolism of pituitary adenylate cyclase activating polypeptide-(1-38), *J Biol Chem*, 278 (2003) 22418-22423.10.1074/jbc.M212355200