

# Novel bifunctional hybrid compounds designed to enhance the effects of opioids and antagonize the pronociceptive effects of nonopioid peptides as potent analgesics in a rat model of neuropathic pain

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## Abstract

The purpose of our work was to determine the role of nonopioid peptides derived from opioid prohormones in sensory hypersensitivity characteristics of neuropathic pain and to propose a pharmacological approach to restore the balance of these endogenous opioid systems. Nonopioid peptides may have a pronociceptive effect and therefore contribute to less effective opioid analgesia in neuropathic pain. In our study, we used unilateral chronic constriction injury (CCI) of the sciatic nerve as a neuropathic pain model in rats. We demonstrated the pronociceptive effects of proopiomelanocortin- and proenkephalin-derived nonopioid peptides assessed by von Frey and cold plate tests, 7 to 14 days after injury. The concentration of proenkephalin-derived pronociceptive peptides was increased more robustly than that of Met-enkephalin in the ipsilateral lumbar spinal cord of CCI-exposed rats, as shown by mass spectrometry, and the pronociceptive effect of one of these peptides was blocked by an antagonist of the melanocortin 4 (MC4) receptor. The above results confirm our hypothesis regarding the possibility of creating an analgesic drug for neuropathic pain based on enhancing opioid activity and blocking the pronociceptive effect of nonopioid peptides. We designed and synthesized bifunctional hybrids composed of opioid (OP) receptor agonist and MC4 receptor antagonist (OP-linker-MC4). Moreover, we demonstrated that they have potent and long-lasting antinociceptive effects after a single administration and a delayed development of tolerance compared with morphine after repeated intrathecal administration to rats subjected to CCI. We conclude that the bifunctional hybrids OP-linker-MC4 we propose are important prototypes of drugs for use in neuropathic pain.

**Keywords:** Chronic pain, Neuropathy, MC3 and MC4 receptors, Proenkephalin system, Bifunctional compounds

## 1. Introduction

Neuropathic pain, which develops as a consequence of damage to the nervous system, is characterized by poor responsiveness to available therapies; therefore, it seriously lowers patients' quality of life.<sup>18,27</sup> Shortly after injury, endogenous antinociceptive factors, such as proopiomelanocortin (POMC), proenkephalin (PENK), and prodynorphin (PDYN), are activated<sup>20,39,49,51,76,81,84</sup> for some time.

Opioid prohormones generate not only antinociceptive peptides ( $\beta$ -endorphins and enkephalins) but also a number of nonopioid pronociceptive peptides, such as melanocortins (MSHs), adrenocorticotropic hormone (ACTH), and des-Tyr-dynorphin, which exert their pronociceptive effects through nonopioid receptors. After searching the UniProt database for PENK-derived nonopioid peptides, we synthesized selected peptides and measured their nociceptive effects and concentration in the spinal cord in a rat model of neuropathic pain. The above information allowed us to hypothesize that some nonopioid peptides derived from opioid prohormones have an important role in weakening opioid activity; therefore, they became the subjects of our study on new drugs for the treatment of neuropathic pain. Currently, opioids are third-line drugs for the treatment of neuropathic pain because of their reduced effectiveness,<sup>15</sup> which can be explained by the pathological activation of pronociceptive systems.<sup>30,68,69</sup> Interestingly, antinociceptive endogenous opioid peptides are derived from the same prohormone molecule as pronociceptive peptides, eg, POMC-derived antinociceptive  $\beta$ -endorphin and pronociceptive melanocortin.<sup>10,43,60</sup> Of the 5 types of melanocortin receptors, only the MC4 receptor is present in the spinal cord, where it modulates nociceptive processes.<sup>34,65,79</sup> The intrathecal (i.t.) administration of MC4 receptor agonists has been shown to cause hypersensitivity in naive rats,<sup>3,61,72</sup> and MC4 receptor antagonists provide analgesic effects, including preventing the development of morphine tolerance after peripheral nerve injury.<sup>14,26,59,73</sup> Considering the abovementioned

Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

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PAIN 162 (2021) 432–445

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<http://dx.doi.org/10.1097/j.pain.0000000000002045>

findings, we hypothesize that the simultaneous activation of the opioid system by an  $\delta$ -opioid peptide (DOP) receptor agonist and antagonism of the pronociceptive system associated with the MC4 receptor will bring an increased antinociceptive effect compared with that of these strategies used separately. In contrast to the simultaneous administration of the 2 individual agents alone, which results in differences in their tissue distribution and metabolism, bifunctional hybrid compounds target the same parts of pain pathways and can effectively enhance their functioning.<sup>7,21,22,54,55</sup>

The goal of our study was to test the hypothesis that reduced opioid effectiveness in neuropathic pain may result from the pathological activation of pronociceptive systems. The results of current studies were the basis for developing a new drug model consisting of 2 pharmacophores, namely, an antinociceptive opioid and an antagonist of the pronociceptive MC4 receptor, linked by specially designed various spacers.<sup>77</sup> We tested the analgesic properties and development of tolerance to 2 hybrids from a series of newly synthesized hybrids with an opioid (OP)-linker-MC4 structure, namely, UW3 and UW5 (which are protected by patent application no. PL422093/P31312PL00/AGR), in rat models of neuropathic pain.

## 2. Methods

### 2.1. Animals

Male Wistar rats (200-260 g) purchased from Charles River Breeding Laboratories, Germany, were housed according to the procedures outlined in our previous articles.<sup>47,50,55</sup> All examinations were conducted in accordance with the recommendations of the International Association for the Study of Pain,<sup>83</sup> the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the Animal Research: Reporting of In Vivo Experiments guidelines.<sup>28</sup> These studies were approved by the Ethical Committee of the Maj Institute of Pharmacology of the Polish Academy of Sciences (Local Ethics Committees: 962/2012, 1213/2015, 212/2019). As to apply the current 3R principle, where the 3R abbreviation stands for replacement (understood as avoiding animal experiments where possible), reduction (minimizing the number of animals), and refinement (limiting the possible suffering of the animals to an absolute minimum), we reduced the number of rats used in the experiments to the minimum. The animals were assigned randomly to individual research groups, based on a single sequence of random assignments (simple randomization—odd/even methods).<sup>2,63</sup> The exact number of animals in each study group is mentioned in the legend below the figures.

### 2.2. Intrathecal catheter implantation

The rats were chronically implanted with i.t. catheters according to the method described by Yaksh and Rudy.<sup>78</sup> The operation was performed under pentobarbital anesthesia (60 mg/kg, intraperitoneally, i.p.), as in previous studies.<sup>47,50,55</sup> A polyethylene tubing catheters (13 cm long, PE 10, intramedic; Clay Adams, Parsippany, NJ) were introduced into the subarachnoid space through the atlanto-occipital membrane. Once it reached the rostral level of the spinal cord lumbar enlargement (L4-L6), it was carefully flushed with 10  $\mu$ L of water for injections. As the last step, the tip was tightened. After catheter implantation, the animals were monitored and possible physical impairments were assessed, so as the rats could recover before the actual experiment (minimum of 1 week). Rats with visible motor deficits (approximately 2%-5%) were eliminated from further study.

### 2.3. Chronic constriction injury of the sciatic nerve

The chronic constriction injury (CCI) model was established according to the procedure developed by Bennett and Xie.<sup>5</sup> The surgical method is routinely performed in our laboratory under sodium pentobarbital anesthesia (60 mg/kg, i.p.).<sup>46,47,55</sup> Seven days after surgery, the animals were examined for their health status and development of neuropathic pain behavior. All rats that underwent the CCI procedure (CCI-exposed rats) developed hypersensitivity to mechanical and thermal stimuli. Behavioral experiments were performed on days 7 to 14 after the CCI procedure. Our previous research indicates insignificant differences between naive and sham-operated animals in behavioral studies measuring the pain threshold, as well as in biochemical studies measuring the level of nociceptive factors.<sup>50</sup>

### 2.4. Drugs

In the experiment, we used the following substances.

#### 2.4.1. Proopiomelanocortin-derived peptides

Adrenocorticotrophic hormone (1, 10, and 50  $\mu$ g/5  $\mu$ L) (3492; Tocris, Bristol, United Kingdom),  $\alpha$ -MSH (1, 10, and 50  $\mu$ g/5  $\mu$ L) (2584; Tocris), corticotropin-like intermediate lobe peptide (CLIP 1, 5, 10, and 50  $\mu$ g/5  $\mu$ L) (MBS659645; MyBioSource, San Diego, CA), and  $\gamma$ -MSH (1, 10, and 50  $\mu$ g/5  $\mu$ L) (4272; Tocris) were used.

#### 2.4.2. Proenkephalin-derived peptides

Met-enkephalin (1, 10, and 50  $\mu$ g/5  $\mu$ L) (MBS659922; MyBioSource), peptide E (1, 10, and 50  $\mu$ g/5  $\mu$ L) (30-3-25; American peptide, Sunnyvale, CA), bovine adrenal medullary peptide (BAM22, 0.5, 1, 10, and 50  $\mu$ g/5  $\mu$ L) (1650; Tocris), BAM8-22 (0.5, 1, 10, and 50  $\mu$ g/5  $\mu$ L) (1763; Tocris), and peptides synthesized by us for these experiments: FAE (0.5, 1, 10, and 50  $\mu$ g/5  $\mu$ L), VGR, known as BAM8-18 (0.5, 1, 10, and 50  $\mu$ g/5  $\mu$ L), and SPQ (1, 10, and 50  $\mu$ g/5  $\mu$ L) were used.

#### 2.4.3. Novel bifunctional opioid-linker-MC4 hybrids

Two active pharmacophores conjugated in a single molecule, UW3 (0.001, 0.01, and 0.1  $\mu$ g/5  $\mu$ L) and UW5 (0.001, 0.01, 0.1, and 1  $\mu$ g/5  $\mu$ L), and single pharmacophores (the active pharmacophores of the hybrids in separate molecules), UW1 (0.001, 0.01, and 0.1  $\mu$ g/5  $\mu$ L)<sup>38</sup> and SHU9119,<sup>24</sup> which are denoted as "parents," were used.

#### 2.4.4. Receptor antagonist

SHU9119 (0.01, 0.1, and 1  $\mu$ g/5  $\mu$ L), an MC3/4 receptor antagonist (3420; Tocris), was used.

#### 2.4.5. Opioid analgesic

Morphine hydrochloride (20  $\mu$ g/5  $\mu$ L) (M; Fagron, Cracow, Poland) and UW1/enkephalin were used.

#### 2.4.6. Synthesis of peptides from proenkephalin

The following 3 fragments of proenkephalin A, which were selected based on target proteolytic enzyme cleavage sites and the lack of the characteristic sequence Tyr-Gly-Gly-Phe for binding to the opioid receptor, were synthesized as follows:

- (1) A 239- to 260-bp fragment with the sequence H-Phe-Ala-Glu-Ser-Leu-Pro-Ser-Asp-Glu-Glu-Gly-Glu-Ser-Tyr-Ser-Lys-Glu-Val-Pro-Glu-Met-Glu-OH (FAE);
- (2) A 219- to 229-bp fragment with the sequence H-Val-Gly-Arg-Pro-Glu-Trp-Trp-Met-Asp-Tyr-Gln-OH (VGR); and
- (3) A 198- to 209-bp fragment with the sequence H-Ser-Pro-Gln-Leu-Glu-Asp-Glu-Ala-Lys-Glu-Leu-Gln-OH (SPQ).

All 3 peptides which we called FAE, VGR, and SPQ, according to the first 3 amino acids of the N-terminus, were prepared by solid phase peptide synthesis using standard Fmoc/tBu methodology. Preloaded Wang resins and common Fmoc-protected amino acid building blocks in the standard Fmoc-Xaa-OH/TBTU/DIPEA protocol (2eq/2eq/4eq, respectively) were used. The final products of the synthesis were purified by reversed-phase high-performance liquid chromatography. The molecular weights of peptides were confirmed by high-resolution electrospray ionization mass spectrometry (ESI-MS) analysis, with results as follow:

FAE: m/z obsd. 830,3571 [M + 3H]<sup>3+</sup>, m/z calcd. 830,3565 [M + 3H]<sup>3+</sup>;  
 VGR: m/z obsd. 733,8333 [M + 2H]<sup>2+</sup>, m/z calcd. 733,8297 [M + 2H]<sup>2+</sup>; and  
 SPQ: m/z obsd. 693,8408 [M + 2H]<sup>2+</sup>, m/z calcd. 693,8410 [M + 2H]<sup>2+</sup>.

#### 2.4.7. Synthesis of the enkephalin analogue UW1 and the hybrid compounds UW3 and UW5

Peptides with the following sequences were synthesized:

Tyr<sup>1</sup>-D-Ala<sup>2</sup>-Gly<sup>3</sup>-Phe<sup>4</sup>-NH<sub>2</sub> (UW1);  
 Tyr<sup>1</sup>-D-Ala<sup>2</sup>-Gly<sup>3</sup>-Phe<sup>4</sup>-Ahx<sup>5</sup>-Nle<sup>6</sup>-cyclo[Asp<sup>7</sup>-His<sup>8</sup>-D-Nal(2')<sup>9</sup>-Arg<sup>10</sup>-Trp<sup>11</sup>-Lys<sup>12</sup>]-NH<sub>2</sub> (UW3); and  
 Tyr<sup>1</sup>-D-Ala<sup>2</sup>-Gly<sup>3</sup>-Phe<sup>4</sup>-Ahx<sup>5</sup>-Ahx<sup>6</sup>-Nle<sup>7</sup>-cyclo[Asp<sup>8</sup>-His<sup>9</sup>-D-Nal(2')<sup>10</sup>-Arg<sup>11</sup>-Trp<sup>12</sup>-Lys<sup>13</sup>]-NH<sub>2</sub> (UW5).

Ahx indicates 6-aminoheptanoic acid, and D-Nal(2') indicates 3-(2'-naphthyl)-D-alanine.

All peptides were synthesized on an MBHA resin (Bachem, 0.27 mmol/g) using the standard Boc strategy and carbodiimide (DIC) as the coupling reagent. The final crude peptides were purified by semipreparative high-performance liquid chromatography in reversed phase and characterized by the electrospray ionization mass spectrometry (ESI-MS) method. The results of ESI-MS analysis are as follows:

UW1: M.W. [u]: 455.5; ion [M + H]<sup>+</sup> m/z calculated: 456.5, m/z observed: 456.2;

UW3: M.W. [u]: 1583.8; ion [M + 3H]<sup>3+</sup> m/z calculated: 528.6, m/z observed: 528.0; and

UW5: M.W. [u]: 1697.0; ion [M + 3H]<sup>3+</sup> m/z calculated: 566.6, m/z observed: 566.6.

A more detailed description of the synthesis of UW3 and UW5 will be presented in a separate article (in preparation).

All abbreviations of the substances used with additional information are summarized in Table 1 in supplementary material (Supplemental Table 1, available at <http://links.lww.com/PAIN/B149>).

Drugs were dissolved in water for injection (Polpharma, Starogard Gdański, Poland) and administered i.t. in a volume of 5 μL through the implanted cannula. The drug-treated groups were compared with a group-administered vehicle (water for injection) in the same fashion. Single-measurement tests (of the effects of POMC- and PENK-derived peptides) were performed 15 to 30 minutes after i.t. drug administration on days 7 to 14 after CCI. Repeated-measurement tests of analgesic effects (of the hybrids UW3 and UW5) were performed 15, 30, 120, and 240 minutes after i.t. administration on days 7 to 14 after CCI. Tolerance

measurement tests were performed 60 minutes after i.t. drug administration on days 7 to 13 after CCI.

## 2.5. Behavioral tests

### 2.5.1. Control (naive) rats

#### 2.5.1.1. Tail-flick test

The tail-flick test was performed with a Tail-Flick Analgesic Meter (Ugo Basile, Comerio, Italy) to assess the pain threshold induced by a thermal stimulus as previously described.<sup>46,55</sup> During the test, the rat was gently restrained by the experimenter. The light beam was focused about 2 to 3 cm from the dorsal part of the tail. When the tail of the animal flicked, the timer was stopped and the time (latency) was automatically recorded. The cutoff time was 9 seconds.

### 2.5.2. Chronic constriction injury-exposed rats

#### 2.5.2.1. von Frey test

The von Frey test was performed with a Dynamic Plantar Anesthesiometer (Cat. No. 37400; Ugo Basile, Gemonio, Italy) to measure mechanical hypersensitivity in rats as described by us in previous publications.<sup>36,47</sup> The animals were placed in cages with a wire mesh floor. The reaction of the ipsilateral and contralateral paws of CCI rats (or both hind paws of naive rats) to the touch stimulator applied through the mesh floor was measured automatically. The maximum strength of stimulation exerted by the von Frey filament was 26 g.

#### 2.5.2.2. Cold plate test

The cold plate test was performed with a Cold/Hot Plate Analgesia Meter (No. 05044; Columbus Instruments, Columbus) to measure thermal hypersensitivity in rats as previously described.<sup>36,47</sup> The rats were placed on a plate (5°C). First, the ipsilateral paw response was recorded, followed by the measurement of the contralateral paw response. In naive rats, the reaction of either hind paw was recorded. The cutoff time was 30 seconds.

## 2.6. Biochemical tests

### 2.6.1. Mass spectrometry

All liquid chromatography–mass spectrometry quantitation analyses (LC-MS) were performed using a triple quadrupole mass spectrometer (LCMS8050; Shimadzu, Kyoto, Japan) coupled to a UPLC Nexera X2 system (Shimadzu). Quantification of particular compounds was performed in Multi Reaction Monitoring mode, and for each compound, 2 transition pairs were selected. One transition pair was used for quantification, and the second pair was used for the identification and confirmation of the structures. The selected transition pairs for that compounds were as follows: FAE (A): 830/505 and 830/120, VGR (B): 734/134 and 734/130, SPQ (C): 694/129 and 694/101, and Met-enkephalin (D): 439/136 and 439/120.

The quantified compounds were separated on an Aeris Peptide chromatographic columns (1.7 μm, 2.1 × 100 mm) (Phenomenex, Torrance, CA). Mobile phase A was a solution of 0.1% formic acid in water (vol/vol), and for mobile phase B, pure acetonitrile was applied. Separation was conducted in gradient mode with the following conditions: 0 to 1 minute: 10% B, 1 to 20 minutes: 36% B, 20 to 22 minutes: 95% B, 22 minutes: 10% B, and 23 to 30 minutes: 10% B.

The mobile phase flow rate was 0.3 mL/minute. The oven temperature was set at 40°C.

The mass spectrometer conditions were set as follows: electrospray ion source operated in positive ionization mode, nebulizing gas flow of 3 L/min, heating gas flow of 10 L/min, drying gas flow of 10 L/min, interface temperature of 300°C, desolvation line temperature of 200°C, heat block temperature of 500°C, collision-induced dissociation gas pressure of 270 kPa, and IS of +2.5 kV.

The standard addition method, in which 20-, 50-, and 100-ng/mL standards of each compound were added to the samples after solid-phase extraction, was used for quantification.

### 2.6.2. Quantitative real-time reverse-transcription polymerase chain reaction

Ipsilateral, the dorsal fragment of the lumbar spinal cord, at approximately L4–L6, was collected immediately after decapitation of the rats on days 2, 7, and 14 after CCI procedure. Total RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the method developed by Chomczynski and Sacchi.<sup>13</sup> The RNA extraction and RNA concentration measurement procedures as well as reverse transcriptase process and quantitative real-time polymerase chain reaction (RT-qPCR) are routinely conducted in our laboratory and thoroughly described in previous publications.<sup>48,50</sup> The CFX96 real-time PCR detection system (Bio-Rad, Hercules, Warsaw, Poland) and Assay-On-Demand TaqMan probes (Applied Biosystems, Foster City, CA) were used for RT-qPCR, and the reaction was performed according to the manufacturer's protocol. The following TaqMan probes were used: Rn00561699\_m1 (Opr1, opioid receptor,  $\delta 1$ ); Rn01491866\_s1 (Mc4r, melanocortin 4 receptor); Rn00567566\_m1 (PENK, proenkephalin); and Rn01527838\_g1 (hypoxanthine-guanine phosphoribosyltransferase, HPRT). The amplification efficiency of each assay was determined (between 1.7 and 2) using a standard dilution curve. The CFX Manager v.2.1 software was used to automatically generate cycle thresholds values. The calculation of RNA concentration was performed using the formula 2 (threshold cycle). HPRT was selected as an appropriate basal metabolism gene because its transcript levels do not change significantly in rats after CCI.<sup>39</sup>

### 2.6.3. Western blot analysis

Ipsilateral, dorsal fragments of the lumbar spinal cord, at approximately L4–L6, were collected immediately after decapitation of the rats on days 2, 7, and 14 after CCI procedure. The procedures for tissue homogenization, measurement of protein concentration in the supernatant, and sample preparation for electrophoresis were developed in our laboratory and described in previous publications.<sup>46–48</sup> The samples were run on 4% to 20% Criterion TGX precast midi protein polyacrylamide gels (Bio-Rad). Next, semidry transfer system was used (Biorad), and proteins were transferred to Immune-Blot PVDF membranes, according to the manufacturer's standard protocol (30 minutes, 25 V). In the following stages, the membranes were blocked by immersion in Tris-buffered saline with 0.1% Tween 20 (TBST) with 5% nonfat dry milk (Bio-Rad) for 1 hour at room temperature. Subsequently, the membranes were washed with TBST and incubated overnight at 4°C with the following primary antibodies: rat anti-MC4 receptor (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, sc-31478), rabbit anti-DOP receptor (1:200, Santa Cruz Biotechnology, sc-9113), rabbit anti-PENK (1:5000, LSBio, Seattle, WA, LS-B15645), and mouse anti-GAPDH (1:5000, Merck Millipore, Darmstadt, Germany, MAB374).

Next, the membranes were incubated with a horseradish peroxidase-conjugated anti-rabbit (1:5000, Vector, Burlingame, CA, PI-1000) or anti-mouse (1:5000, Vector, PI-2000) secondary antibodies for at least 1 hour. Then, the blots were washed in TBST. Immune complexes were visualized with Clarity Western ECL Substrate (Bio-Rad), Fujifilm LAS-4000 FluorImager system and analyzed with the Fujifilm Multi Gauge software. After visualization, membranes were washed in TBST and reprobbed with an antibody, this time against GAPDH as internal load control. The proteins levels of the DOP and MC4 receptors and PENK were normalized to internal references and are presented as the ratio of DOP receptor, MC4 receptor, or PENK signal to the GAPDH signal.

### 2.7. Statistical analysis

The behavioral data obtained in the experiments are presented as %MPE  $\pm$  SEM, which stands for the percentage of the maximal possible antinociceptive effect  $\pm$  SEM. The values were calculated as follows: %MPE = [(TL – BL)/(cutoff value – BL)]  $\times$  100% (BL = baseline latency; TL = latency obtained after drug injection). The obtained data were analyzed statistically using 1-way analysis of variance. Bonferroni post hoc tests were used to further analyze the differences between the treatment groups. An animal was excluded from the experiments if its basal response (baseline latency) in the behavioral tests was abnormal, or if a technical error, such as i.t. catheter obstruction making the injection of the drug impossible, occurred.

## 3. Results

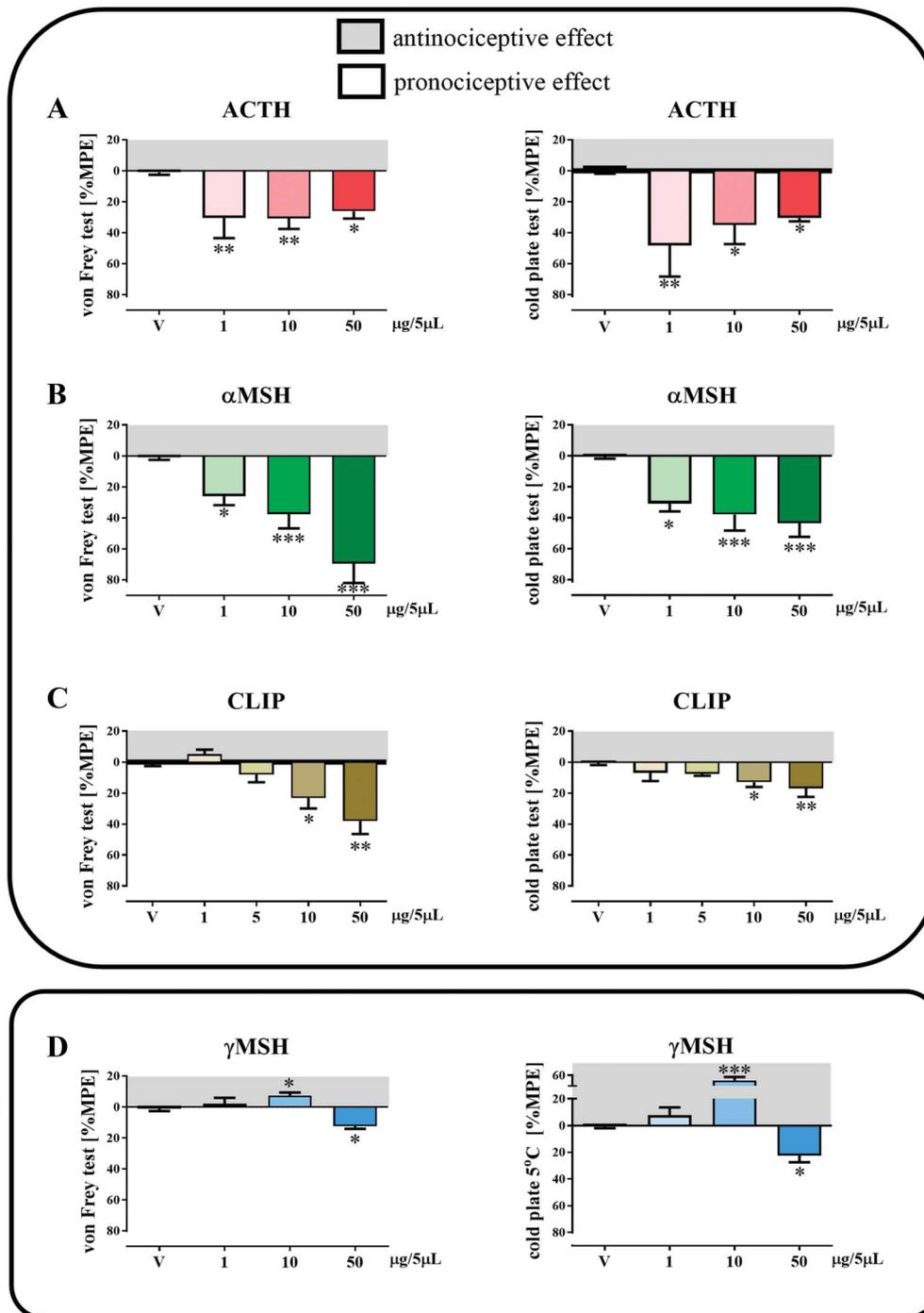
### 3.1. Dose-dependent pronociceptive effect of a single intrathecal administration of proopioidmelanocortin-derived peptides (ACTH, $\alpha$ -melanocortin, CLIP, and $\gamma$ -melanocortin) on nociceptive transmission in chronic constriction injury-exposed rats

Single i.t. administration of different doses of ACTH (1, 10, and 50  $\mu$ g/5  $\mu$ L),  $\alpha$ -MSH (1, 10, and 50  $\mu$ g/5  $\mu$ L), and CLIP (10 and 50  $\mu$ g/5  $\mu$ L) increased mechanical and thermal hypersensitivity, as measured 7 to 14 days after CCI using the von Frey and cold plate tests (Figs. 1A–C), respectively ( $P < 0.05$ ,  $P < 0.01$ ,  $P < 0.001$ ). In the von Frey and cold plate tests, no significant pronociceptive effects of the low dose of  $\gamma$ -MSH (1  $\mu$ g/5  $\mu$ L) were observed (Fig. 1D). However, one of the higher doses (10  $\mu$ g/5  $\mu$ L) diminished pain-related behavior ( $P < 0.05$  in the von Frey test,  $P < 0.001$  in the cold plate test). Interestingly, an even higher dose evoked mechanical and thermal hypersensitivity ( $P < 0.05$ ) (Fig. 1D).

### 3.2. Dose-dependent antinociceptive effect of a single intrathecal administration of PENK-derived peptides (Met-enkephalin, peptide E, BAM22, and BAM8-22) on nociceptive transmission in chronic constriction injury-exposed rats

Single i.t. administration of different doses of Met-enkephalin (1, 10, and 50  $\mu$ g/5  $\mu$ L), peptide E (1, 10, and 50  $\mu$ g/5  $\mu$ L), BAM22 (0.5, 1, 10, and 50  $\mu$ g/5  $\mu$ L), and BAM8-22 (0.5, 1, 10, and 50  $\mu$ g/5  $\mu$ L) significantly decreased mechanical and/or thermal hypersensitivity, as measured 7 days after CCI using the von Frey and cold plate tests (data not shown), respectively ( $P < 0.05$ ,  $P < 0.01$ ,  $P < 0.001$ ). In the von Frey test, but not the cold plate test, no significant antinociceptive effects were observed after the administration of the low doses of BAM8-22 (0.5, 1, and 10  $\mu$ g/5  $\mu$ L) (data shown in supplementary materials—Supplemental Figure 1, available at <http://links.lww.com/PAIN/B149>).

# Peptides synthesized from the POMC



**Figure 1.** Dose-dependent pronociceptive effects (top panel) or pronociceptive and/or antinociceptive effects (bottom panel) of intrathecal (i.t.) administration of ACTH (A),  $\alpha$ MSH (B), CLIP (C), and  $\gamma$ MSH (D) measured by the von Frey and cold plate tests 15 minutes after administration in CCI-exposed rats (6–10 animals per group) were normalized and are expressed as the %MPE (mean  $\pm$  SEM). Intergroup differences were analyzed using 1-way ANOVA followed by the Bonferroni multiple comparisons test. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs vehicle (V)-treated CCI-exposed rats. ACTH, adrenocorticotropic hormone; ANOVA, analysis of variance; CCI, chronic constriction injury; MSH, melanocortin.

### 3.3. Dose-dependent pronociceptive and/or antinociceptive effect of a single intrathecal administration of selected and synthesized for our experiments proenkephalin-derived peptides (SPQ, FAE, and VGR) on nociceptive transmission in chronic constriction injury-exposed rats

Single i.t. administration of all doses of SPQ (1, 10, and 50  $\mu$ g/5  $\mu$ L) produced significant mechanical and thermal hypersensitivity, as

measured 7 days after CCI using the von Frey and cold plate tests (Fig. 2A), respectively ( $P < 0.05$ ,  $P < 0.01$ ,  $P < 0.001$ ). In addition, single i.t. administration of low doses of FAE (0.5 and 1  $\mu$ g/5  $\mu$ L) and VGR (1  $\mu$ g/5  $\mu$ L) produced mechanical and thermal hypersensitivity in both tests (Figs. 2B and C) ( $P < 0.05$ ). In the von Frey test and cold plate test, no significant effects were observed after the administration of a higher dose of FAE or VGR (10  $\mu$ g/5  $\mu$ L) (Figs. 2B

and C). However, the highest dose of FAE or VGR (50  $\mu\text{g}/5\ \mu\text{L}$ ) significantly reduced pain-related behavior in both tests (Figs. 2B and C) ( $P < 0.01$ ,  $P < 0.001$ ).

**3.4. Effect of a single intrathecal administration of the MC4 receptor antagonist SHU9119 on the pronociceptive effect of the proenkephalin-derived peptide SPQ in chronic constriction injury-exposed rats**

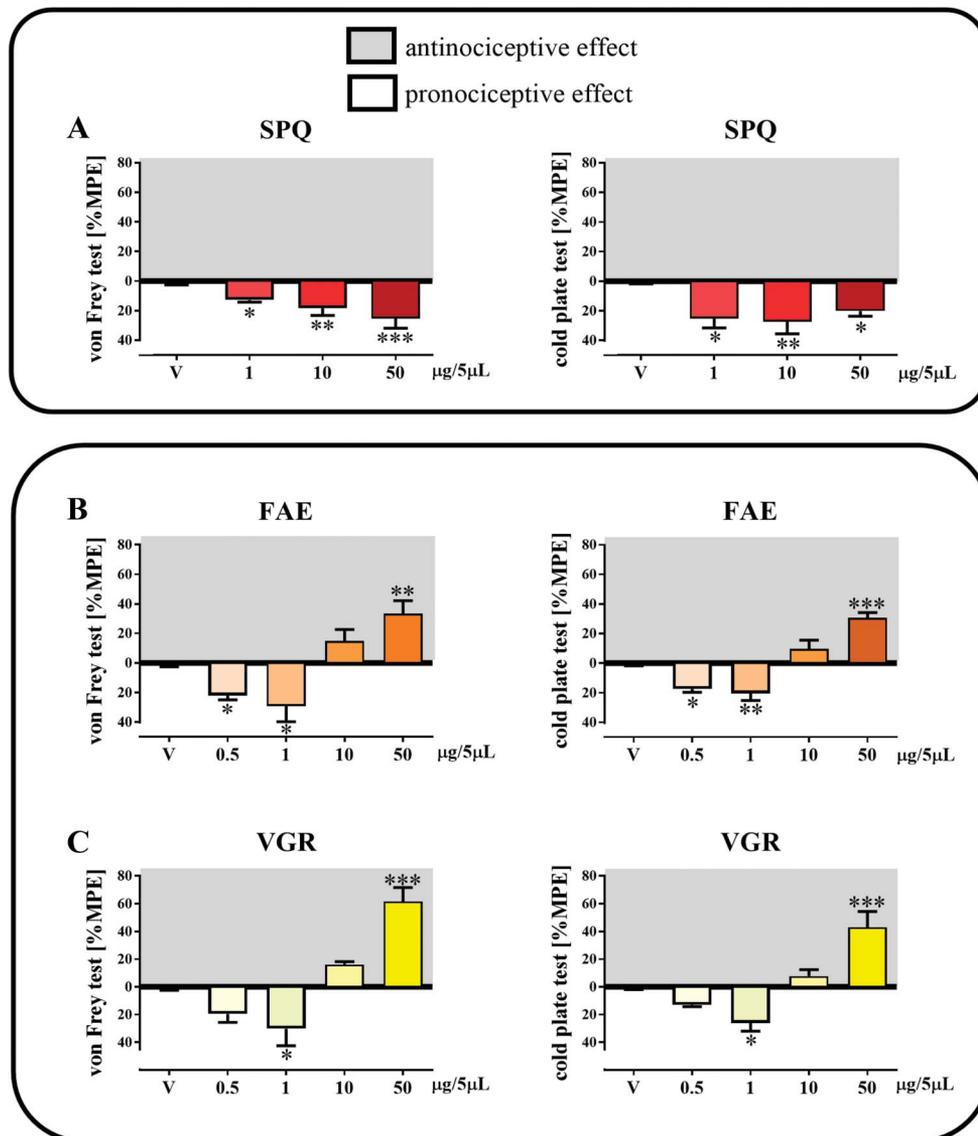
To evaluate the involvement of the MC4 receptor in SPQ activity, an experiment was performed as shown in the diagram (Fig. 3A). Single i.t. administration of 2 doses of SPQ (1 and 10  $\mu\text{g}/5\ \mu\text{L}$ ) produced significant mechanical hypersensitivity, as measured 7 days after CCI using the von Frey test (Figs. 3B and C,  $P < 0.05$ ). We observed a similar result in the cold plate test, with significant differences being found for both doses of SPQ (Figs. 3B and C,  $P < 0.05$ ). Earlier administration of SHU9119, an MC4 receptor antagonist, produced an analgesic effect and completely blocked

the pronociceptive effect of SPQ, causing significant differences in performance in both tests between the animals treated with SPQ alone and those treated with SPQ after SHU9119 in both tests (Figs. 3B and C,  $P < 0.001$ ).

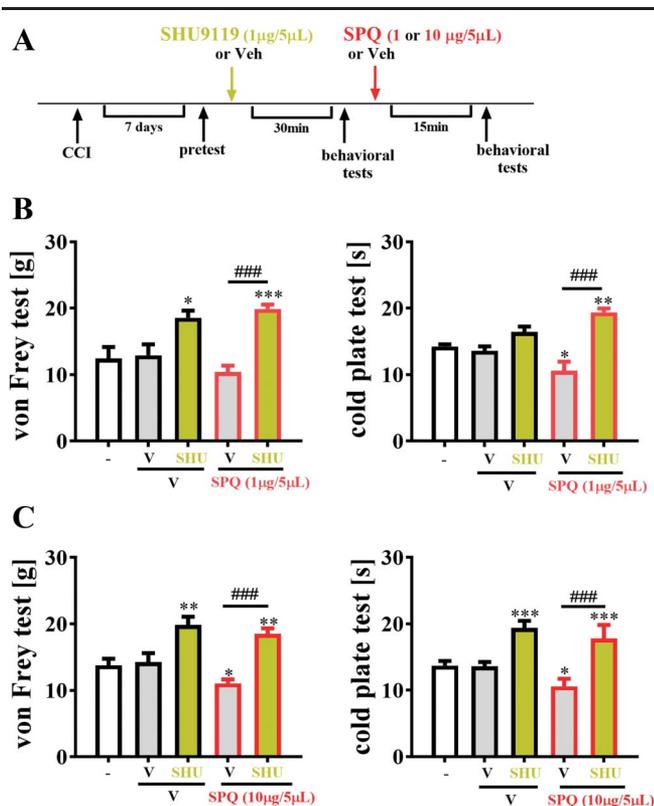
**3.5. Changes in the concentration of FAE, VGR, SPQ, and Met-enkephalin (ng/mL) in ipsilateral and contralateral dorsal lumbar spinal cord tissue from chronic constriction injury-exposed rats**

Mass spectrometry analysis of the spinal cord showed that the concentration of FAE remained unchanged after CCI (Fig. 4A). On the ipsilateral side, the concentration of VGR was significantly enhanced only on the 14th day in comparison with that in naive mice (Fig. 4B) ( $P < 0.01$ ). In addition, on day 14 after CCI, the concentration of VGR was significantly enhanced on the ipsilateral side in comparison with the contralateral side (Fig. 4B) ( $P < 0.01$ ). Similarly, on the

**Peptides synthesized from the PENK**



**Figure 2.** Dose-dependent pronociceptive effects (top panel) or pronociceptive and/or antinociceptive effects (bottom panel) of intrathecal (i.t.) administration of PENK-derived peptides: SPQ (A), FAE (B), and VGR (C) measured in the von Frey and cold plate tests 15 minutes after administration in CCI-exposed rats (6-10 animals per group) were normalized and are expressed as the %MPE (mean  $\pm$  SEM). Intergroup differences were analyzed using 1-way ANOVA followed by the Bonferroni multiple comparisons test. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs vehicle (V)-treated CCI-exposed rats. ANOVA, analysis of variance; CCI, chronic constriction injury; PENK, proenkephalin; V, vehicle-treated group.



**Figure 3.** Influence of single administration of SHU9119 on the effects of a single injection of SPQ and nociceptive transmission in CCI-exposed rats (B and C). Schematic of drug administration 7 days after CCI in rats (A). Single intrathecal injection of SHU9119 (1 µg/5 µL) or vehicle (V) was administered 30 minutes before the administration of single intrathecal injection of SPQ or V (1 or 10 µg/5 µL). The effects of drug administration on mechanical hypersensitivity (von Frey test; B and C) and thermal hypersensitivity (cold plate test; B and C) were measured 30 minutes after SHU9119 injection and then 15 minutes after SPQ injection (as shown in scheme A). The data are presented as the mean ± SEM (5–6 rats per group). The results were evaluated using 1-way ANOVA followed by the Bonferroni test for comparisons of selected pairs. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  indicate differences in comparison with V + V-treated animals. #### $P < 0.001$  indicate differences in comparison with V + SPQ-treated animals. ANOVA, analysis of variance; SHU, SHU9119; V, vehicle.

ipsilateral side, the concentration of SPQ was significantly enhanced on the 14th day in comparison with that in naive mice (Fig. 4C) ( $P < 0.001$ ). In addition, on days 7 and 14 after CCI, the concentration of SPQ was strongly increased on the ipsilateral side in comparison with the contralateral side (Fig. 4C) ( $P < 0.001$ ). On the ipsilateral side, the concentration of Met-enkephalin was significantly increased in the spinal cord on day 14 after CCI in comparison with that in naive mice (Fig. 4D) ( $P < 0.05$ ). The concentration of Met-enkephalin was enhanced on the ipsilateral side in comparison with the contralateral side 14 days after CCI (Fig. 4D) ( $P < 0.05$ ).

### 3.6. Time course of changes in the mRNA and protein levels of the $\delta$ -opioid peptide and MC4 receptors and proenkephalin in the spinal cord on the 2nd, 7th, and 14th days after chronic constriction injury in rats

Reverse transcriptase process and quantitative real-time polymerase chain analysis of the spinal cord showed that the mRNA level of DOP receptor was downregulated 2, 7, and 14 days after CCI (Fig. 5A) ( $P < 0.001$ ,  $P < 0.001$ , and  $P < 0.05$ , respectively).

The mRNA level of the MC4 receptor in the spinal cord was decreased on the 7th and 14th days (Fig. 5B) ( $P < 0.05$ ). The mRNA level of PENK was strongly decreased 2 and 7 days after CCI (Fig. 5C) ( $P < 0.001$ ).

Western blot analysis of the spinal cord showed that the protein level of the DOP receptor was increased 7 and 14 days after CCI (Fig. 5D) ( $P < 0.05$ ). No significant changes in the protein level of the MC4 receptor were observed after CCI (Fig. 5E). However, an increase in the protein level of PENK was detected on the 2nd ( $P < 0.05$ ) and 14th ( $P < 0.01$ ) days after CCI (Fig. 5F).

### 3.7. Influence of single intrathecal administration of UW1, SHU9119, UW3, and UW5 on nociceptive transmission in naive rats and pain-related behaviors in chronic constriction injury-exposed rats

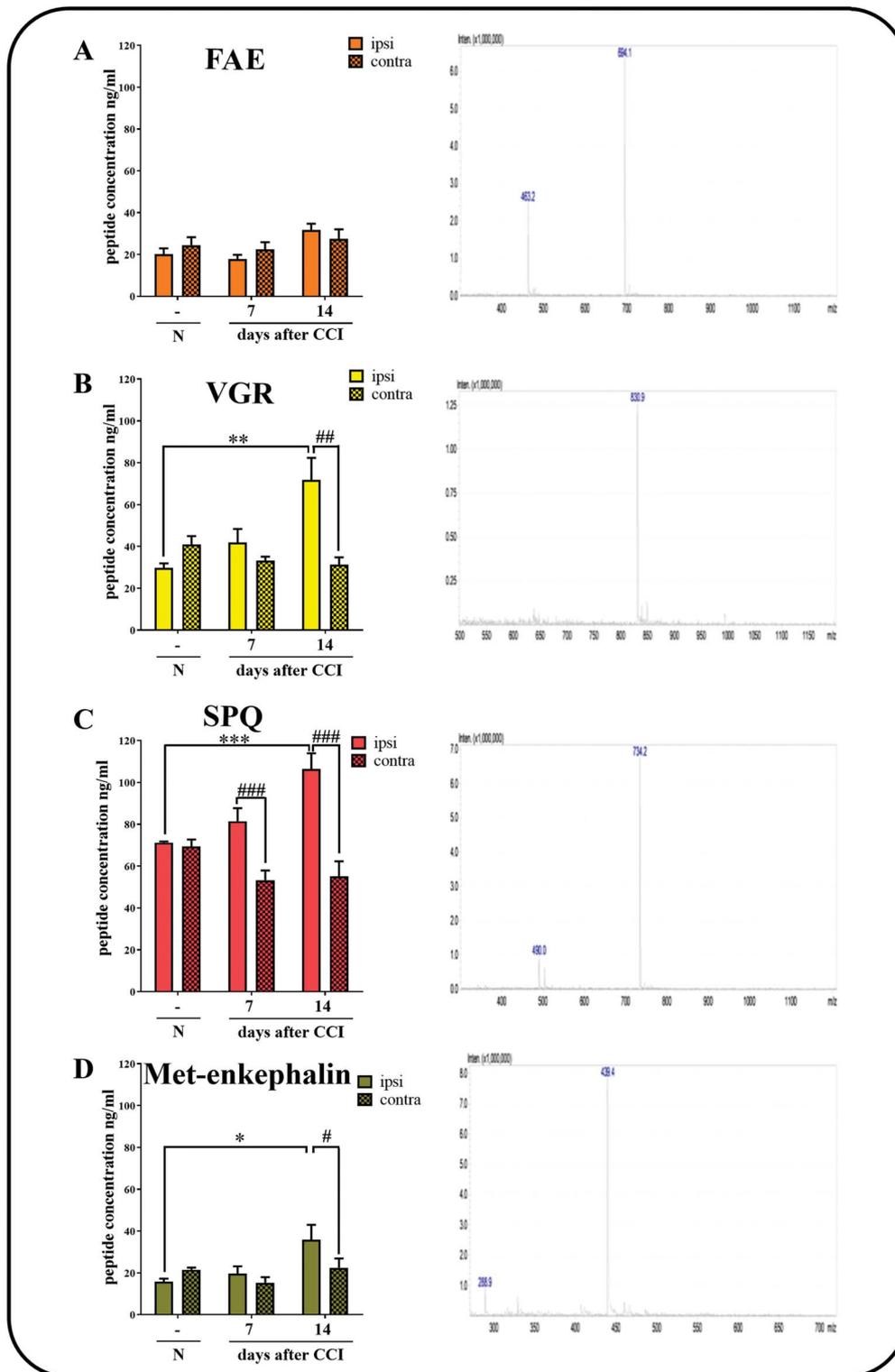
Single i.t. administration of UW1, SHU9119, and UW5, but not UW3 (0.1 µg/5 µL), increased the nociceptive threshold of naive rats, as measured using the tail-flick test (Figs. 6A, B, and D) ( $P < 0.05$ ,  $P < 0.001$ ). Injection of the lowest dose of UW1 and SHU9119 did not diminish the mechanical or thermal hypersensitivity (Figs. 6E and F, respectively) of rats with CCI-induced neuropathic pain. Only 1 dose of UW1 and SHU9119 (1 µg/5 µL) diminished mechanical and thermal hypersensitivity after CCI (Figs. 6E and F, respectively) ( $P < 0.05$ ,  $P < 0.01$ ,  $P < 0.001$ ). Interestingly, the administration of both hybrids, UW3 and UW5, showed significant analgesic effects at all doses tested (0.001, 0.01, and 0.1 µg/5 µL) 15, 30, 120, and even 240 minutes after injection, as measured by the von Frey and cold plate tests (Figs. 6G and H, respectively) ( $P < 0.05$ ,  $P < 0.01$ ,  $P < 0.001$ ).

### 3.8. Influence of repeated intrathecal administration of UW3, UW5, and morphine on the development of tolerance to analgesic effects in chronic constriction injury-exposed rats

In the von Frey and cold plate tests, the development of tolerance was observed after the repeated administration of the hybrids UW3 and UW5 (0.1 µg/5 µL; selected based on the above experiments), morphine (the dose was chosen on the basis of preliminary experiments), and vehicle (water for injection for control animals), which were injected i.t. for 9 days (once daily) from day 7 after CCI to day 15 after CCI. The difference between the analgesic effects produced by UW3, UW5, and morphine in both the von Frey and cold plate tests on the first day of administration under neuropathic pain conditions was not statistically significant (Figs. 7A and B, respectively). The tolerance curves of the hybrids were longer than that of morphine in the von Frey and cold plate tests. The efficacy of morphine (compared to vehicle) in the cold plate test was weaker than the efficacy of UW5 on days 7 to 9 after the start of the test for tolerance to analgesic effects (Figs. 7A and B). In addition, on day 9 (15 days after CCI), morphine completely lost its analgesic properties, and the animals exhibited similar mechanical and thermal hypersensitivity as that exhibited after administration of the vehicle. However, on the same day, the UW3 and UW5 hybrids showed an antinociceptive effect in both the von Frey ( $P < 0.01$ ) and cold plate tests (Figs. 7A and B) ( $P < 0.01$ ,  $P < 0.001$ , respectively).

## 4. Discussion

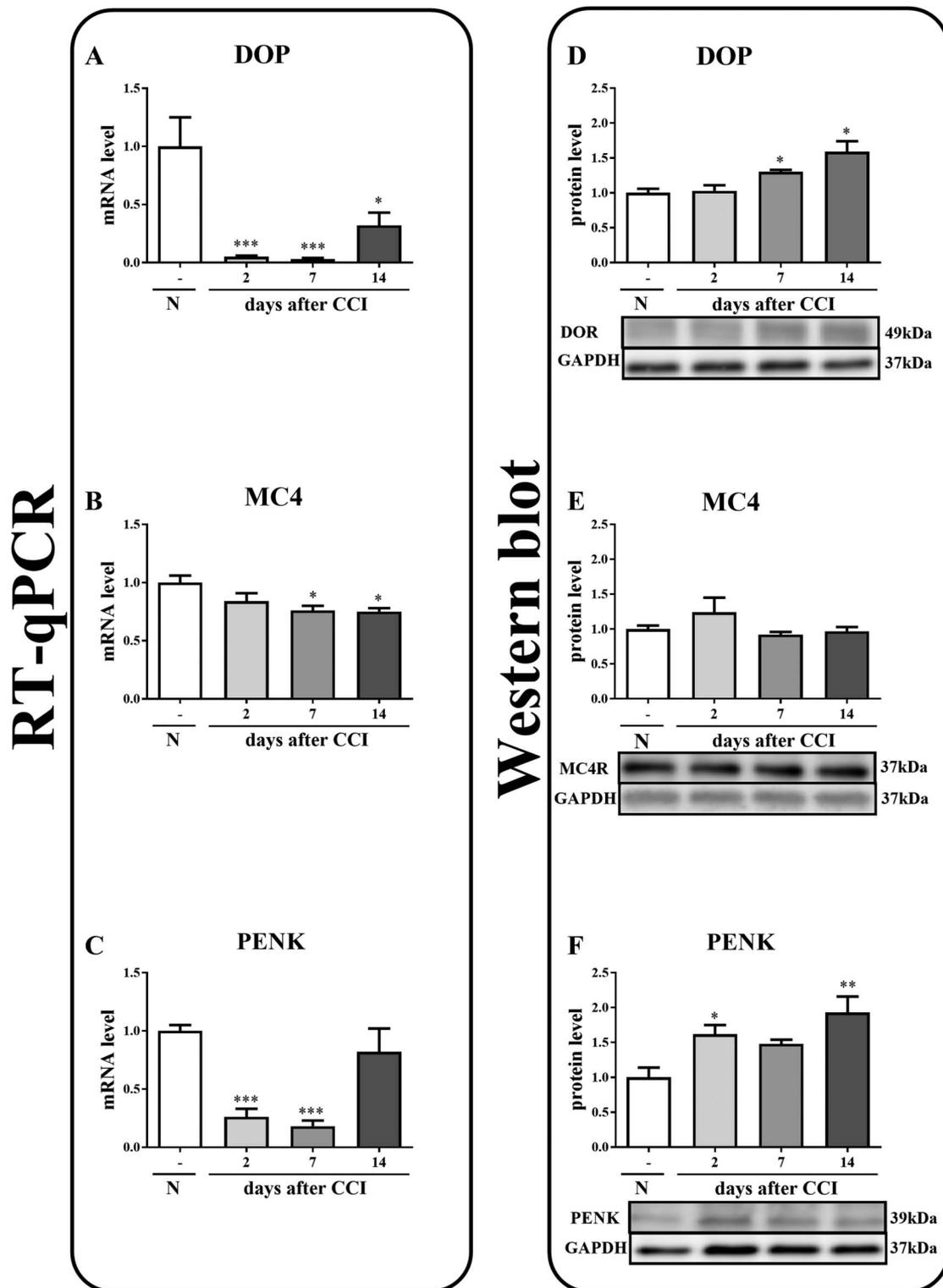
The lack of effective therapy for neuropathic pain and the limited effectiveness of opioids in this condition justify research into the



**Figure 4.** Mass spectrometry analysis of the PENK-derived peptides: FAE (A), VGR (B), SPQ (C), and Met-enkephalin (D) concentrations (ng/mL) in ipsilateral (ipsi) and contralateral (contra) dorsal lumbar spinal cord tissue of rats (A–D) tissue on the 7th and 14th days after chronic constriction injury (CCI). The data are presented as the mean ± SEM of 3 samples per group (5 animals per group). Intergroup differences were analyzed using 1-way ANOVA followed by the Bonferroni multiple comparisons test. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  indicate significant differences vs the dorsal lumbar spinal cord of naive rats. # $P < 0.05$ , ## $P < 0.01$ , and ### $P < 0.001$  indicate significant differences vs the ipsilateral dorsal lumbar spinal cord of rats 7 days after CCI or 14 days after CCI. The charts on the right side of the columns show the mass spectra acquired for FAE, VGR, SPQ, and Met-enkephalin, which were recorded using a triple quadrupole mass spectrometer equipped with an electrospray ion source. ANOVA, analysis of variance; CCI, chronic constriction injury; N, naive; PENK, proenkephalin.

mechanisms of this phenomenon. Our data reveal a dualistic role of POMC and PENK in nociceptive transmission, as peptides cleaved from these prohormones have both pronociceptive and

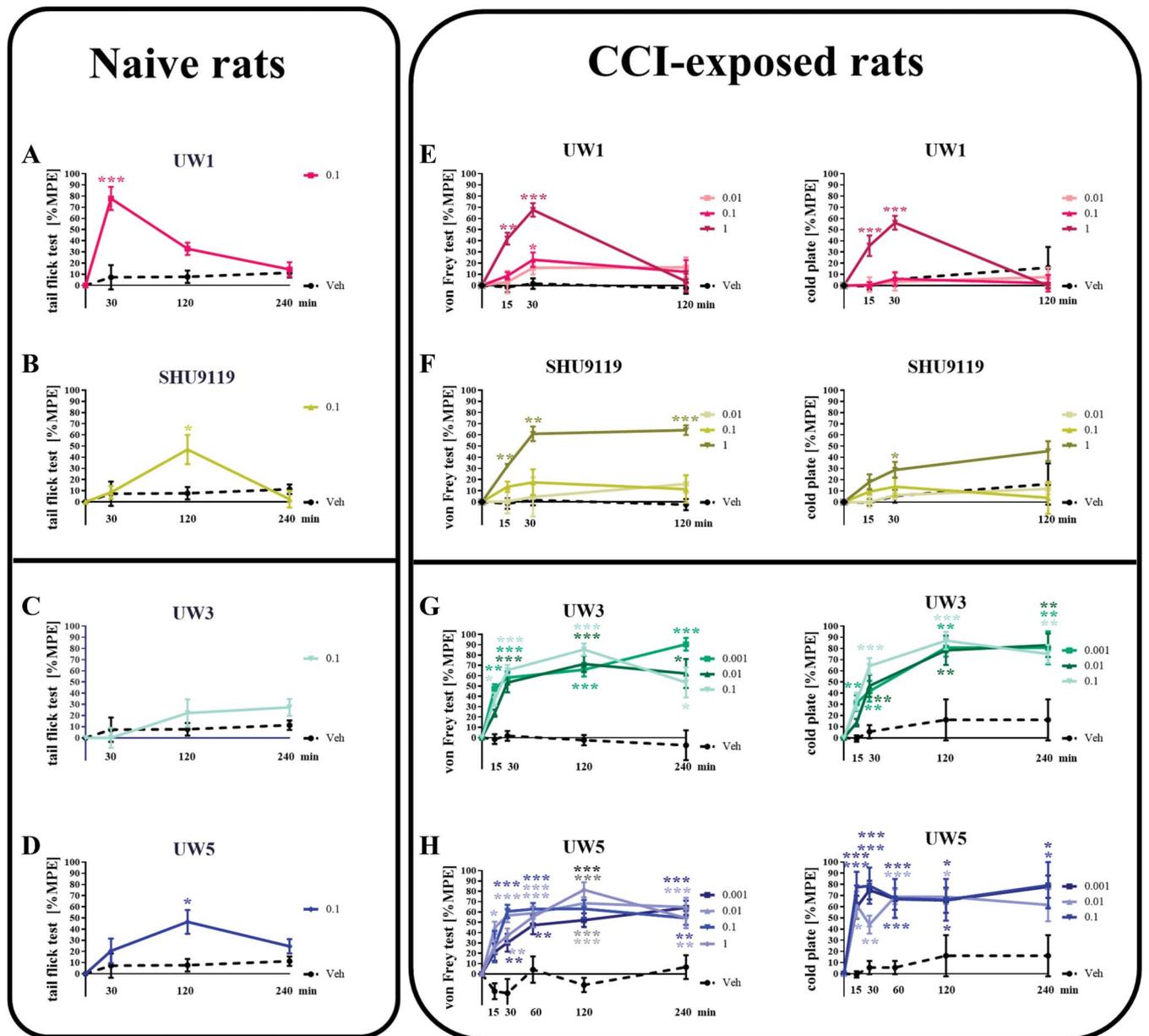
antinociceptive properties. In addition to assessing the pronociceptive properties of commercially available POMC-derived nonopioid peptides, such as  $\alpha$ -MSH, ACTH, and others, we



**Figure 5.** The time course of changes in the mRNA (A–C) and protein levels (D–F) of the DOP and MC4 receptors and PENK in the spinal cord tissues of rats on the 2nd, 7th, and 14th days after CCI. The RT-qPCR and Western blot data are presented as the mean  $\pm$  SEM of 6 to 9 and 4 to 5 samples per group, respectively. Intergroup differences were analyzed using ANOVA with the Bonferroni multiple comparisons test. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$  indicate differences vs naive rats. ANOVA, analysis of variance; CCI, chronic constriction injury; DOP,  $\delta$ -opioid peptide; N, naive; PENK, proenkephalin; RT-qPCR, reverse transcriptase process and quantitative real-time polymerase chain.

synthesized structure-based PENK-derived peptides, and, verified their pronociceptive properties in a rat model of neuropathic pain. In addition, we showed that compared with that of Met-enkephalin, the concentration of PENK-derived nonopioid

peptides was high in spinal cord tissue from the injured side. Consecutively, basing on the obtained results, our previous research, and the available literature, we designed potential drugs, specifically bifunctional ligands (hybrids), which likely act

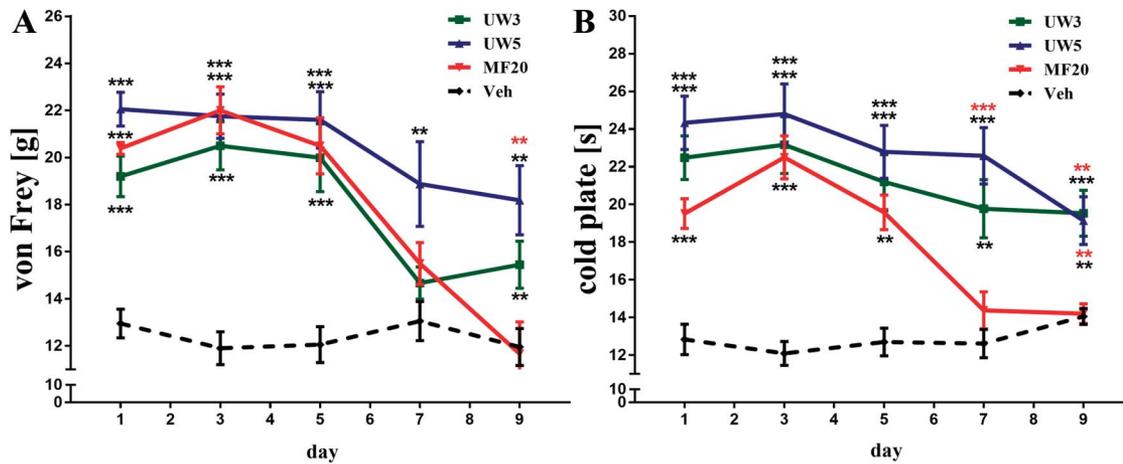


**Figure 6.** Effect of single intrathecal (i.t.) administration of the opioid parent UW1 (A and E) and nonopioid parent SHU9119 (B and F) and the hybrids UW3 (C and G) and UW5 (D and H) on pain threshold, as measured by the tail-flick test in naive rats (left panel), and hypersensitivity, as measured by the von Frey and cold plate tests in CCI-exposed rats (right panel). The tests were performed on rats (4-9 animals per group) from the 7th to 14th day after CCI. Intergroup differences were analyzed by ANOVA with the Bonferroni multiple comparison post hoc test. The results are presented as the percentage of the maximal possible effect (%MPE). The doses are presented as  $\mu\text{g}/5 \mu\text{L}/\text{animal}$ . \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs the vehicle-treated group. ANOVA, analysis of variance; CCI, chronic constriction injury; Veh, vehicle-treated group.

as agonists of opioid receptors and antagonists of the MC4 receptor, the activation of which in the spinal cord leads to pronociceptive activity. Then, we confirmed the analgesic properties of these hybrids and their ability to reduce the symptoms of neuropathic pain at much lower doses than those of their individual components. Moreover, tolerance to the analgesic effects of these hybrids developed more slowly than tolerance to the analgesic effects of morphine, which may be an important indicator of the potential efficacy of drugs developed on the basis of this hybrid group.

Unlike that of POMC, the PENK prohormone system is widespread throughout the central and peripheral nervous system, and PENK neurons have been found in the spinal cord, cranial sensory system, and major pain signaling network.<sup>71</sup> Our

data demonstrated a significant decrease in PENK mRNA levels in the spinal cord 7 days after sciatic nerve injury and a simultaneous increase in PENK protein levels. We also observed that among the PENK-derived peptides, Met-enkephalin, peptide E, BAM22, and BAM8-22 had analgesic effects (supplementary material, available at <http://links.lww.com/PAIN/B149>), which is in agreement with other studies.<sup>9,12,16,25,53</sup> A characteristic feature of Met-enkephalin, peptide E, BAM22 is the occurrence of the Tyr-Gly-Gly-Phe-Met sequence, which is the fundamental endogenous opioid pentapeptide Met-enkephalin. Using the UniProt database, we selected PENK-derived peptides for our study that do not possess this characteristic sequence and their roles in neuropathic pain have not yet been determined. For this



**Figure 7.** Effect of repeated once-daily intrathecal (i.t.) administration of the hybrids UW3 and UW5, morphine, and vehicle (green, blue, red, and black, respectively) on the development of tolerance to analgesic effects, as measured by the von Frey and cold plate tests (A and B, respectively) in CCI-exposed rats. The tests were performed on rats (9–10 animals per group) from day 7 after CCI (for 9 days). Intergroup differences were analyzed by ANOVA with the Bonferroni multiple comparison post hoc test.  $^{**}P < 0.01$ ,  $^{***}P < 0.001$  vs the vehicle-treated group or morphine-treated group. ANOVA, analysis of variance; CCI, chronic constriction injury; MF, morphine-treated group; Veh, vehicle-treated group.

purpose, we synthesized 3 peptides cleaved from PENK that terminated with a pair of basic amino acids constituting an enzymatic cleavage site, which we called SPQ, FAE, and VGR. The i.t. administration of low doses of FAE and VGR induced mechanical and thermal hypersensitivity in rats after sciatic nerve injury, and surprisingly, they had an analgesic effect at higher doses. This may have been due to enzymatic degradation processes and the formation of shorter peptides with other properties; eg, VGR, known as BAM8-18, is part of the BAM8-22 sequence, which has an antinociceptive effect through MAS-related G protein-coupled receptors.<sup>12,75</sup> In turn, SPQ intensified the symptoms of neuropathic pain at each of the tested doses. In addition, mass spectrometry showed an increase in SPQ and VGR concentrations after nerve injury on the ipsilateral side of the spinal cord, suggesting that they have important roles in the pathomechanism of neuropathic pain. Comparing the levels of SPQ and VGR to the Met-enkephalin levels on days 7 and 14 after sciatic nerve injury, we can conclude that one of the causes of the development of neuropathic pain is disturbed homeostasis of endogenous opioid systems towards the formation of pronociceptive peptides. The cause of this disturbed homeostasis of the PENK system is not yet clear. We hypothesize that injury to peripheral nerves causes an increase in the levels of nonopioid peptides and that the cleavage of these peptides is limited, probably due to changes in the activity of proteolytic enzymes. However, the action of Met-enkephalin, which compensates for the action of nonopioid peptides under normal conditions, may not be sufficient in pathological states, especially since Met-enkephalin is a very unstable peptide that undergoes rapid metabolism.<sup>17,19</sup>

It has been shown that in nociceptive transmission, nonopioid POMC-derived peptides also play an important role.<sup>1,66</sup> Consisting of 39 amino acids, ACTH, the major hormone secreted by the anterior pituitary in response to severe pain and other stressors, is a precursor of  $\alpha$ -MSH and CLIP.<sup>31,67,80</sup> Despite the fact that POMC-derived  $\beta$ -endorphin has opioid activity, ACTH and  $\alpha$ -MSH exhibit opioid antagonist-like effects, and their pronociceptive effects are associated with the activation of melanocortin receptors.<sup>8,9,11,37</sup> It has been indicated that, at the level of the spinal cord, only one of the melanocortin receptor subtypes,

namely, the MC4 receptor, is expressed.<sup>34,65</sup> Coherently, we observed exacerbation of neuropathic pain symptoms in rats on day 7 after sciatic nerve injury after the i.t. administration of endogenous ligands of this receptor, such as ACTH,  $\alpha$ -MSH, and CLIP.

Because the nonopioid effect of POMC-derived peptides is related to MC4 receptor activity and because PENK-derived peptide, SPQ, has a similar amino acid chain structure such as ACTH and  $\alpha$ -MSH, we decided to evaluate how the blockade of the MC4 receptor affects SPQ activity. We proved that an antagonist of these receptors, in addition to having analgesic effects, suppressed the pronociceptive properties of PENK-derived SPQ in a neuropathic pain model. Thus, the MC4 receptor seems to be responsible for the pronociceptive effect of PENK-derived peptides. However, this supposition must still be confirmed by receptor affinity studies.

Our results did not indicate significant differences in the mRNA and protein levels of the MC4 receptor in the ipsilateral lumbar spinal cord dorsal horn in neuropathic pain, and the literature data provide conflicting information.<sup>4,57</sup> This suggests that changes in the distribution of the MC4 receptor during neuropathic pain may be limited to changes in its density in specific layers of the spinal cord, which has been confirmed by immunohistochemical analysis.<sup>58</sup> However, it is undeniable that blocking the MC4 receptor produces analgesic effects, as this has been well documented in many models of pain in mice and rats,<sup>3,6,26,85</sup> including in a rat sciatic nerve injury model.<sup>14,57,58,62,72–74</sup> Thus, the MC4 receptor seems to be a good molecular target for effective pharmacological treatment of neuropathic pain.

Based on the obtained results regarding the role of endogenous opioid systems in the pathomechanism of neuropathic pain, the idea of constructing a hybrid compound, containing an opioid agonist with an enkephalin structure (UW1, Tyr-D-Ala-Gly-Phe-NH<sub>2</sub>) and the MC3/4 receptor antagonist SHU9119, arose. The basis for designing such a hybrid was our team's long-term work and reports from other authors who synthesized compounds containing several functional groups with different biological activities,<sup>45</sup> including opioid ligands.<sup>21–23,52,54,55</sup> Melanocortin receptor

antagonists were conjugated trivalent ligands targeting opioids and cholecystokinin by Lee et al.<sup>32</sup> but have never been studied in an animal model of neuropathic pain. We find the projecting of multifunctional drugs vitally important in the modern chronic pain pharmacology, since several drugs designed by adding adjunctive pharmacophores to opioids are in clinical trials.<sup>29,35</sup> The clear advantage of the hybrids we constructed is the unique linker between the opioid agonist and MC3/4 receptor antagonist, which strengthens the structure. Moreover, we constructed a stable hybrid structure, which was shown by *in vivo* studies to act much longer than the individual pharmacophores of the hybrid. In addition, we selected enkephalin as the opioid pharmacophore because it is a potent agonist of the DOP receptor and, to a lesser extent, the  $\mu$ -opioid peptide receptor, and agonism of these receptors may be critical for the treatment of neuropathic pain.<sup>40,41,44,82</sup> Our results indicated that treating the control naive animals with the UW3 or UW5 hybrid brought no effect or elicited similar nociceptive pain-related behaviour as in the treatment with parent compounds. The results were completely different in the neuropathic pain model, in which both hybrids produced a strong analgesic effect and reduced mechanical and thermal hypersensitivity at very low doses at which the parent compounds were ineffective. Undoubtedly, the satisfactory antinociceptive effects of the hybrids are associated with their better simultaneous achievement for their molecular targets than the individual pharmacophores. In our experiments on the mouse model of neuropathic pain, we have shown the superiority of OP-MC4 hybrids over physical mixture of parents in terms of analgesic effect provided.<sup>56</sup>

In addition, our results comparing the effects of both hybrids with those of morphine indicated that UW5 (like UW3 in the cold plate test) did not lose its analgesic effectiveness in CCI-exposed rats after *i.t.* administration for 9 days. By contrast, morphine lost its analgesic effect on day 6. These beneficial properties of the hybrids may be associated with DOP receptor activity because the effectiveness of DOP receptor ligands does not change in neuropathic pain.<sup>33,40,42,70</sup> In addition, a multimodal approach has been used in the treatment of neuropathic pain,<sup>64</sup> and SHU9119 causes a delay in the development of analgesic morphine tolerance, which is important for overcoming therapeutic restrictions.<sup>26,59,62</sup> Thus, the use of hybrids seems to be an interesting and promising direction for innovative therapies for the treatment of chronic pain.

### Conflict of interest statement

The authors have no conflicts of interest to declare.

### Acknowledgements

This work was funded by the National Science Centre, Poland, MAESTRO 2012/06/A/NZ4/00028 and OPUS 2018/29/B/NZ7/00082 grants, and statutory funds from the Maj Institute of Pharmacology at the Polish Academy of Sciences. A. Piotrowska and J. Starnowska-Sokół are recipients of the KNOW scholarship sponsored by the Ministry of Science and Higher Education, Poland. A. Piotrowska thanks the Foundation for Polish Sciences (FNP), the L'Oréal-UNESCO for Women in Science Program in Poland, and Ministry of Science and Higher Education Republic of Poland for funding. Project performed with the use of CePT infrastructure (University of Warsaw) financed by the European Union—the European Regional Development Fund within the Operational Programme “Innovative economy” from 2007 to

2013. The English was corrected by American Journal Experts (certificate no. DE68-254A-7023-AF32-E3EB).

Ethics statement: The experiments conducted as part of this publication were realized on a minimum number of animals and in accordance with the IASP (Zimmerman 1983) and NIH Guide for the Care and Use of Laboratory Animals recommendations. In addition, the consent of the 2nd Local Ethical Committee on Animal Testing of the Maj Institute of Pharmacology, Polish Academy of Sciences (12 Smetna Str., 31-343 Krakow, Poland; permission numbers: 962/2012, 1213/2015 and 212/2019) was obtained for their conduct.

### Appendix A. Supplemental digital content

Supplemental digital content associated with this article can be found online at <http://links.lww.com/PAIN/B149>.

### Article history:

Received 3 March 2020

Received in revised form 20 July 2020

Accepted 29 July 2020

Available online 20 August 2020

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