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CNS penetration of the opioid glycopeptide MMP-2200: A microdialysis study

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Abstract

Endogenous opioid peptides enkephalin and dynorphin are major co-transmitters of striatofugal pathways of the basal ganglia. They are involved in the genesis of levodopa-induced dyskinesia and in the modulation of direct and indirect striatal output pathways that are disrupted in Parkinson's disease. One pharmacologic approach is to develop synthetic glycopeptides closely resembling endogenous peptides to restore their normal functions. Glycosylation promotes penetration of the blood-brain barrier. We investigated CNS penetration of the opioid glycopeptide MMP-2200, a mixed δ/μ-agonist based on leu-enkephalin, as measured by *in vivo* microdialysis and subsequent mass spectrometric analysis in awake, freely moving rats. The glycopeptide (10 mg/kg) reaches the dorsolateral striatum (DLS) rapidly after systemic (*i.p.*) administration and is stably detectable for the duration of the experiment (80 min). The detected level at the end of the experiment (around 250 pM) is about 10-fold higher than the level of the endogenous leu-enkephalin, measured simultaneously. This is one of the first studies to directly prove that glycosylation of an endogenous opioid peptide leads to excellent blood-brain barrier penetration after systemic injection, and explains robust behavioral effects seen in previous studies by measuring how much glycopeptide reaches the target structure, in this case the DLS.

Keywords

striatum; leu-enkephalin; glycopeptide; blood-brain barrier penetration; mass spectrometry

Introduction

Parkinson's disease (PD) is the 2nd most common neurodegenerative disorder. The cardinal motor symptoms are tremor, rigidity, and bradykinesia [38]. The underlying pathology is the loss of dopaminergic neurons with cell bodies located in the substantia nigra and axonal

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projections to the striatum. The gold standard treatment for PD is dopamine replacement therapy utilizing levodopa, the dopamine precursor, or utilizing dopamine receptor agonists. These therapies become unsatisfactory as the disease progresses due to a variety of effects that occur with dose escalation, including levodopa-induced dyskinesias (LID). Therefore, there is an urgent need to develop non-dopaminergic therapies, especially adjunct therapies that may extend the use of levodopa [38].

Although classic opioids have not found utility in the treatment of PD, they have a profound effect on locomotion and reward behavior mediated by the basal ganglia by acting as modulators of glutamate, GABA and dopamine neurotransmission [17]. High levels of μ- and δ-opioid receptors are present in the striatum [35] and striatal levels of the endogenous opioid peptides dynorphin and met-enkephalin are greatly altered in PD [42]. Striatopallidal projection neurons of the indirect striatal output pathway express enkephalins derived from the precursor preproenkephalin A (PPE-A, Penk), while the striatonigral neurons of the direct striatal output pathway express opioids derived from preproenkephalin B (PPE-B, Pdyn) [10,20]. Following long-term levodopa therapy that eventually produces dyskinesia, both the levels of opioid peptides and the mRNA encoding their precursors are elevated in PD animal models [16,13,7,22]. Postmortem studies in PD patients who have had motor fluctuations due to long-term levodopa use confirm increased striatal Penk and Pdyn expression [23,37,6]. These alterations suggest that the increased expression of enkephalin may be a compensatory mechanism for dopamine depletion in PD. Studies of opioid antagonists in MPTP-lesioned primates with dyskinesia have produced contradictory results [21,24,27,41]. In phase IIa clinical trials low-dose oral naltrexone failed to show any effect [40], whereas high-dose naltrexone had a minimal effect [34]. A trial using naloxone at a dose known to block central opioid receptors also failed to demonstrate reduction in dyskinesia, but did show an extension in duration of action of levodopa [18]. This disparity has led to conflicting concepts that opioids represent either a cause of dyskinesia or a compensatory mechanism. In our view, μ and δ-opioid systems have differing, complex, and opposing effects in PD so that non-selective opioid antagonists may have no net effect. Given this background, novel opioid compounds could provide an important non-dopaminergic therapy for PD and other movement disorders. Selective μ-opioid antagonists have shown promise in preclinical trials of LID [28] and δ-agonists have potent effects in vertebrate models of dopamine denervation [25,26,8,33]. These cyclic alkaloidal drugs may have side effects and toxicities independent of their opioid pathway activity that are not present in their peptide counterparts [1]. The probability of side effects due to the production of active metabolites is less with glycopeptide-based drugs versus alkaloids, since the opioid glycopeptides are degraded to inactive di- and tri-peptides and sugars [19].

We previously showed that systemic administration of MMP-2200, a glycosylated leu-enkephalin analog, Tyr-D-Thr-Gly-Phe-Leu-Ser(β-O-Glc)-$CONH_2$ [30], has potent behavioral effects in 2 rodent models of striatal dopamine depletion [44]. It is also worth noting that when compared to morphine, MMP-2200 shows a better side effect profile as shown in several pain and dependence models, including naloxone precipitated withdrawal [30]. In the current study we expand these findings by measuring the amount of MMP-2200 that reaches the DLS with *in vivo* microdialysis and mass spectrometry after systemic injection in rats to quantify the amount of glycopeptide in the target structure, the DLS in this case.

Materials and Methods

Glycopeptide Agonist

MMP-2200 was GMP-compliant material synthesized by PolyPeptide Labs in Torrence, CA, and has been described in detail previously [30,44], leu-ENK for standard preparation

was purchased from Phoenix Pharmaceuticals (Burlingame, CA). Water and methanol for mobile phases are Burdick & Jackson HPLC grade purchased from VWR (Radnor, PA). All other salts and chemicals were from Sigma Aldrich (St. Louis, MO) unless otherwise noted.

Microdialysis

Adult male Sprague–Dawley rats (Harlan, Indianapolis, IN) weighing between 250 and 350 g were used for all experiments (n=7). Rats were housed in a temperature and humidity controlled room with 12 h light/dark cycles with food and water available *ad libitum*. All animals were treated as approved by the University of Michigan Unit for Laboratory Animal Medicine (ULAM) and in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals.

Prior to surgery, rats were anesthetized with an intraperitoneal (*i.p.*) injection of a ketamine (65 mg/kg) and dexdormitor (0.25 mg/kg) mixture prepared in an isotonic salt solution. Concentric microdialysis probes with 2 mm long (AN69 polyacrylonitrile membrane,Hospal, Bologna, Italy) and 0.3 mm diameter active membranes were implanted using an ultra precise model 963 stereotaxic instrument (David Kopf Instruments, Tujunga, CA), into the DLS according to the following coordinates from bregma: AP +1.0, ML ±3.5 DV −5.5 [40]. Although *in vitro* recoveries were not performed for every probe as is sometimes done with microdialysis studies, we generally achieve ~30–40% *in vitro* recovery of enkephalin peptides with 2 mm long polyacrylonitrile probes at a flow-rate of 0.9 μl/min. Thus, it is concluded that concentrations of actual peptide content in the brain is ~3 X the reported values for analyzed dialysates. Probes were secured to the skull by acrylic dental cement and metallic screws. Following surgery, rats were allowed to recover and experiments were run 24 h after probe implantation. Microdialysis probes were flushed at a flow rate of 1.5 μL/min with a modified Ringer solution (composition in mM: $CaCl_2$ 1.2; KCl 2.7, NaCl 148 and $MgCl_2$ 0.85) for 2 h using a Chemyx Fusion 400 syringe pump (Chemyx, Stafford, TX). Perfusion flow rate was then reduced to 0.9 μL/min and samples were collected every 10 min into tubes containing 0.5 μL acetic acid to preserve peptide stability as previously described [29,32]. Basal samples were collected for 40 min and then subjects were injected with MMP-2200 (10 mg/kg *i.p.*). Samples were then collected for an additional 80 min. Samples were immediately injected on the LC-MS system following collection.

MMP-2200 and leu-ENK detection with capillary LC-MS[3]

MMP-2200 and Leu-ENK were measured using a modified version of a method previously used for enkephalins *in vivo* [29,32]. Chromatography columns were 50 μm inner diameter (i.d.) fused silica capillary packed in-house to 4 cm length with 5 μm Alltima C18 reversed-phase particles (Alltech, Deerfield, IL) using a high pressure reservoir. ESI emitter tips were also prepared in-house from a 3 cm length of 40 μm i.d. fused silica capillary using a laser puller (P-2000, Sutter Instruments, Novatao, CA). Columns and tips were joined using a 2 cm PTFE 1/16″ x .010″ sleeve adapter. An air driven fluid pump (DHSF-151, Haskel Inc., Burbank, CA) was used for sample loading and desalting (4000 psi), and a micro HPLC pump (MicroPro, Eldex Laboratories, Napa, CA) for gradient elution (700 psi). Samples were injected using a WPS-3000TPL autosampler (Dionex, Sunnyvale, CA), in partial loop injection mode (5 μL loop).

The 5 μL samples were loaded over 8 min followed by 2 min rinse with H_2O to desalt the column at 2.5 μL/min. Following loading and desalting, the injector valve was switched to the gradient pump to elute the peptides at 300 nL/min. Mobile phase A consisted of LC-MS grade water with 0.5% acetic acid and mobile phase B consisted of LC-MS grade methanol with 0.5% acetic acid. The gradient program began with an isocratic step of 10% B then a

linear increase to 80% B over 4 min, followed by a linear increase to 100% B in 1 min. An isocratic step at 100% B for 2 min was followed by a linear decrease down to 10% B over 0.5 min. Finally, 10% B was maintained isocratically for 2 min to re-equilibrate the LC system before the next injection. All valve switching and runs were controlled automatically with Xcalibur software (Thermo Fisher Scientific).

The column and emitter tip were coupled to a PV-550 nanospray ESI source (New Objective, Woburn, MA) interfaced to an LTQ XL linear ion trap (LIT) MS (Thermo Fisher Scientific, Waltham, MA). A +2.5 kV potential was applied to a liquid junction prior to the column for electrospray. The MS^3 ion transition pathways set on the LIT for MMP-2200 and leu-ENK (both singly charged) were: 1010→686→582,651,689 and, 556→397→278,323,380, respectively.

Detection limits for both leu-ENK and MMP-2200 were around 1 pM. This is in accordance with previous detection limits of leu-ENK using variations of this LC-MS method from our group [29,32]. Detection limit was determined by visual inspection of peak area at a range of concentrations when performing calibration curves. Generally, we consider the limit of detection to be equal to 3 X the signal-to-noise ratio of a blank (aCSF) injection. This can be interpreted as the standard deviation of the blank multiplied by 3, divided by the slope of the calibration curve. Leu-ENK, and to a greater extent MMP-2200, were measured at levels considerably higher than detection limits.

Statistical analysis of the data

Repeated measures ANOVAs were used to evaluate statistical differences over the course of the experiment for both MMP-2200 and leu-ENK measurements using GraphPad Prism 5 (GraphPad, La Jolla, CA). One tail Fisher LSD post hoc tests were used to evaluate the statistical difference between baseline and post-injection of MMP-2200 at different time points. In two animals one data point each in the leu-ENK experiment was more than 3 standard deviations greater than the mean and with Cook's distance above $D_i = 4/n$. These two animals were excluded from analysis in both measurements of the experiment. The null hypothesis was rejected when $p < 0.05$.

Results and Discussion

LC-MS measurements of leu-ENK and MMP-2200

Both MMP-2200 and leu-ENK were readily detectable by the current LC-MS method (Figure 1). Following a 10 min loading and desalting phase, both peptides chromatographically eluted in about 5 min. Total run time was 17 min per sample injection.

CNS penetration of MMP-2200

MMP-2200 was systemically injected (10 mg/kg, *i.p.*) and the uptake into the DLS was measured by *in vivo* microdialysis and LC-MS analysis. These experiments were performed in awake, freely moving rats, implanted with microdialysis probes in the DLS 24 hr prior to the experiments. It has been demonstrated that the blood-brain barrier (BBB) integrity is established after such a short period following probe implantation [4,43,2,11]. Although some controversy remains regarding the integrity of the BBB following microdialysis surgeries [36], performing experiments 24 h after probe implantation is likely the most suitable method for maintaining BBB integrity. Other factors such as lowering the probe slowly, using narrow (~300 μm) concentric probes and a low flow rate (0.9 μl/min) likely prevented BBB disruption [11]. The animals were fully recovered, their wounds healed, and the probes were fixed in place with dental cement. A highly significant (repeated measures ANOVA: $p < 0.01$; $n = 5$) level of MMP-2200 (post hoc tests at single time points: *p <

0.05; $^{\#}p < 0.09$) can be measured from dialysate of the DLS at the first time point (10 min) after injection (arrow) at approximately 1200 pM and remains at active levels (around 250 pM) until the end of the experiment at 80 min, as shown in Figure 2. It must be noted that dialysate concentrations are reported here, and not actual brain peptide levels. Since *in-vitro* recoveries do not necessarily reflect true *in vivo* conditions such as active transport of peptide, temperature, and enzymatic degradation, we choose to omit this step and estimate concentrations instead based on past experience (data not shown). Thus, we estimate that our reported values are ~1/3 less than actual concentrations reaching the brain. For comparative reasons, the endogenous leu-ENK level was measured at the same time as MMP-2200 and is depicted in Figure 3. The basal dialysate concentration of leu-ENK is around 25 pM in concordance with the literature [3,32], and remains unchanged during the experiment (repeated measures ANOVA: $p = 0.8$), further supporting that the BBB was not compromised by the experimental procedure. No quantitative evaluation of brain concentrations of other opioid peptide analogs has been published to date. The rapid rise and fall of MMP-2200 levels between 10 and 20 min in the brain post *i.p* administration is noteworthy. The pharmakodynamics of this class of drug is not well studied, but membranes may be regarded as an additional compartment that complicates distribution [9].

Centrally-mediated behavioral effects of MMP-2200 and other glycosylated opioid peptides

The high level of MMP-2200 that reaches the DLS after systemic injection explains the strong behavioral effects seen in preclinical models of striatal dopamine depletion [44]. Amphetamine-induced rotations were reduced by 50%, and the apomorphine-induced overshoot in movement of reserpine-treated akinesia rats was reversed by MMP-2200 in that study, both indicators that MMP-2200 reduces downstream effects of dopamine depletion. Additionally, strong behavioral effects in preclinical rodent models of pain that are centrally-mediated were reported by systemically administered (*i.v., i.p., s.c.*) glycopeptides [5,14,30,31]. Like its unglycosylated parent peptide, MMP-2200 displays similar nanomolar affinities for both δ- and μ-opioid receptors [15]. Combined, these data indicate that glycosylation of endogenous peptides is a valid strategy to achieve BBB-penetration. One previous study in rhesus monkeys suggested that CNS distribution of MMP-2200 was very limited after *i.m.* injection [12]. The glycopeptide did produce anti-allodynic effects, comparable to morphine, but was not very effective in a tail flick model of thermal nociception. This may reflect differences in route of administration (*i.m.* vs *s.c.* or *i.v.*), differences in pharmacokinetics and brain penetration due to species (rhesus monkey vs rodent), or may even reflect ambiguity in interpretation of the tail flick response in rhesus monkey (antinociception vs euphoria).

Conclusion

This is the first study to directly show that glycosylation of an acyclic endogenous opioid peptide leads to excellent BBB penetration after systemic injection and explains the robust behavioral effects seen in our previous studies in models of dopamine depletion by quantifying the amount of glycopeptide in the target structure, the DLS in this case. We believe that further development of opioid glycopeptides with more receptor specificity, particularly δ-agonists and μ-antagonists could lead to novel therapeutic options for the treatment of LID. This proof-of-principle study substantiates that glycopeptides based on endogenous peptides are valid targets for drug development.

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Highlights

- Novel glycosylated opioid peptide MMP-2200 is opioid mu and delta receptor agonist.
- MMP-2200 reached dorsolateral striatum (DLS) after systemic administration (*i.p.*).
- MMP-2200 concentration in DLS reached nM range within 10 minutes.
- Endogenous leu-enkephalin levels were unchanged after MMP-2200 administration.

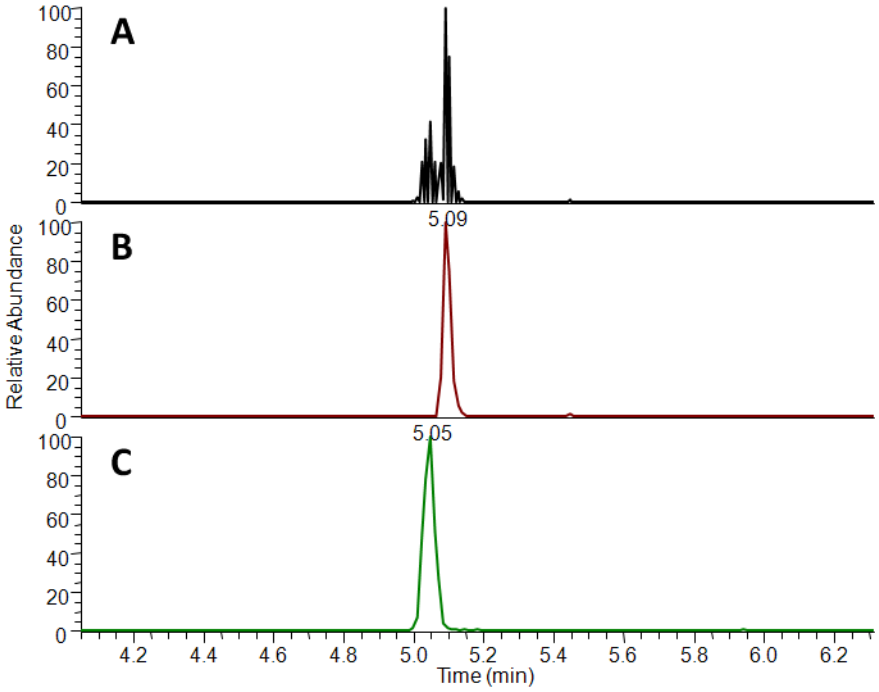

Figure 1. LC-MS analysis of dialysate peptide content

Total ion chromatogram from MS^3 analysis of a 10 pM standard including both leu-ENK and MMP-2200 (A). Reconstructed ion chromatograms for MMP-2200 (B) and leu-ENK (C). Run time for each sample was 17 min including 10 min for sample loading and desalting and 7 min for MS analysis.

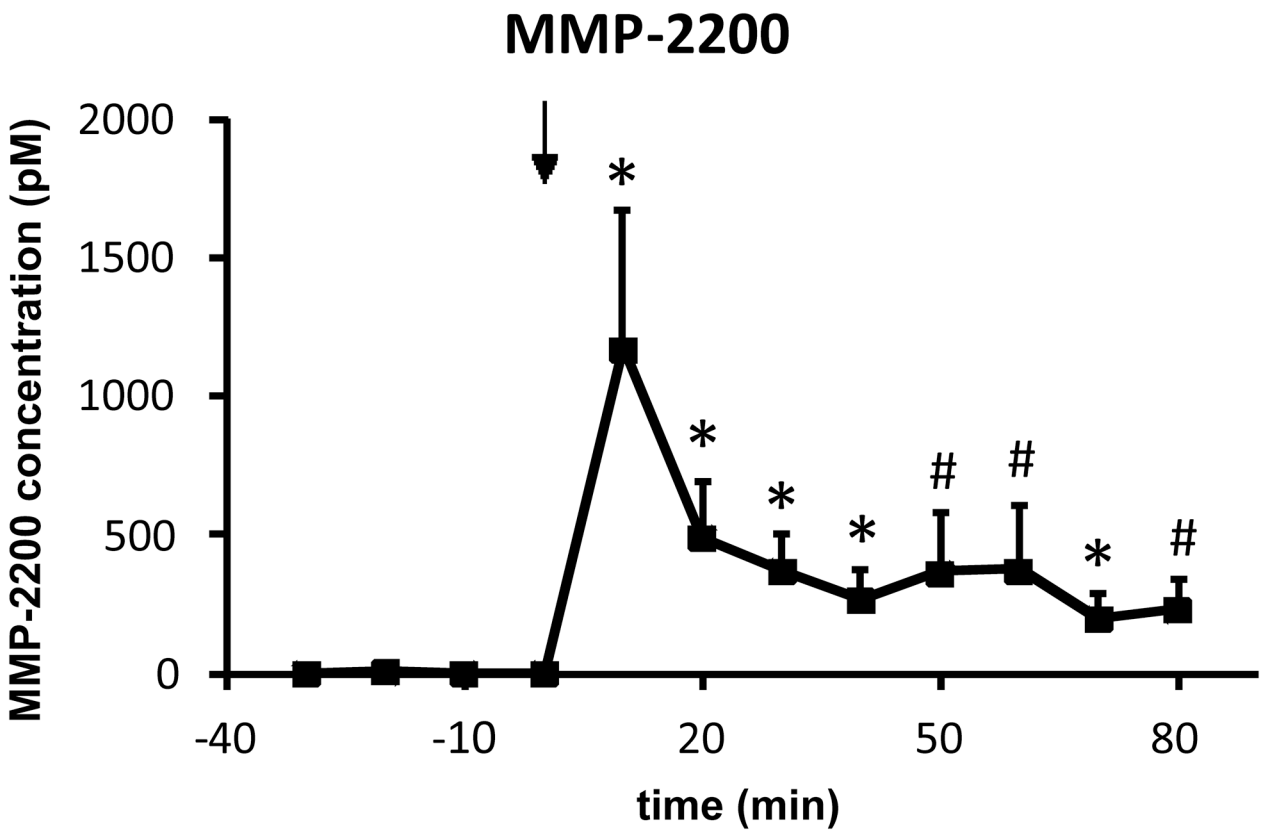

Figure 2. Proof of blood-brain barrier penetration of the opioid glycopeptide MMP-2200 after systemic administration

MMP-2200 (10 mg/kg, *i.p.*) rapidly reaches the DLS as measured by *in vivo* microdialysis and subsequent mass spectrometric analysis in awake, freely moving rats, implanted 24 hr prior to the experiments (fully recovered, wounds healed, covered by a stage that holds the probe in place; n = 5). A highly significant (repeated measures ANOVA: $p < 0.01$) level of MMP-2200 (mean±SEM; post hoc tests at each time point post-injection vs. time point 0: *$p < 0.05$, $^{\#}p < 0.09$) can be measured in the dialysate from the first time point after injection (arrow) and remains at active levels (around 250 pM) until the end of the experiment.

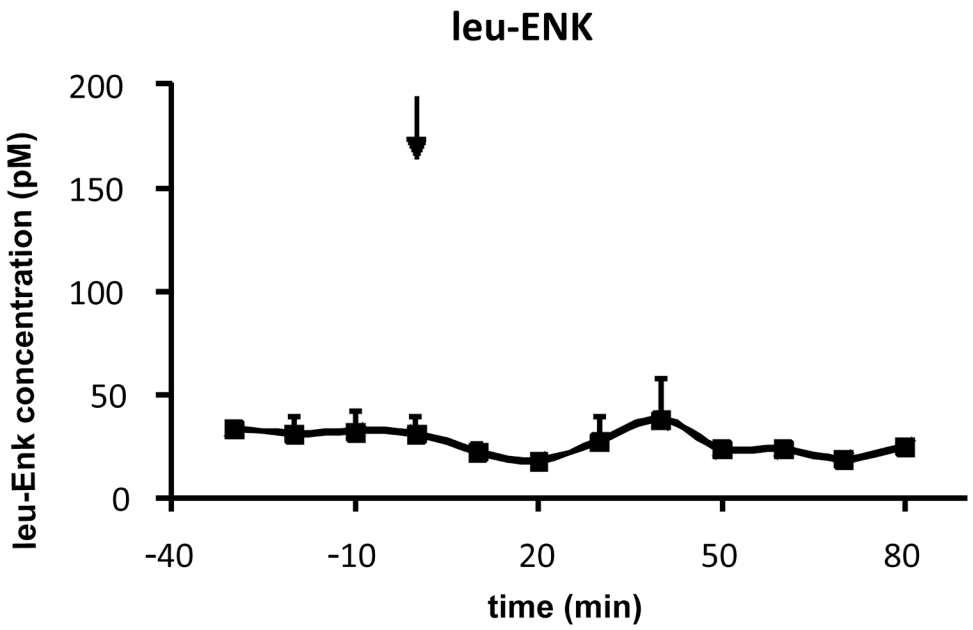

Figure 3. Level of endogenous leu-enkephalin in DLS is unchanged

The endogenous leu-ENK level (mean±SEM), measured by *in vivo* microdialysis and subsequent mass spectrometric analysis at the same time as in Figure 2 (n = 5; repeated measures ANOVA: p = 0.8), is unchanged before and after the MMP-2200 (10 mg/kg, *i.p.*) injection (arrow). The average leu-ENK level remains stable at around 25 pM at both baseline and post-injection.