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Full paper

Neuropeptide oxytocin enhances μ opioid receptor signaling as a positive allosteric modulator

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ABSTRACT

Oxytocin (OT) is a 9-amine neuropeptide that plays an essential role in mammalian labor, lactation, maternal bonding, and social affiliation. OT has been reported to exert an analgesic effect in both humans and animals, and the results of certain animal experiments have shown that the analgesic effect of OT is partially blocked by opioid receptor antagonists. To investigate the relationship between OT and μ opioid receptor (MOR), we evaluated how OT affects MOR in vitro by performing an electrical impedance-based receptor biosensor assay (CellKey™ assay), an intracellular cAMP assay, and a competitive receptor-binding analysis by using cells stably expressing human MOR and OT receptor. In both the CellKey™ assay and the intracellular cAMP assay, OT alone exerted no direct agonistic effect on human MOR, but treatment with 10^{-6} M OT markedly enhanced the MOR signaling induced by 10^{-6} M endomorphin-1, β-endorphin, morphine, fentanyl, and DAMGO. Moreover, in the competitive receptor-binding assay, 10^{-6} M OT did not alter the affinity of endomorphin-1 or morphine for MOR. These results suggest that OT could function as a positive allosteric modulator that regulates the efficacy of MOR signaling, and thus OT might represent a previously unrecognized candidate analgesic agent.

1. Introduction

Oxytocin (OT) is a 9-amine neuropeptide synthesized in the paraventricular nucleus (PVN) and the supraoptic nucleus of the hypothalamus. OT is secreted from the posterior pituitary into the systemic circulation, where it plays an essential role in mammalian

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labor and lactation through its peripheral actions,[1] and suggested to modulate numerous functions including maternal bonding and social affiliation through its central actions.[2,3]

Recently, the effect of OT on pain sensitivity in both humans and animals has been increasingly investigated by several research groups including ours.[4–11] The first crucial mechanism linking OT and pain involves hypothalamo-spinal oxytocinergic pathways. Stimulation of the PVN or administration of OT activates presynaptic OT receptors (OTRs) located superficially in the dorsal horn (Laminae I and II) and subsequently excites inhibitory GABAergic interneurons. Activation of GABAergic interneurons, in turn, presynaptically inhibits A δ-fiber and C-fiber signals at nociceptive-specific and wide-dynamic-range neurons in Laminae I and II.[7,12–14] These effects can be reversed by selective OTR antagonists.[13] The second potential mechanism linking OT and pain involves an indirect pathway through the endogenous opioid system. Injection of opioid receptor (OR) antagonists has been reported to partially block the analgesic effects of OT.[4–6] In terms of the relationship between OT and endogenous opiates, at least two potential mechanisms exist: OT can stimulate the release of endogenous opioids in the brain[15,16]; and OT can bind to ORs and act as an orthosteric agonist or an allosteric modulator.

μORs (MORs) as key targets in pain management are among the most studied G protein-coupled receptors (GPCRs). MORs are grouped in the Class A family of GPCRs and signal through the Gi/o family of heterotrimeric G proteins, which results in the inhibition of adenylate cyclase to cause cAMP level reduction, modulation of ion channel activity (through G protein βγ subunits), and transcriptional changes in cells.[17] Although widely recognized for their therapeutic values, opioid drugs frequently cause serious side effects, including respiratory suppression, constipation, allodynia, tolerance, dependence, and withdrawal symptoms, as well as produce rewarding effects and hold abuse potential.[18] Recently, research attention has been focused on MOR positive allosteric modulators (μ-PAMs), which were first described by Burford et al., in 2013[19] and followed by us.[20] GPCR allosteric ligands bind to a site on the receptor distinct from the site that binds the orthosteric agonist,[21] and an allosteric modulator can change a range of activities at the target protein. PAMs might present no intrinsic efficacy in terms of receptor activation, whereas enhances the binding affinity and/or efficacy of orthosteric agonists when bound to a receptor. PAMs thus offer the specific advantage of acting as modulators of receptor activity only when orthosteric agonists occupy the targeted receptors. This advantageous feature of PAMs could emerge as a novel target in the discovery of drugs that present improved side-effect profiles or fewer tolerance and dependence problems as compared with orthosteric OR agonists.[21]

In this study, we evaluated the effect of OT on MOR by performing an electrical impedance-based receptor biosensor assay (CellKey™, MDS Sciex, Ontario, Canada),[22] an intracellular cAMP assay, and a competitive receptor-binding analysis by using cells stably expressing human MOR (hMOR).

2. Materials and methods

2.1. Construction of plasmids and generation of stable cell lines

The hMOR cDNA (NM_000914) with or without a stop codon was amplified from a Flexi ORF clone (Promega, Madison, WI, USA), and the amplified MOR fragment was introduced into pcDNA3.1 (+) vector (Life Technologies, Carlsbad, CA, USA). Human Embryonic Kidney 293 (HEK293) cells were obtained from American Type Culture Collection (ATCC®, Manassas, VA, USA), and HEK293 cells stably expressing hMOR were generated through transfection of the constructed plasmids by using Lipofectamine reagent (Life Technologies) and selection based on MOR activity measured using the CellKey™ assay. OTR-expressing HEK293 cells were also generated using human OTR (hOTR) cDNAs (Promega) as in the case of hMOR. Moreover, pGloSensor™-22F plasmid (Promega) was transfected into cells that already stably expressed hMOR, and stable cell lines expressing both hMOR and pGloSensor™-22F were generated for monitoring intracellular cAMP levels.

2.2. Cell culture

HEK293 cells (stably expressing hMOR, both hMOR and GloSensor™, or hOTR) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 U/mL), streptomycin (100 mg/mL), and genistein (700 μg/mL) in a humidified atmosphere containing 95% air and 5% CO_2 at 37 °C (in a cell-culture incubator).

2.3. Chemicals

The following reagents were purchased from the listed sources: human OT, Peptide Institute Inc. (Osaka, Japan); endomorphin-1, Tocris Bioscience (Bristol, UK); DAMGO (D-Ala(2)-N-Me-Phe(4)-Gly-ol(5)-enkephalin), β-endorphin, forskolin, 3-isobutyl-1-methylxanthine (IBMX), and Ro 20-1724, Sigma–Aldrich (St. Louis, MO, USA); morphine hydrochloride, Takeda Pharmaceutical Co., Ltd. (Tokyo, Japan); and fentanyl, Janssen Pharmaceutical K.K. (Tokyo, Japan). Forskolin, IBMX, and Ro 20-1724 were diluted with dimethyl sulfoxide (DMSO), whereas other reagents were diluted with H_2O.

2.4. Functional analysis of human ORs by using the CellKey™ system

The CellKey™ assay has been described previously.[22–24] Briefly, cells stably expressing hMOR and hOTR were seeded at densities of 6.0×10^4 and 5.0×10^4 cells/well, respectively, in CellKey™ 96-well microplates containing an electrode at the bottom of each well, and then incubated for 21–24 h according to the method of Miyano et al.[22] The wells were washed with CellKey™ buffer composed of Hanks' balanced salt solution (1.3 mM $CaCl_2$nbsp;$2H_2O$, 0.81 mM $MgSO_4$, 5.4 mM KCl, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 136.9 mM NaCl, 0.34 mM Na_2HPO_4, and 5.6 mM D-glucose) containing 20 mM HEPES and 0.1% bovine serum albumin, and then the cells were allowed to equilibrate in the CellKey™ buffer for 30 min at 29 °C before assays. Changes in the impedance of an induced extracellular current (dZiec) in each well were monitored, using the CellKey™ system, once every 10 s for up to 30 min; a 5-min baseline was recorded, after which drugs were added and then the dZiec was measured for 25 min. The extent of changes in dZiec values was expressed as the ΔZiec (ΔZiec = dZiec maximum - dZiec minimum) after drug application. The ΔZiec value of each sample was normalized by that of the control sample.

2.5. Intracellular cAMP assay

The GloSensor™ cAMP biosensor (Promega) includes a modified form of firefly luciferase harboring a cAMP-binding motif.[25] cAMP accumulation was analyzed using HEK293 cells stably expressing both GloSensor™-22F protein and hMOR. The cells were seeded (at 3×10^4/well) into poly-D-lysine-coated 96-well, white, clear-bottomed plates (Corning, Corning, NY, USA), and intracellular cAMP accumulation after 24 h was assayed using the GloSensor™ reagent. Cells were incubated with diluted GloSensor™ reagent at room temperature for 2 h, and then treated with a test compound diluted with CellKey™ buffer containing 250 μM IBMX and 50 μM

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Ro 20-1724. Test compounds were added 10 min before treatment with 3×10^{-6} M forskolin, and the baseline luminescence intensity was measured before forskolin injection. After treatment with forskolin, luminescence intensity was measured every 2.5 min for 40 min by using a Synergy™ H1 system (BioTek Instruments Inc., Winooski, VT, USA) and time-luminescence curves were traced (supplementary Fig. 1). The responses were expressed as the area under the time-luminescence intensity curve (AUC) of each sample.

2.6. OR binding assay

Protein pellets from Chinese hamster ovary (CHO) cells expressing hMOR were resuspended in 50 mM Tris-HCl buffer containing 5 mM $MgCl_2$, 1 mM EGTA, pH 7.4. The cell suspensions were disrupted using a glass-Teflon homogenizer and centrifuged at 48,000 × *g* for 15 min, and after discarding the supernatant, the pellets were resuspended in the buffer at a concentration of 5 mg protein/mL and stored at −80 °C until use. CHO cell membranes expressing hMOR were incubated for 2 h at 25 °C in 0.25 mL of the buffer containing various concentrations of each tested compound (OT, endomorphin-1, or morphine) or 0.5 nM [^{3}H]diprenorphine (PerkinElmer, Norwalk, CT, USA). The incubation was terminated by collecting membranes on Filtermat B filters (PerkinElmer) by using a FilterMate™ harvester (PerkinElmer), and the filters were washed thrice with 50 mM Tris-HCl, pH 7.4 and the radioactivity was measured using a MicroBeta scintillation counter (PerkinElmer). Nonspecific binding was measured in the presence of 1 μM unlabeled diprenorphine. Ki values were calculated using GraphPad Prism software version 5.0 (GraphPad Software, La Jolla, CA, USA).

2.7. Statistics

Statistical analyses and concentration–response curve fittings were performed using the GraphPad Prism software. The statistical significance of ΔZiec measurements and the data on the inhibition of cAMP accumulation was assessed by performing one- or two-way analysis of variance (ANOVA) followed by Bonferroni's test. Groups of data that failed tests for normality and equal variance according to Bartlett's test were analyzed using the nonparametric Kruskal–Wallis statistic followed by Dunn's test (Figs. 1 and 2a, c, and 4d). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of OT on MOR activity in HEK293 cells expressing hMOR, measured using CellKey™ assay

First, the effects of DAMGO and OT were tested on hMOR-expressing HEK293 cells by using the CellKey™ assay. Relative to vehicle, DAMGO at 10^{-5} M significantly increased ΔZiec, whereas OT at 10^{-10}–10^{-5} M did not (Fig. 1), suggesting that OT by itself exerted no direct agonistic effect on hMOR.

3.2. Effect of OT on MOR activity induced by opioid agonists in hMOR-expressing HEK293 cells using CellKey™ assay

We next determined whether OT exhibits PAM activity toward MOR. We performed the CellKey™ assay with cells expressing hMOR, and measured the MOR activity induced by several opioid agonists (at 10^{-6} M) in the absence or presence of OT at various concentrations (Fig. 2). OT enhanced MOR activation by all tested opioid agonists: endomorphin-1-, morphine-, and DAMGO-induced

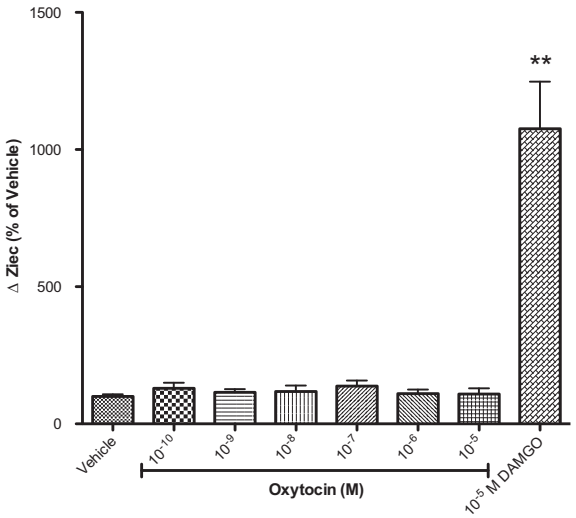

Fig. 1. Effect of OT on impedance in HEK293 cells stably expressing hMOR. Cells were treated with OT at concentrations of 10^{-10}–10^{-5} M or with 10^{-5} M DAMGO as a positive control. All data are presented as means ± standard error of the mean (S.E.M.) of 3 independent experiments (n = 9). Whereas DAMGO at 10^{-5} M significantly increased ΔZiec relative to vehicle, OT did not at any tested concentration. ΔZiec: extent of change in the impedance of an induced extracellular current (maximum−minimum) after treatment with test compounds.

increases of ΔZiec were significantly enhanced by OT used at 10^{-6}, 10^{-6} or 10^{-5}, and 10^{-6} M, respectively (Fig. 2a–c).

Concentration-response curves for endomorphin-1, β-endorphin, morphine, fentanyl, and DAMGO in the absence or presence of OT (at 10^{-6} M) were next generated, which revealed that OT significantly enhanced the ΔZiec induced by endomorphin-1 (used at 10^{-7}–10^{-5} M), β-endorphin (10^{-6}–10^{-5} M), morphine (10^{-7}–10^{-5} M), fentanyl (10^{-7}–10^{-6} M), and DAMGO (10^{-7}–10^{-5} M) (Fig. 3a–e). The E_{max} values (agonist only, agonist + 10^{-6} M OT) were the following: endomorphin-1: 100.8, 118.4; β-endorphin: 103.9, 136.3; morphine: 100.3, 138.0; fentanyl: 98.77, 110.1; and DAMGO: 99.07, 130.4. The EC_{50} values were nearly the same with all opioids tested regardless of the absence or presence of 10^{-6} M OT (Fig. 3a–e).

3.3. OT effect on opioid agonist-induced inhibition of cAMP levels in cells coexpressing hMOR and cAMP biosensor protein, measured using GloSensor™ cAMP assay

MOR activation induced a reduction in the intracellular cAMP levels (Fig. 4), and the effect of OT on MOR activation was evaluated using this assay. Whereas 10^{-6} M OT alone did not alter cAMP production as compared with vehicle, the OT treatment significantly enhanced the inhibition of cAMP production induced by 10^{-6} M endomorphin-1, β-endorphin, morphine, fentanyl, and DAMGO (Fig. 4a–e).

3.4. Competitive assay to examine OT effect on OR binding by agonists

Considering the results obtained from the CellKey™ and the intracellular cAMP assays, we investigated whether OT modulates the affinity of endomorphin-1 or morphine for MOR by performing competitive receptor-binding assays. OT alone (10^{-11}–10^{-5} M) did not bind to MOR (Fig. 5a), and the affinities with which endomorphin-1 and morphine bound to MOR in the presence and absence of 10^{-6} M OT did not differ (Fig. 5b and c).

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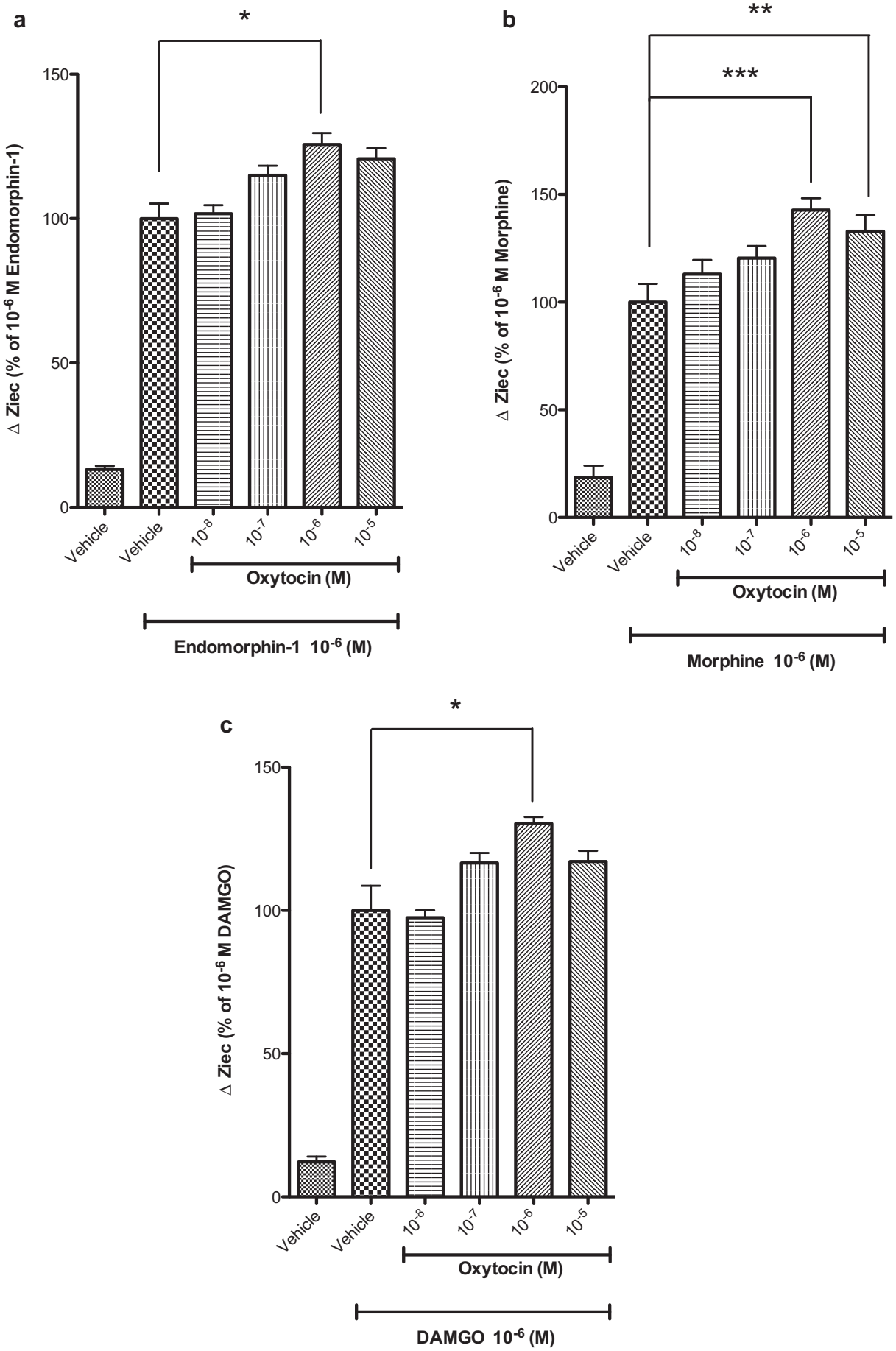

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3.5. Effect of endomorphin-1 on OT-induced increase of ΔZiec in OTR-expressing HEK293 cells, using CellKey™ assay

Lastly, we tested whether opioids could exert PAM effects on OTR. The results of CellKey™ assays performed using hOTR-expressing HEK293 cells showed that OT treatment increased ΔZiec in a dose-dependent manner (Fig. 6). However, endomorphin-1 at 10^{-6} M did not affect the OT-induced increase of ΔZiec (Fig. 6).

4. Discussion

In this study, we investigated the effects of OT on hMOR in vitro. Although previous studies have suggested a relationship between the analgesic effects of OT and MOR, in these studies, in vivo pain-withdrawal tests were performed using rats.[4–6] To the best of our knowledge, our study has provided the first direct evidence indicating that OT by itself strongly affects the activity of hMOR in vitro by using hMOR-expressing cells.

Studies conducted by others and us[22,26] have led to the acceptance that the CellKey™ impedance assay for GPCR activation is as representative as traditional assays for GPCR signaling, such as cAMP and $[Ca^{2+}]_i$ measurement. Moreover, the CellKey™ system has been reported to demonstrate similar or higher sensitivity as compared to traditional assays such as the GTPγS binding, cAMP and Ca^{2+} imaging assays.[26] Collectively, our results affirmed that the CellKey™ system can be used to generate high-quality pharmacological data in GPCR studies.

We first showed that OT alone failed to increase ΔZiec in HEK293 cells expressing hMOR by using the CellKey™ (Fig. 1); indicating that OT does not exert an agonistic effect on hMOR. We next considered the possibility that the analgesic effect of OT is associated with allosteric modulation upon the binding of an endogenous opioid to MOR. We found that endomorphin-1, morphine, and DAMGO induced ΔZiec even in the presence of OT, and further that OT at 10^{-6} M or higher significantly enhanced these opioid-induced ΔZiec values (Fig. 2). Concentration-response curves generated for endomorphin-1-, β-endorphin-, morphine-, fentanyl-, and DAMGO-induced ΔZiec in the absence and presence of 10^{-6} M OT showed that OT enhanced the ΔZiec induced by high concentration of the opioids (Fig. 3); by contrast, endomorphin-1 at 10^{-6} M did not enhance the ΔZiec induced by OT applied at any concentration in cells expressing hOTR (Fig. 6). Thus, OT appears to enhance opioid-induced ΔZiec by augmenting MOR signaling, and not through a nonspecific mechanism, and these results agree closely with the results of the intracellular cAMP assay performed in this study. Further, the allosteric cooperativity can be different depending on which particular orthosteric agonists (probe) are used. This is referred to as probe dependence and considered an important feature of PAM.[21] Differences of amino acids acting on the binding pocket of MOR in each agonist may cause a difference in the conformational change of MOR, which may affect the degree of the PAM efficacy.

Conn et al. proposed a model for allosteric modulation,[27] in which allosteric ligands bind to a topographically distinct site on the receptor as compared with the orthosteric agonist, and these ligands can modulate the orthosteric agonist's binding affinity and efficacy, and might exhibit intrinsic agonist activity. Therefore, we evaluated whether the PAM activity of OT affects the binding affinities of opioid agonists; however, we found that OT failed to alter the affinity of opioids (endomorphin-1 and morphine) for hMOR (Fig. 5). Thus, OT specifically modulates the efficacy of MOR signaling, which might correlate well with the Conn et al. model.[27]

ORs represent key cellular targets because of their therapeutic values in pain management; however, OR-targeted therapy has raised concerns because of the adverse side effects produced, such as respiratory suppression, constipation, allodynia, and dependence.[18] Previous studies on ORs have focused on developing ligands that can act as (a) orthosteric agonists that exhibit defined selectivity profiles for distinct ORs (μ, δ, κ); (b) partial agonists, which show reduced efficacy as compared with full agonists; or (c) agonists, when used together in combination therapy.[28,29] However, these ligands targeting the orthosteric binding sites present the common drawback of inability to maintain spatial and temporal precision in their action (i.e., to act where and when required). When these orthosteric ligands are administered systematically, they affect the receptors not only in pain-related sites, but also in other non-targeted tissues. Moreover, continuous exposure of opioid agonists to an orthosteric binding site might lead to receptor desensitization and cause tachyphylaxis, as well as produce toxic side effects mediated by long-term exposure of the drug at the receptor sites.[19,21] A novel strategy for pain management is μ-PAM administration, which enhances the activity of endogenous opioids and maintains native spatial and temporal activity where MOR activation is necessary.

As for optimum doses of OT, our result suggest that OT at 10^{-6} M showed the most strong PAM effect on MOR. Neurotransmitters including OT are packaged in the vesicles in cells in high concentrations before releasing; OT is secreted from axonal terminals or dendrites by exocytosis and released into the narrow synaptic clefts or extracellular fluid of the neuron.[30] In addition, we reported that during a condition of pain, OT levels in rats were increased in the blood and in Laminae I and II of the dorsal horn of the spinal cord.[10,11] Considering these facts, the OT concentration at 10^{-6} M could be likely concentration just in the local area where the MOR exist. Also, we must consider the possibility by the differences between in vivo and in vitro using recombinant receptor expressing cells. Endogenous opioids has been reported to increase during pain.[31] Our results in this study showed that OT enhanced the MOR signaling induced by high concentrations of endomorphin-1 and β-endorphin (Fig. 3a and b). These phenomena could potentially represent the expected inherent effect of endogenous opioids and OT for pain modulation in vivo. Given these features, endogenous OT by itself might produce analgesic effects not only by functioning through OTR, but also by acting on MOR through the PAM effect during pain.

In the pain treatment, exogenously added high concentrations of OT directly enters into the brain via extraneuronal/perineuronal routes through trigeminal or olfactory nerve[32] and could keep high concentration in the just local area before diffusely spread in the whole cerebrospinal fluids and subsequent degradation. In this regard, exogenously added OT could offer specific advantages; when administered alone in a pain condition, OT effectively enhances the opioid signaling induced by high concentrations of endogenous opioids, which results in adequate pain control, demonstrating spatial and temporal fidelity in vivo. Moreover, when used together with exogenous opioids for pain control, exogenously added OT as well as the endogenous OT that has already been released, might enhance the action of high doses of the exogenous opioids to produce further strengthened analgesic effects.

Fig. 2. Effect of OT on ΔZiec induced by distinct opioids in HEK293 cells stably expressing hMOR. Cells were treated with 10^{-6} M (a) endomorphin-1, (b) morphine, or (c) DAMGO in the absence or presence of OT at concentrations of 10^{-8}–10^{-5} M. All data are presented as means ± S.E.M. of 3–4 independent experiments (n = 9–15); *P < 0.05, **P < 0.01, ***P < 0.001, compared to each vehicle treatment (with opioid alone).

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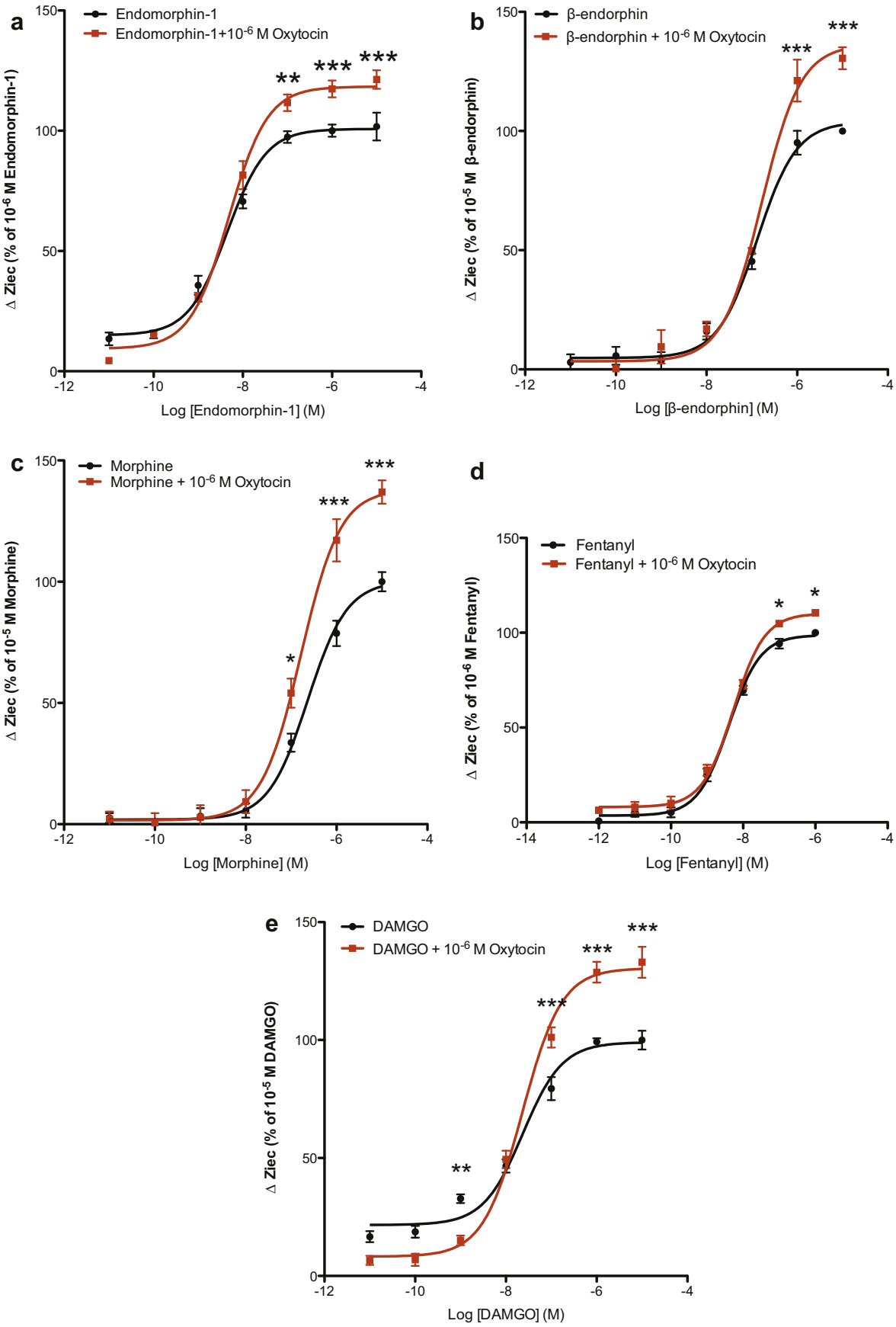

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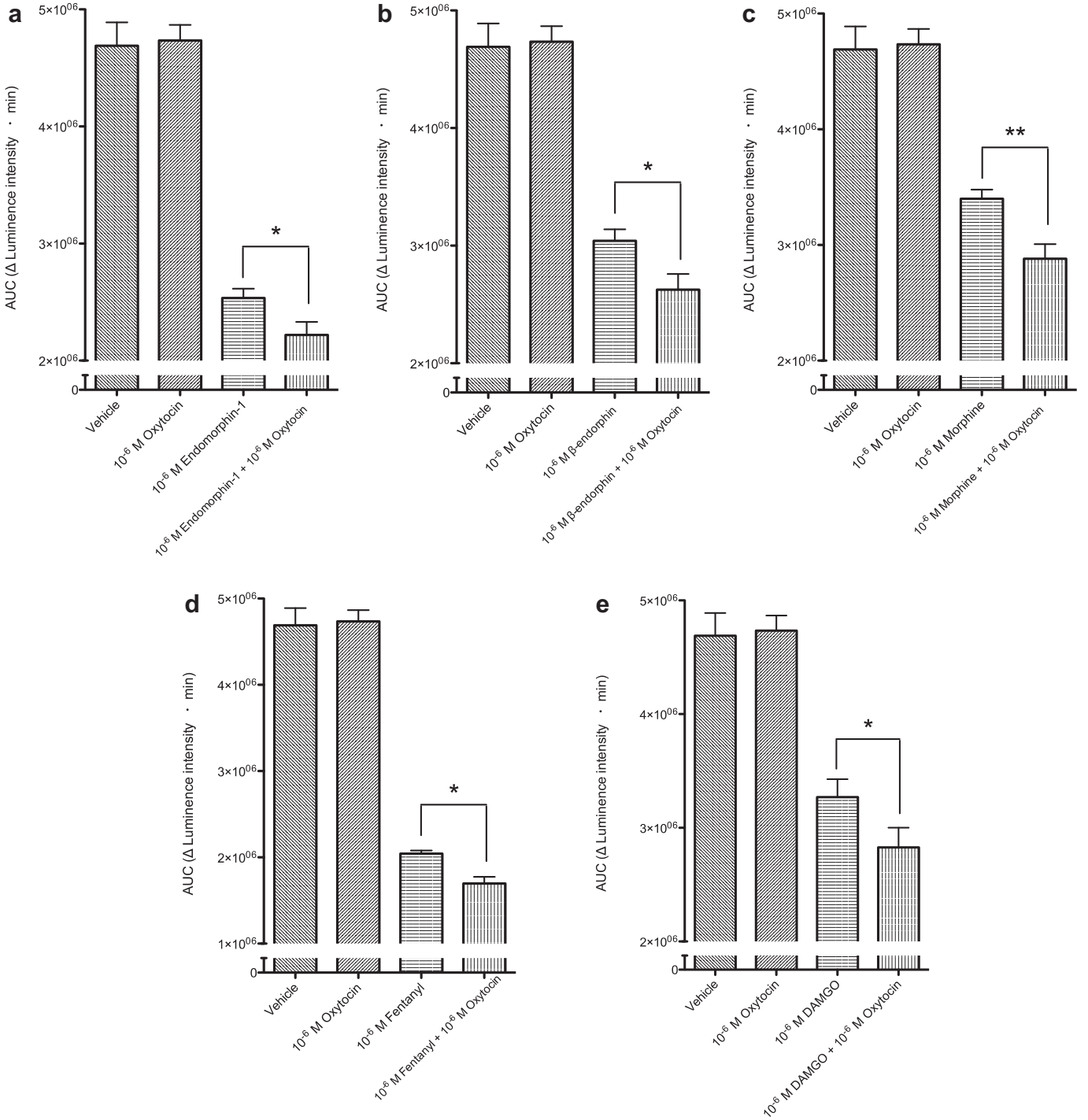

Fig. 4. OT effect on opioid-induced decrease in intracellular cAMP in HEK293 cells stably coexpressing both hMOR and GloSensor™ cAMP biosensor protein. Cells were treated with 10^{-6} M (a) endomorphin-1, (b) β-endorphin, (c) morphine, (d) fentanyl, and (e) DAMGO in the absence or presence of 10^{-6} M OT. All data are presented as means ± S.E.M. of 6 independent experiments (n = 9–18); *P < 0.05, **P < 0.01, compared to each opioid treatment alone.

Lastly, regarding the efficacy and safety of OT as a therapeutic compound, OT is naturally present in humans and has long been employed as a uterotonic agent in the field of obstetrics. Recently, clinical studies have generated unprecedented interests in the therapeutic targets, and potential benefits of intranasal OXT application for neuropsychiatric disorders characterized by social dysfunction, such as social anxiety, autism, and schizophrenia.[32] Okamoto et al. reviewed previous clinical trials investigating the efficacy and safety of OT intranasal single-dose and long-term administration for ASD patients[33]; all studies considered in this

Fig. 3. Concentration-response curves for opioid-induced ΔZiec in the absence and presence of 10^{-6} M OT. Cells were treated with (a) endomorphin-1 (10^{-11}–10^{-5} M), (b) β-endorphin (10^{-11}–10^{-5} M), (c) morphine (10^{-11}–10^{-5} M), (d) fentanyl (10^{-12}–10^{-6} M), and (e) DAMGO (10^{-11}–10^{-5} M) in the absence or presence of 10^{-6} M OT. All data are presented as means ± S.E.M. of 3–7 independent experiments (n = 9–21); *P < 0.05, **P < 0.01, ***P < 0.001, compared to each opioid treatment alone.

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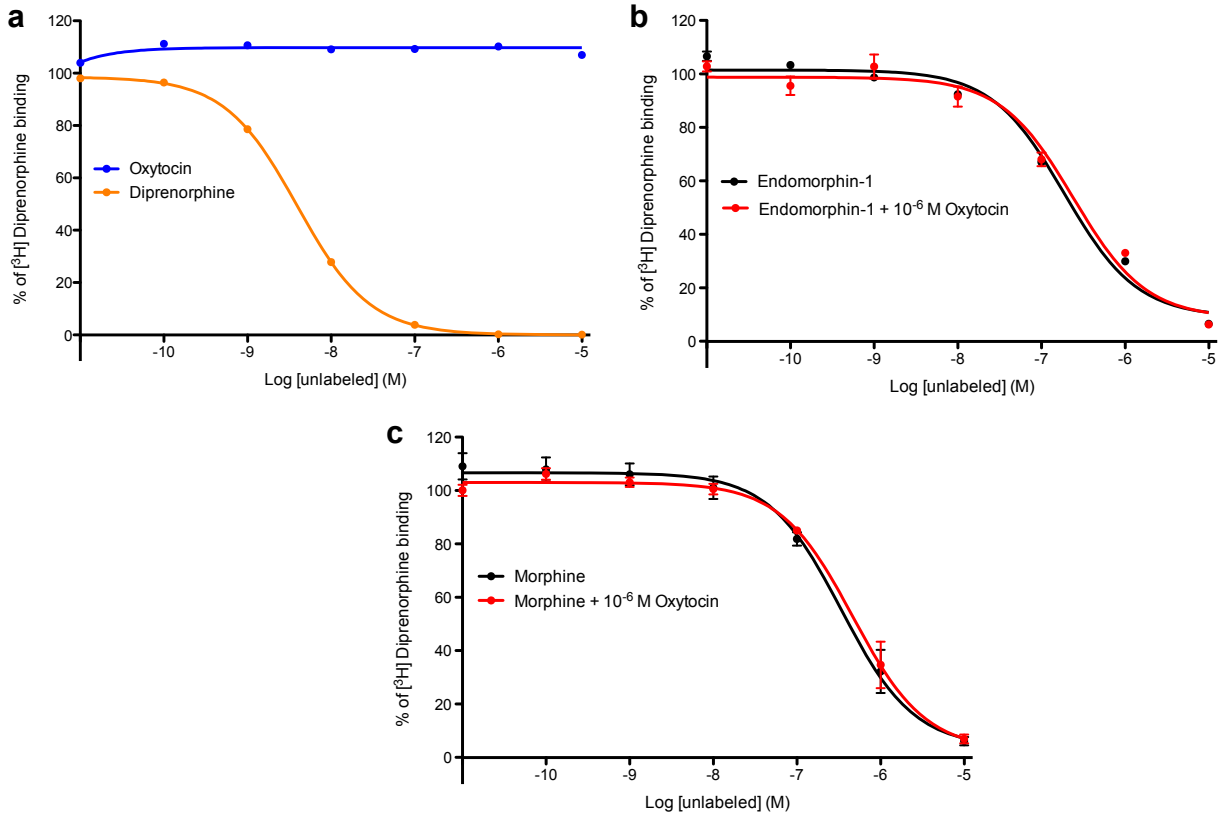

Fig. 5. Competitive OR-binding assays performed using membranes prepared from CHO cells expressing hMOR. All data are presented as means ± S.E.M. of 2 independent experiments (n = 6). (a) OT neither binds to MOR nor alters the affinity of (b) endomorphin-1 or (c) morphine for MOR.

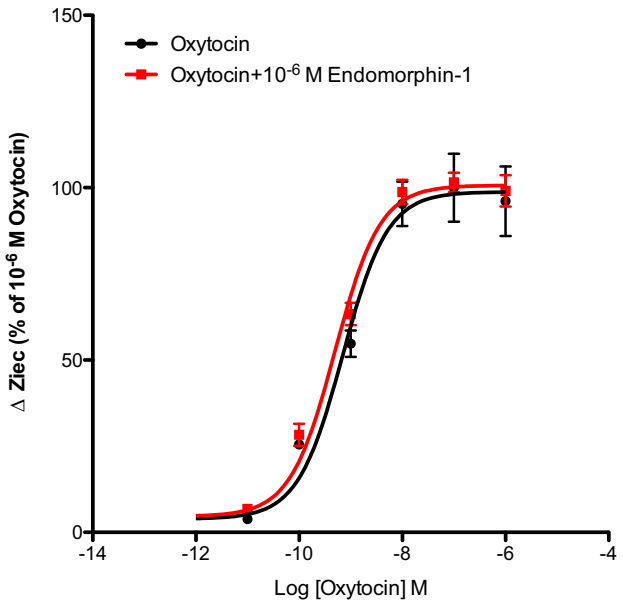

Fig. 6. Concentration-response curves for OT-induced ΔZiec in the absence and presence of 10^{-6} M endomorphin-1, measured with HEK293 cells stably expressing hOTR. Cells were treated with OT (10^{-12}–10^{-6} M) in absence or presence of 10^{-6} M endomorphin-1. All data are presented as means ± S.E.M. of 3 independent experiments (n = 6).

review suggested that single-dose and long-term administration of OT were well tolerated, and no severe adverse events were reported. Further report suggested the efficacy and safety of OT when used intranasally as an analgesic for acute pain.[9] Considering these findings, we propose that intranasal administration of OT might offer the therapeutic advantage of safety, and that OT could represent a potential candidate for drug repositioning as a clinically effective analgesic agent. However, further investigation into the clinical use of OT in pain control is required.

In conclusion, our present in vitro study suggest that OT might function as a PAM for MOR.

Conflict of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jphs.2018.04.002

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