A caged DAMGO for selective photoactivation of endogenous mu opioid receptors

Authors

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ABSTRACT:
Photoactivatable drugs and peptides are valuable tools that can drive quantitative studies into endogenous receptor signaling in relatively intact tissue preparations with high spatiotemporal resolution. We previously developed photoactivatable or "caged" variants of the opioid neuropeptides [Leu^5]-enkephalin and dynorphin A (1-8), but these reagents activate multiple opioid receptors simultaneously upon photolysis. To achieve selective engagement of mu opioid receptors (MORs), here we describe a photoactivatable derivative of the MOR-selective peptide agonist [D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin (DAMGO). Starting from commercially available DAMGO, we appended a negatively charged carboxynitroveratryl (CNV) caging group to the N-terminal tyrosine side chain (Y) to produce CNV-Y-DAMGO. Dose-response curves in a heterologous functional assay revealed that CNV-Y-DAMGO is ~1,000-fold less potent than DAMGO at MORs. In acute hippocampal brain slices, CNV-Y-DAMGO photoactivation with ultraviolet light resulted in rapid, transient suppression of inhibitory synaptic transmission, and this was blocked by the MOR-selective antagonist CTAP. These results validate CNV-Y-DAMGO as a photopharmacological reagent for manipulating endogenous MOR signaling in brain tissue with light.

KEYWORDS: caged compounds, photopharmacology, opioid, synaptic transmission, neurophysiology

INTRODUCTION

Photoactivatable or "caged" ligands are powerful tools for probing receptor signaling in living systems (Ellis-Davies, 2007; Paoletti, Ellis-Davies and Mourot, 2019). Because photorelease is typically faster than the biological processes under study, pre-equilibration of the caged molecule followed by photolysis can provide a robust stimulus that facilitates quantitative analysis of downstream processes. Caged molecules are compatible with more intact *ex vivo* tissue preparations such as brain slices, and can even work *in vivo* in intact organisms (Font *et al.*, 2017; Taura *et al.*, 2018).

The mu opioid receptor (MOR) is an inhibitory, $G_{i/o}$-coupled G protein-coupled receptor (GPCR) that is widely expressed in the nervous system. MORs are the primary target of opioid analgesics such as morphine and fentanyl, and are activated by the endogenous opioid neuropeptides enkephalin, beta-endorphin and dynorphin (Toll *et al.*, 1998; Gomes *et al.*, 2020). Activation of MORs typically leads to the suppression of neuronal excitability and synaptic output. Because MORs are often expressed in inhibitory interneurons, MOR agonists can lead to an increase in circuit output through the disinhibition of projection neurons (Nicoll, Alger and Jahr, 1980; Banghart *et al.*, 2015). In response to sustained activation, MORs undergo acute desensitization on the timescale of minutes, as well as long-term downregulation on the timescale of days (Quillinan *et al.*, 2011). As these molecular processes are relevant to clinical opioid use, either for treating pain or in the context of opiate drug abuse, understanding the basic molecular mechanisms of MOR signaling and their adaptations to chronic activation are of great significance. Furthermore, MORs can interact with other receptors in the nervous system, including other GPCRs such as the delta opioid receptor (DOR), and the N-methyl-D-Aspartate receptor (Kow *et al.*, 2002; Rodríguez-Mũoz *et al.*, 2012; Cahill and Ong, 2018).

The ability to detect changes in MOR signaling in response to processes such as chronic activation or functional interactions with other receptors can be greatly facilitated by the availability of photopharmacological probes such as caged ligands (Williams, 2014; Arttamangkul *et al.*, 2018; He *et al.*, 2021). Ideal tools would enable endogenous MORs to be stimulated in relatively intact brain tissue preparations such as brains slices in a

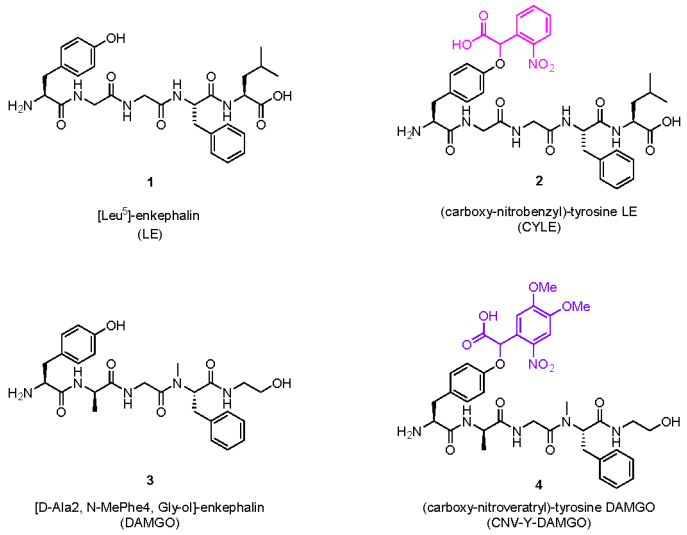
manner that is highly reproducible, temporally precise (*i.e.* not diffusion limited), readily varied in magnitude (*i.e.* concentration), and compatible with experimental approaches such as electrophysiology and fluorescence imaging. To facilitate studies into opioid receptor signaling in the nervous system, we have developed several caged opioid ligands, including the neuropeptide agonists [Leu^5]-enkephalin (LE) and dynorphin A (1-8) (Dyn8), as well as the antagonist naloxone (Banghart and Sabatini, 2012; Banghart *et al.*, 2013; Banghart, He and Sabatini, 2018). Although LE activates MORs with nanomolar affinity, it also activate DORs in a similar concentration range (Toll *et al.*, 1998; Banghart and Sabatini, 2012; Gomes *et al.*, 2020). Therefore, selective stimulation of MORs using caged LE requires the inclusion of a DOR-selective antagonist (Banghart, He and Sabatini, 2018). This requirement adds cost and technical challenges, as optimal antagonists are not inexpensive and can interfere with biological processes mediated by endogenous peptidergic tone. Furthermore, it greatly complicates experiments that intend to specifically probe functional interactions between MORs and DORs, as MORs can only be probed selectively when DORs are blocked (He *et al.*, 2021).

To simplify experiments involving the activation of MORs with caged peptide agonists, we developed a caged analogue of the MOR-selective peptide agonist [D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin (DAMGO) (Handa *et al.*, 1981). As DAMGO is an enkephalin derivative that was designed to resist proteolysis, it shares a cageable pharmacophore with LE and Dyn8. This new reagent, dubbed CNV-Y-DAMGO, can be photoactivated with millisecond flashes of ultraviolet (UV) light in brain tissue to stimulate endogenous mu opioid receptors with high spatiotemporal precision.

RESULTS AND DISCUSSION

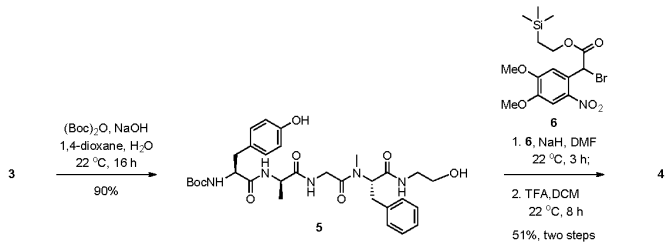
Our caged DAMGO design is based on our prior success in caging LE (**1**) by appending a carboxy-nitrobenzyl caging group to the N-terminal tyrosine phenol to produce (carboxy-nitrobenzyl)-tyrosine-LE or CYLE (**2**, **Scheme 1**) (Banghart and Sabatini,

2012). The presence of a phenolic proton at this position facilitates high affinity interactions between opioid ligands and opioid receptors through formation of a hydrogen bond in the orthosteric binding site (Manglik *et al.*, 2012). Given the high structural similarity between DAMGO (**3**) and LE, we reasoned that the presence of a caging group at this site would similarly reduce the affinity of DAMGO for MOR. In this case, we chose to use the carboxy-nitroveratryl (CNV) caging group, as it includes a negatively charged carboxylic acid to facilitate solubility, and because it exhibits an absorbance tail that enables photolysis with 405 nm light. Importantly, we previously found that caging the *N*-terminal tyrosine in LE with the CNV group (CNV-Y-LE) afforded rapid photorelease in brain slices to activate MORs with good sensitivity to UV light (Banghart, He and Sabatini, 2018).



Scheme 1. Design of a caged DAMGO based on caged [Leu5]-enkephalin.

We therefore took an analogous approach to synthesize CNV-Y-DAMGO (**4**). Synthesis commenced with Boc-protection of the *N*-terminal amine of commercially available DAMGO, which proceeded in high yield (**Scheme 2**). Without protecting the C-terminal glyol, *N*-Boc-protected DAMGO was then selectively alkylated on the tyrosine phenol using (TMSE)-carboxynitroveratryl bromide (**6**), followed by simultaneous deprotection of the *N*-terminus and carboxylic acid using TFA in DCM. This three-step synthesis readily afforded milligram quantities of CNV-Y-DAMGO in ~45% overall yield.



Scheme 2. Synthesis of CNV-Y-DAMGO.

We next evaluated the activity of CNV-Y-DAMGO at MORs using the GloSensor assay of G_α signaling in HEK293T cells (**Figure 1**). Whereas we found the EC_{50} of DAMGO to be 1.5 nM in this assay, the EC_{50} of CNV-Y-DAMGO was 1.7 μM. This ~1,000-fold reduction in potency is similar to that observed when caging LE in the same way, suggesting that CNV-Y-DAMGO is sufficiently inactive to produce strong photoactivation of MORs with light.

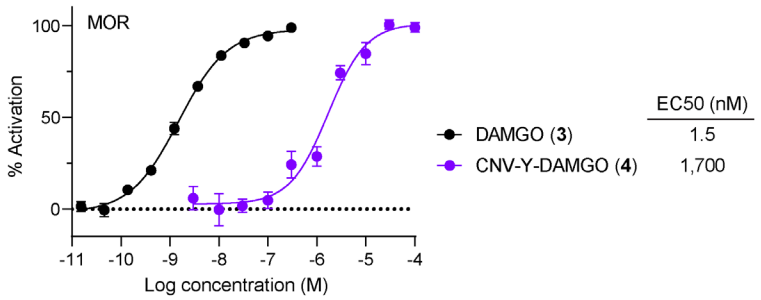


Figure 1. Activity of CNV-Y-DAMGO at the mu opioid receptor. Dose response-curves were obtained using a Glo-Sensor assay of cAMP signaling in HEK293T cells (n=12 wells per data point). Data were normalized to the maximal response to DAMGO (1 μM) and are expressed as the mean ± SEM.

To validate the utility of CNV-Y-DAMGO for probing native opioid receptors in brain tissue, we performed whole cell voltage clamp recordings from hippocampal neurons in acute brain slices taken from adolescent mice (**Figure 2A**). We previously reported that electrically-evoked synaptic inhibition in the perisomatic region of CA1 pyramidal neurons is strongly suppressed by both MOR and DOR agonists (Banghart, He and Sabatini, 2018). We first verified the inactivity of CNV-Y-DAMGO by recording inhibitory post-synaptic currents (IPSCs) during bath application of either DAMGO (1 μM) or CNV-Y-DAMGO (1 μM). Whereas DAMGO suppressed IPSC amplitude by ~70%, CNV-Y-DAMGO had no discernable effect on synaptic transmission (**Figure 2B**) (DAMGO: 0.70 ± 0.02; CNV-Y-DAMGO: -0.046 ± 0.05; $p < 0.0001$, Mann-Whitney test). We next probed the effects of illumination with 5 ms flashes of light emitted from a 355 nm laser, applied 2 seconds prior to an electrical stimulus. Similar to caged LE, photolysis of CNV-Y-DAMGO produced a rapid suppression of inhibitory synaptic transmission that reversed over the course of several minutes (**Figure 2C**). Presumably, the rate of reversal reflects diffusional clearance of the uncaged DAMGO from the photolysis site. Consistent with effect of DAMGO photorelease being mediated entirely

by MORs, the optically-evoked suppression of synaptic transmission was completely blocked by the inclusion of the selective MOR antagonist CTAP in the bath (**Figure 2D**) (+UV: 0.61 ± 0.05; +UV +CTAP: -0.05 ± 0.10, p = 0.001, Mann-Whitney test).

Taken together, these results indicate that CNV-Y-DAMGO can be used to reliably activate endogenous MORs in brain tissue using light. Importantly, because DAMGO is selective for MORs, its use does not require the addition of antagonists for other receptors. Because the photolysis involves cleavage of a phenolic ether, the kinetics of photorelease are fast, similar to CYLE and CNV-Y-LE (Banghart, He and Sabatini, 2018). Like other photoactivatable probes based on dialkoxynitrobenzyl caging groups, CNV-Y-DAMGO should be amenable to photoactivation with low-cost LEDs that emit wavelengths ranging from 365-405 nm. It should also interface well with

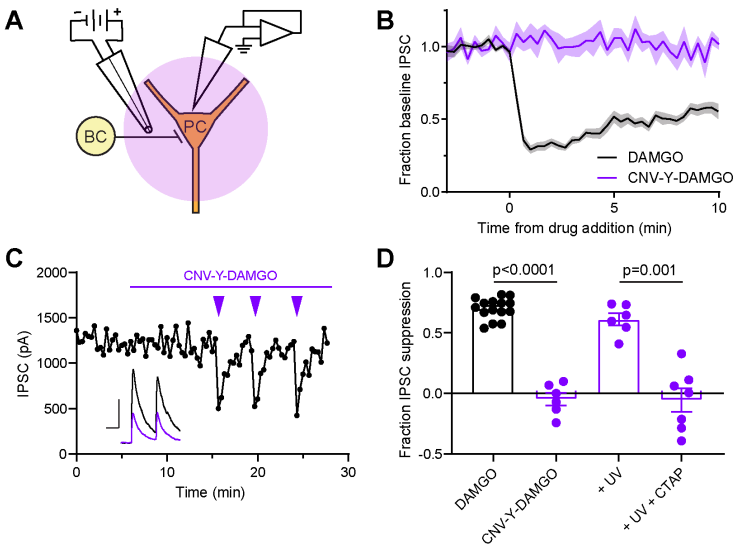


Figure 2. CNV-Y-DAMGO uncaging suppresses synaptic transmission in hippocampal brain slices via activation of the mu opioid receptor. A. Schematic of the experimental configuration for photo-uncaging of CNV-Y-DAMGO while recording electrically-evoked inhibitory synaptic transmission from basket cells (BC) impinging on pyramidal cells (PC). **B.** Baseline-normalized, average inhibitory post-synaptic current (IPSC) amplitude over time during bath application of DAMGO (n = 15 cells from 14 mice) or CNV-Y-DAMGO (n = 6 cells from 4 mice). **C.** Representative example of IPSC amplitude over time during bath application of CNV-Y-DAMGO (1 μM) and repeated photolysis using 355 laser flashes (84 mW, 5 ms). **Inset:** Example IPSCs immediately before (black) and after (purple) CNV-Y-DAMGO uncaging. Scale bars: x = 25 ms, y = 500 pA. **D.** Summary data comparing the fraction of baseline IPSC suppression in response to either DAMGO (1 μM) bath application, CNV-Y-DAMGO (1 μM) bath application, CNV-Y-DAMGO uncaging, and CNV-DAMGO uncaging in the presence of the mu-selective antagonist CTAP (1 μM) (DAMGO (n = 15 cells from 14 mice), CNV-DAMGO (n = 6 cells from 4 mice), +UV (n = 6 cells from 4 mice), +UV + CTAP (n = 7 cells from 2 mice)).

experiments involving fluorescence imaging, as it will not be photolyzed by the longer wavelengths used to excite green and red fluorophores, which includes two-photon excitation. Future efforts will explore modifications designed to improve compatibility with *in vivo* uncaging, which may involve further reducing residual activity in the caged form, and the use of caging groups that respond to longer wavelengths of light that better penetrate tissue.

METHODS

Chemical Synthesis and Characterization. High-resolution mass spectrometry data were obtained at the UCSD Chemistry and Biochemistry Mass Spectrometry Facility on an Agilent 6230 time-of flight mass spectrometer (TOFMS). CNV-Y-DAMGO was purified by reverse-phase chromatography to >99.9% purity, used as a mixture of diastereomers, and found to be stable in the dark for at least 24 h in phosphate-buffered saline at pH 7.2. DAMGO was obtained from HelloBio (HB2409) and TMSE-CNV-bromide (**6**) was synthesized according to a published procedure (Gilbert *et al.*, 2007).

N-Boc-DAMGO (**4**): To a stirred solution of DAMGO (50 mg, 97 μmol) in 1,4-dioxane/water (3/1, 300 μL) in an amber glass vial at 22 ^{0}C, 1 M NaOH in water (97 μL, 97 μmol) was added followed by $(Boc)_2O$ (21 mg, 96 μmol) in 1,4-dioxane (50 μL). The reaction was monitored by HPLC. After 16 h, the reaction mixture was concentrated under vacuum and the residual was purified by C18 column chromatography (water/CH_3CN, 95%/5% → 100%). Relevant fractions were combined and concentrated under vacuum to give *N*-Boc-DAMGO as a white solid (54 mg, 90%). LR-MS (ESI) *m/z* 636 [$(M+Na)^+$, 100%]. HR-MS *m/z* 636.3005 $[M+Na]^+$ (calcd for $C_{31}H_{43}N_5O_8Na$, 636.3004).

N-Boc-TMSE-CNV-Y-DAMGO: To a stirred solution of *N*-Boc-DAMGO (50 mg, 81 μmol) in anhydrous DMF (500 μL), NaH (60% in mineral oil, 3.3 mg, 82 μmol) was added slowly. The mixture was stirred at 0 ^{0}C for 0.5 h followed by the addition of TMSE-CNV-bromide (**6**, 34 mg, 81 μmol) in anhydrous DMF (100 μL) dropwise. The mixture was stirred under nitrogen and warmed up to 22 ^{0}C. The reaction was monitored by HPLC. After 3 h, a drop of MeOH was added to quench the reaction. The reaction mixture was concentrated under vacuum and the residual was purified by C18 column

chromatography (water/CH_3CN, 95%/5% → 100%). Relevant fractions were combined and concentrated under vacuum to give *N*-Boc-TMSE-CNV-DAMGO as a white solid (67 mg, 87%). LR-MS (ESI) *m/z* 975 [$(M+Na)^+$, 100%]. HR-MS *m/z* 975.4145 $[M+Na]^+$ (calcd for $C_{46}H_{64}N_6O_{14}SiNa$, 975.4142).

CNV-Y-DAMGO (**6**): To a stirred solution of *N*-Boc-TMSE-CNV-DAMGO (60 mg, 63 μmol) in DCM (200 μL), TFA (400 μL) was added. The reaction was monitored by HPLC. After 8 h, the reaction mixture was concentrated under vacuum and the residual was purified by C18 column chromatography (water/CH_3CN, 95%/5% → 100%). Relevant fractions were combined and concentrated under vacuum to give CNV-Y-DAMGO as a white solid (27 mg, 58%). LR-MS (ESI) *m/z* 753 [$(M+H)^+$, 60%], 775 [$(M+Na)^+$, 100%]. HR-MS *m/z* 753.3086 $[M+H]^+$ (calcd for $C_{36}H_{45}N_6O_{12}$, 753.3090).

GloSensor Assay. Human embryonic kidney 293T cells were grown in complete DMEM (Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) containing 5% fetal bovine serum (Corning), 50 U/mL Penicillin-Streptomycin (Invitrogen), and 1 mM sodium pyruvate (Corning) and maintained at 37 °C in an atmosphere of 5% CO_2 in 10 cm TC dishes. Media in 10 cm TC dishes with HEK 293T cells (at around 70% confluence) were replaced with Opti-MEM (Invitrogen). Then the SSF-MOR plasmid (Tanowitz and Von Zastrow, 2003), cAMP-dependent GloSensor reporter plasmid (-22F cAMP plasmid, Promega, E2301), and Lipofectamine 2000 (Invitrogen) in Opti-MEM were added. The dishes with transfection media were incubated at 37 °C in an atmosphere of 5% CO_2 for 6 h before replacing media with complete DMEM. After incubating at 37 °C in an atmosphere of 5% CO_2 for 16 h, transfected cells were plated in ploy-*D*-lysine coated 96-well plates at ~40,000 cells/well and incubated at 37 °C in an atmosphere of 5% CO_2 for 16 h. On the day of assay, media in each well were replaced with 50 μL of assay buffer (20 mM HEPES, 1x HBSS, pH 7.2, 2 g/L *D*-glucose), followed by addition of 25 μL of 4x drug solutions for 15 min at 37 °C. To measure agonist activity for MOR, 25 μL of 4 mM luciferin (Assay Reagent, Promega, E1291) supplemented with isoproterenol at a final concentration of 200 nM was added, and luminescence counting was done after 25 min.

Brain Slice Preparation. Animal handling protocols were approved by the UC San Diego Institutional Animal Care and Use Committee. Male and female postnatal day 15–35 mice on a C57/Bl6 background were anesthetized with isoflurane and decapitated, and the brain was removed, blocked, and mounted in a VT1000S vibratome (Leica Instruments). Horizontal slices (300 μm) were prepared in ice-cold choline-ACSF containing (in mM) 25 $NaHCO_3$, 1.25 NaH_2PO_4, 2.5 KCl, 7 $MgCl_2$, 25 glucose, 0.5 $CaCl_2$, 110 choline chloride, 11.6 ascorbic acid, and 3.1 pyruvic acid, equilibrated with 95% O_2/5% CO_2. Slices were transferred to a holding chamber containing oxygenated artificial cerebrospinal fluid (ACSF) containing (in mM) 127 NaCl, 2.5 KCl, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 2 $CaCl_2$, 1 $MgCl_2$, and 10 glucose, osmolarity 290. Slices were incubated at 32 °C for 30 min and then left at room temperature until recordings were performed.

Electrophysiology. All recordings were performed within 5 h of slice cutting in a submerged slice chamber perfused with ACSF warmed to 32 °C and equilibrated with 95% O_2/5% CO_2. Whole-cell voltage clamp recordings were made with an Axopatch 700B amplifier (Axon Instruments). Data were filtered at 3 kHz, sampled at 10 kHz, and acquired using National Instruments acquisition boards and a custom version of ScanImage written in MATLAB (Mathworks). Cells were rejected if holding currents exceeded −200 pA or if the series resistance (<25 MΩ) changed during the experiment by more than 20%. Patch pipets (2.5–3.5 MΩ) were filled with an internal solution containing (in mM) 135 $CsMeSO_3$, 10 HEPES, 1 EGTA, 3.3 QX-314 (Cl − salt), 4 Mg-ATP, 0.3 Na-GTP, and 8 Na 2 phosphocreatine (pH 7.3, 295 mOsm/kg). Cells were held at 0 mV to produce outward currents. Excitatory transmission was blocked by the addition to the ACSF of NBQX (10 μM) and CPP (10 μM).To electrically evoke IPSCs, stimulating electrodes pulled from theta glass with ~5 μm tip diameters were placed at the border between stratum pyramidale and stratum oriens nearby the recorded cell (~50–150 μm) and a two brief pulses (0.5 ms, 50–300 μA, 50 ms interval) were delivered every 20 s.

UV Photolysis. Uncaging was carried out using 5 ms flashes of collimated full-field illumination with a 355 nm laser, as previously described. Light powers in the text

correspond to measurements of a 10 mm diameter collimated beam at the back aperture of the objective. Beam size coming out of the objective onto the sample was 3900 μm^2.

Data Analysis. Electrophysiology data were analyzed in Igor Pro (Wavemetrics). Peak current amplitudes were calculated by averaging over a 2 ms window around the peak of the IPSC. To determine magnitude of modulation by DAMGO uncaging (%IPSC suppression), the IPSC peak amplitude immediately after a flash was divided by the average peak amplitude of the three IPSCs preceding the light flash. The effects of drugs on IPSC suppression were calculated as the average %IPSC suppression 2-3 minutes after drug addition. Summary values are reported as mean ± SEM. All data are treated as non-parametric and specific statistical tests and corrections are described for each figure in the text and figure legends.

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Author contributions

X.M, X.J.H and M.R.B. conceived of the project. X.M. designed and synthesized CNV-Y-DAMGO and performed the GloSensor assays. X.J.H. designed and conducted the brain slice electrophysiology experiments and analyzed the data. All authors contributed to the writing of the manuscript.

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Notes

The authors declare no competing financial interests.

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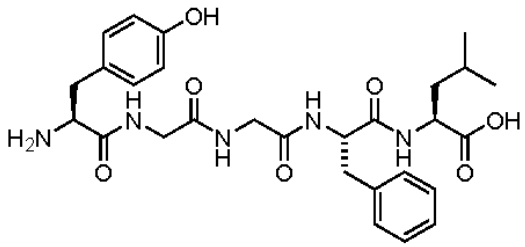
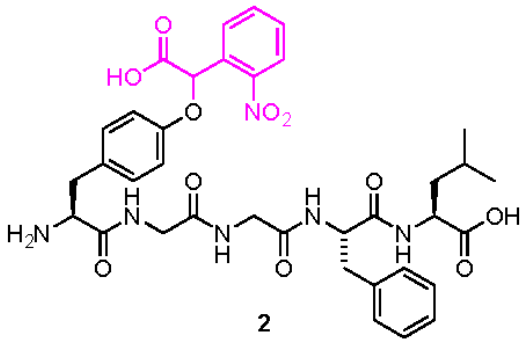
FIGURES



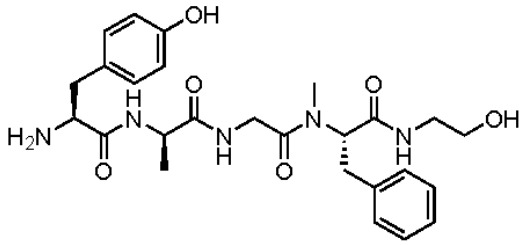
1

[Leu^5]-enkephalin
(LE)



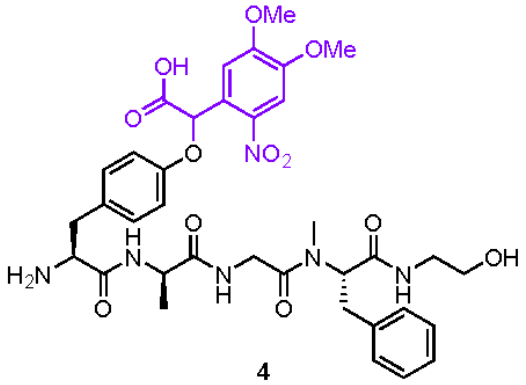
2

(carboxy-nitrobenzyl)-tyrosine LE
(CYLE)



3

[D-Ala2, N-MePhe4, Gly-ol]-enkephalin
(DAMGO)



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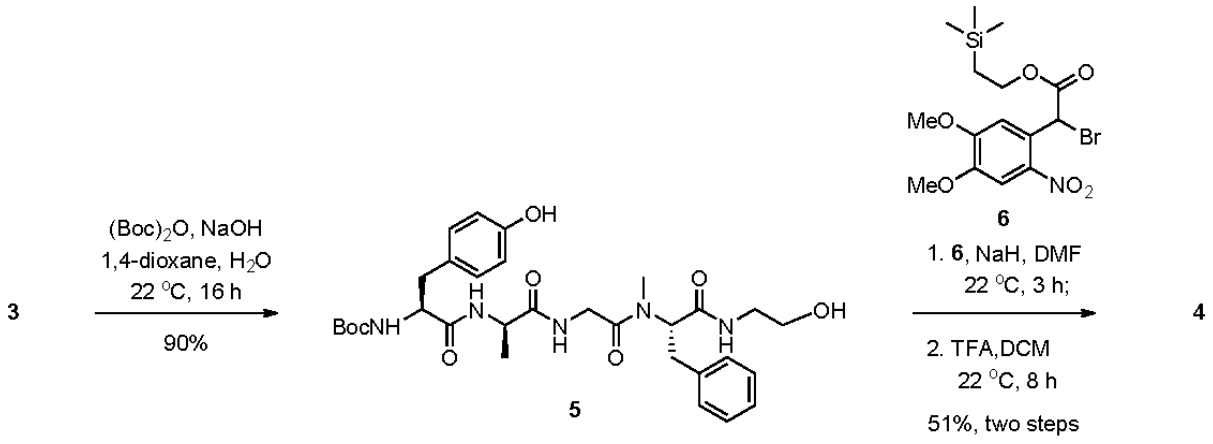
(carboxy-nitroveratryl)-tyrosine DAMGO
(CNV-Y-DAMGO)

Scheme 1. Design of a caged DAMGO based on caged [Leu^5]-enkephalin.



Scheme 2. Synthesis of CNV-Y-DAMGO.

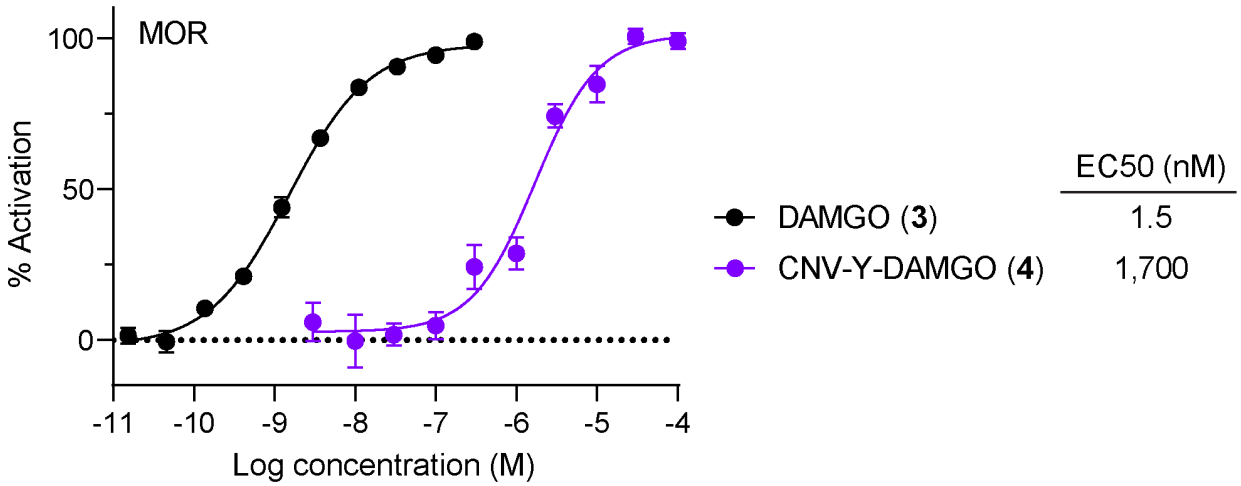


Figure 1. Activity of caged DAMGO at the mu opioid receptor. Dose response-curves were obtained using a Glo-Sensor assay of cAMP signaling in HEK293T cells (n=12 wells per data point). Data were normalized to the maximal response to DAMGO (1 μM) and are expressed as the mean ± SEM.

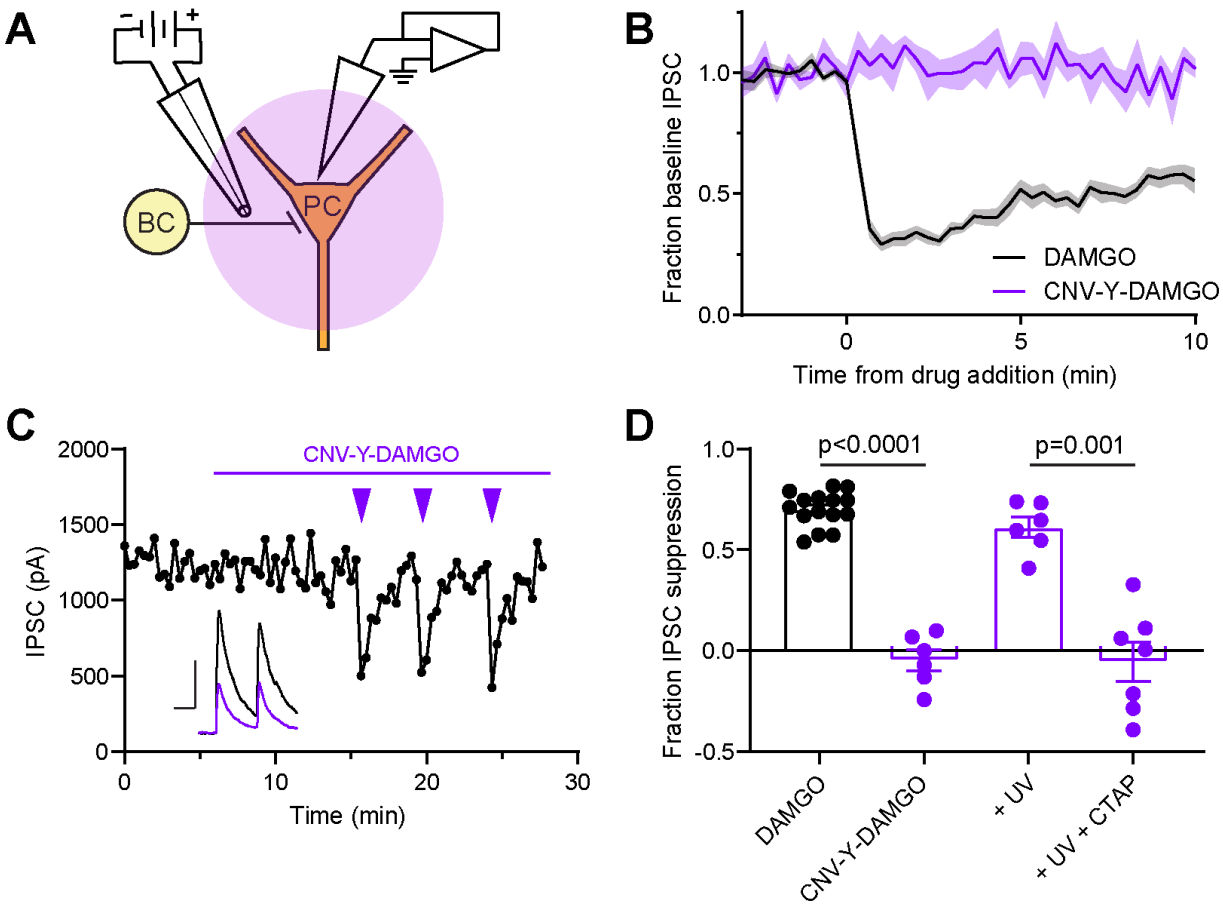


Figure 2. CNV-Y-DAMGO uncaging suppresses synaptic transmission in hippocampal brain slices via activation of the mu opioid receptor. A. Schematic of the experimental configuration for photo-uncaging of CNV-Y-DAMGO while recording electrically-evoked inhibitory synaptic transmission from basket cells (BC) impinging on pyramidal cells (PC). **B.** Baseline-normalized, average inhibitory post-synaptic current (IPSC) amplitude over time during bath application of DAMGO (n = 15 cells from 14 mice) or CNV-Y-DAMGO (n = 6 cells from 4 mice). **C.** Representative example of IPSC amplitude over time during bath application of CNV-Y-DAMGO (1 μM) and repeated photolysis using 355 laser flashes (84 mW, 5 ms). **Inset:** Example IPSCs immediately before (black) and after (purple) CNV-Y-DAMGO uncaging. Scale bars: x = 25 ms, y = 500 pA. **D.** Summary data comparing the fraction of baseline IPSC suppression in response to either DAMGO (1 μM) bath application, CNV-Y-DAMGO (1 μM) bath application, CNV-Y-DAMGO uncaging, and CNV-DAMGO uncaging in the presence of the mu-selective antagonist CTAP (1 μM) (DAMGO (n = 15 cells from 14

mice), CNV-DAMGO (n = 6 cells from 4 mice), +UV (n = 6 cells from 4 mice), +UV + CTAP (n = 7 cells from 2 mice)).