Evaluation of Different Photocaging Strategies to Develop Next-Generation Photoprobes

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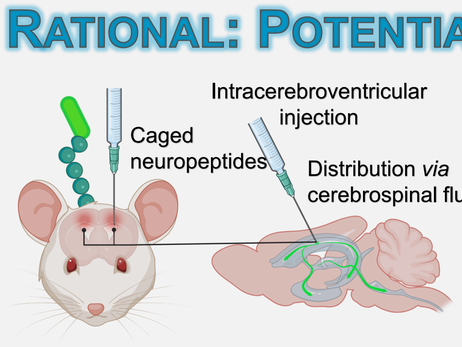
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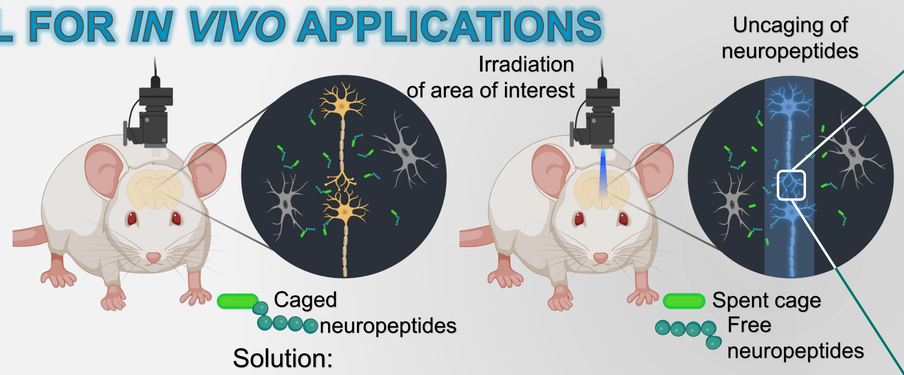
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Neuropeptides and Photopharmacology

Peptide signalling pathways govern diverse physiological and behavioural processes, yet their complex spatial and temporal dynamics remain difficult to study due to widespread receptor expression, long peptide half-lives, and extensive diffusion in neuronal tissue. To address these limitations and to enable precise control over neuropeptide activity, we systematically evaluated three classes of photolabile protecting groups – coumarin, nitrophenylpropyl, and boron-dipyrromethene – to establish a broadly applicable photocaging strategy for peptides. These cages support controlled one-photon (365-527 nm) and two-photon (730-780 nm) activation while avoiding cytotoxic by-products. Using the neuropeptides oxytocin and vasopressin as a proof-of-concept model system, we synthesised and characterised the photopharmacological profiles of these photocaged neuropeptide probes at their neuronal receptors (OTR, $V_{1a}R$, $V_{1b}R$) and in acute mouse cortical slices. The experiments identified the coumarin derivative DCMAC as the most effective photocage, providing robust bioactivity masking while maintaining biocompatibility. Rapid release of the native peptides and receptor activation could be achieved upon irradiation with both low-cost LED setups and high-precision two-photon lasers. Guided by these findings, we extended the DCMAC-based strategy to somatostatin and vasoactive intestinal peptide, demonstrating its broad applicability to other peptide families. Together, this generalised photocaging platform enables on-demand modulation of peptide signalling with high spatiotemporal precision, opening new avenues for mechanistic studies across diverse peptide systems in health and disease.

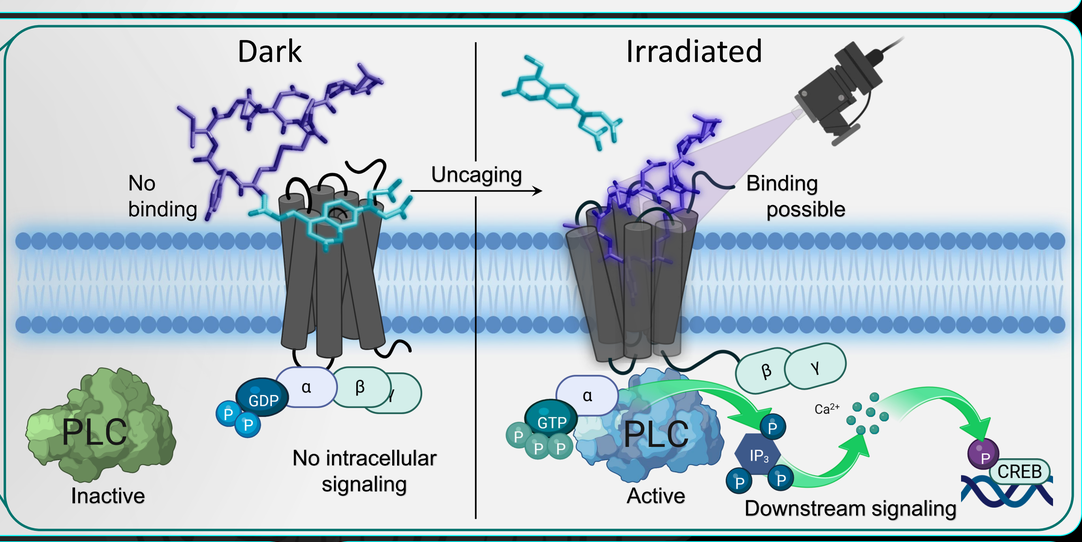
Rational: Potential for *in vivo* Applications



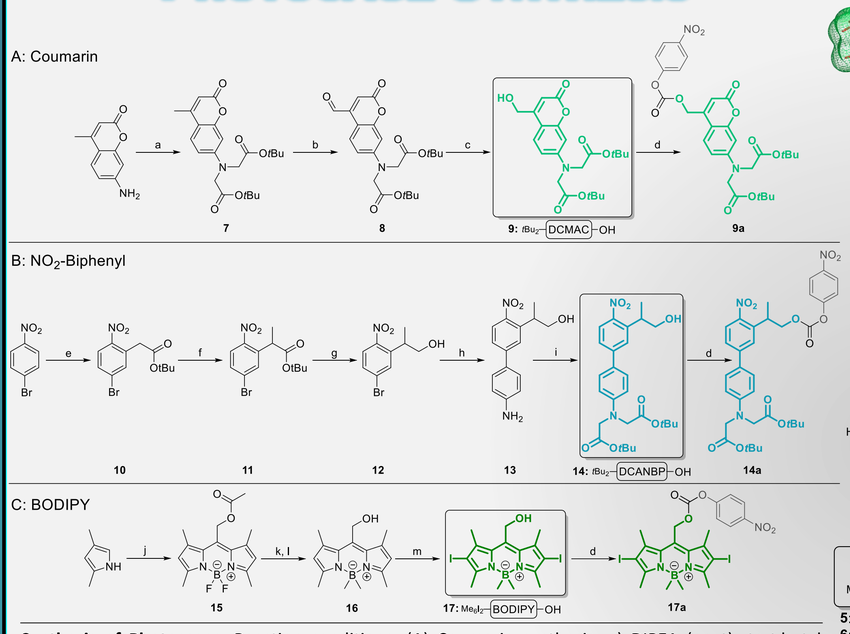


Problem:
- Injection stressful for the animal
- Limited time window for behavioural experiments
- Diffusion away from original point of injection
- Interactions in non-targeted areas

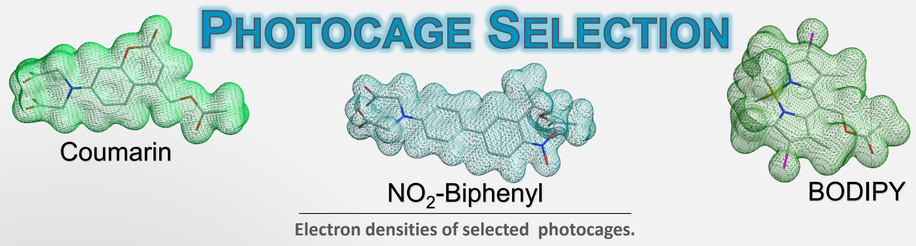
Solution:
- Combination with Photopharmacology
- Caged neuropeptides biologically inert
- Uncaging releases high concentration of native peptides
- High spatiotemporal control of receptor activation



Photocage Synthesis

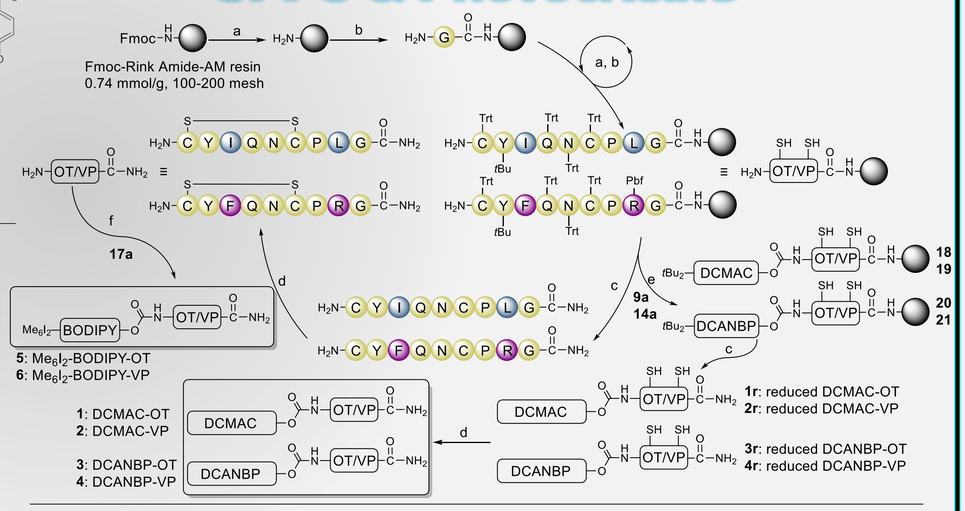


Photocage Selection



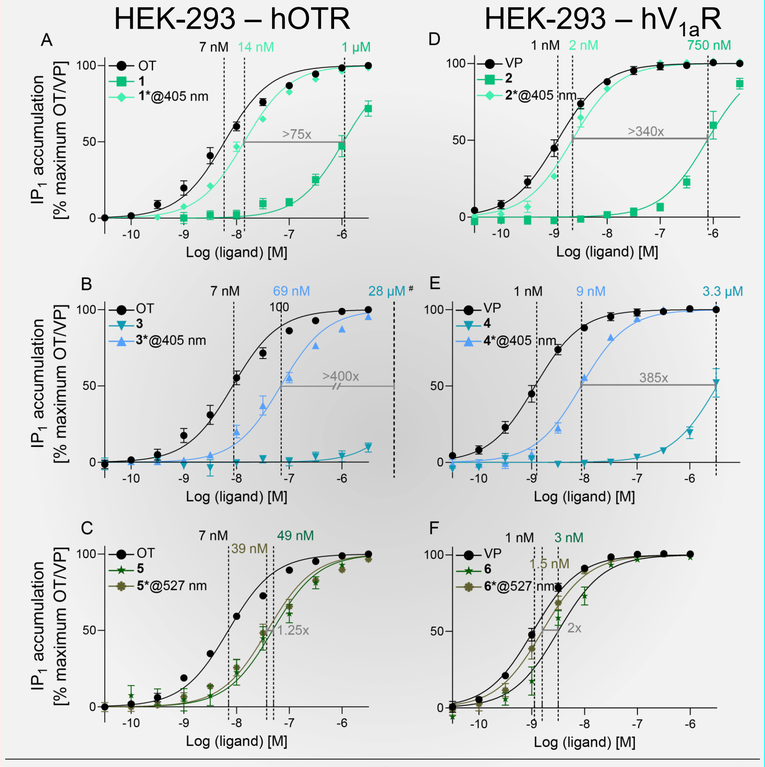
Electron densities of selected photocages.

SPPS & Photocaging



SPPS and caging of OT and VP. Reaction conditions: a) 1) 1 min DMF flow-wash, 2) 2x 1 min piperidine/DMF (1:1), 3) 1 min DMF flow-wash; b) HATU (0.5 M in DMF)/amino acid/DIPEA (3.95/4/4 with respect to resin loading), RT, 15 min; c) 1) 1 min DCM flow-wash, 2) TFA/ddH_2O/TIPS (90/5/5), RT, 2 h, 3) N_2-stream, Et_2O, 0 °C; d) 1) 0.1 M NH_4HCO_3 buffer (pH=8.2), RT, 24 h, 2) TFA; Yields after folding and RP-HPLC: 18% **1**, 17% **2**, 28% **3**, 12% **4**; e) DMF, pyridine, **9a** or **14a**; f) THF, DMF, pyridine, **17a**; Yields after photocaging and RP-HPLC: 56% **5**, 24% **6**; HATU: 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate, TIPS: triisopropylsilane.

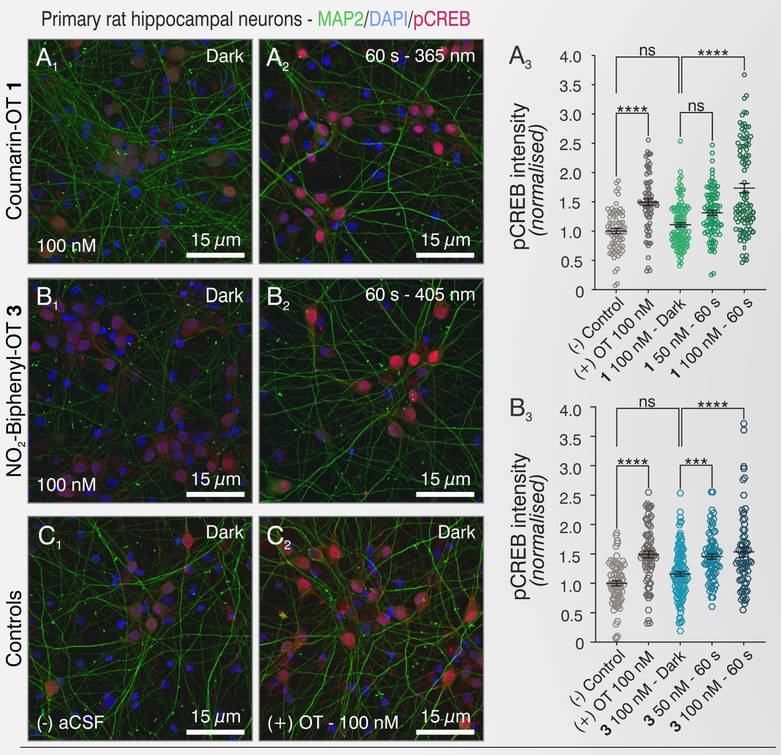
Pharmacology



***In vitro* pharmacologically evaluation of photocaged and *in situ* uncaged (*) neuropeptides**. Cellular functional IP_1-assay performed on stable HEK-293 cells, overexpressing hOTR and $hV_{1a}R$ (N≥3, n=3). Results normalised to OT/VP (100%) and negative control (buffer; 0%) activity. Error bars indicate standard error of the mean. (A-C) hOTR, (D-F) $hV_{1a}R$. EC_{50} values shown above with same color coding for the corresponding compounds, asterisks indicate 60 s *in situ* uncaging, # indicate extrapolated values values, grey numbers and lines indicate x-fold difference of EC_{50} values before and after uncaging.

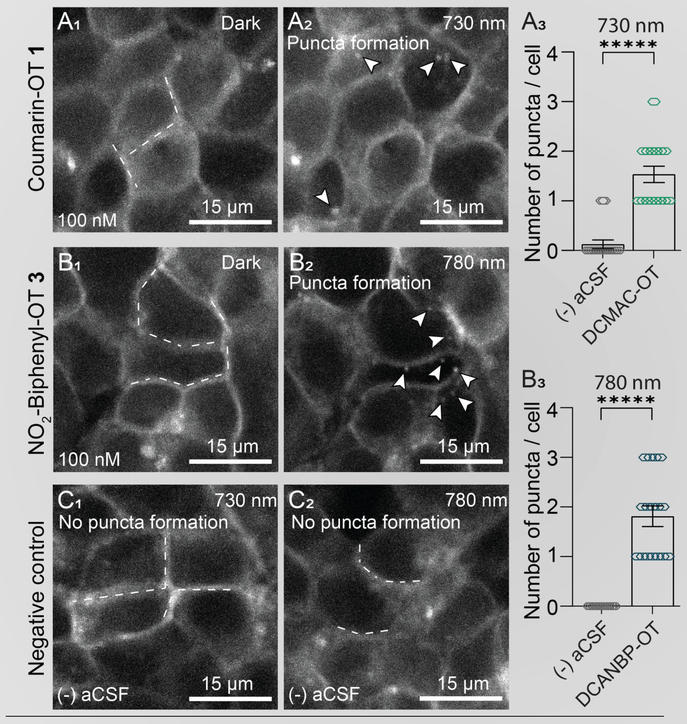
Biological Evaluation

1PE Uncaging – Neurons



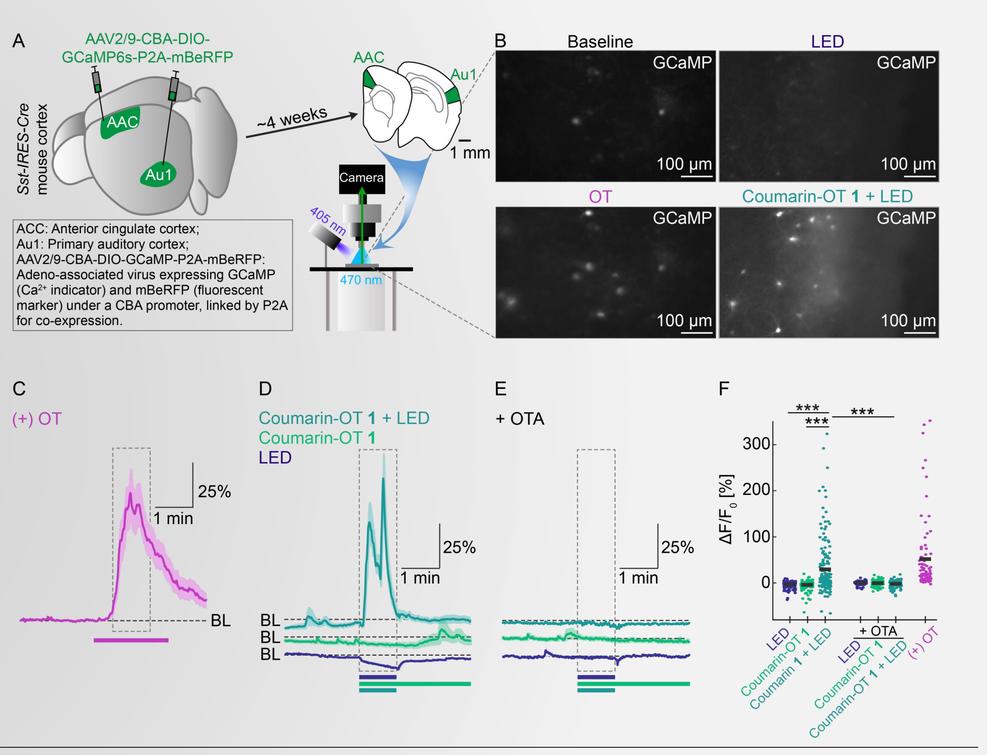
***In vitro* evaluation of the bioactivity of OT photoprobes, caged and uncaged**. Right on primary rat hippocampal neurons. Nuclei were stained with DAPI (blue), and dendrites with antibodies for MAP2 (green). Phosphorylation of CREB (red) was quantified after immunocytochemistry of n=~100 cells over two coverslips and normalised to the negative control (aCSF). (A_{1-3}) Coumarin-OT **1**, 100 nM, dark and irradiated at 365 nm for 60 s. (B_{1-3}) NO_2-Biphenyl-OT **3**, 100 nM, dark and irradiated at 405 nm for 60 s. (C_{1-2}) Negative control (aCSF) and positive control (OT, 100 nM) dark; ns, non-significant; *=p<0.05; ***=p<0.001; ****=p<0.0005; *****=p<0.0001

2PE Uncaging – HEK-293



2PE OT photoprobe uncaging during live-cell imaging. HEK-293 cells stably overexpressing hOTR-GFP, Ti:Sapphire laser tuned to 730 nm or 780 nm. (A_{1-3}) Coumarin-OT **1** 100 nM in the dark and 10 min after local irradiation (dashed lines) at 730 nm. (B_{1-3}) NO_2-Biphenyl-OT **3** 100 nM in the dark and 10 min after local irradiation (dashed lines) at 780 nm. (C_{1-2}) aCSF 10 min after local irradiation (dashed lines) at 730/780 nm. Arrowheads indicate puncta; puncta formation was quantified for n=15 cells; *****=p<0.0001

1PE Uncaging – Acute Cortical Slices



***Ex vivo* evaluation of Coumarin-OT 1 *via* epifluorescence GCaMP imaging**. (A) Schematic of cortical injection sites. (B) Example images of *ex vivo* epifluorescence GCaMP imaging. (C-E) Normalised GCaMP fluorescence changes ($\Delta F/F_0$) shown as solid lines with ± SEM as translucent areas: (C) Pink: 1 µM OT, N=3 mice, n=4 slices; (D) Blue: 405 nm LED control; green: 1-5 µM Coumarin-OT **1** in the dark; turquoise: 1-5 µM Coumarin-OT **1** and 60 s uncaging with 405 nm LED, N=3 mice, n=5 slices; (E) In the presence of 10 µM OTR antagonist (OTA: $d(CH_2)_5[yITNCP(Orn)]$-COOH). Blue: 405 nm LED control; green: 1 µM Coumarin-OT **1** in the dark; turquoise: 1 µM Coumarin-OT **1** and 60 s uncaging with 405 nm LED, N=2 mice, n=4 slices. (F) Quantification of normalised GCaMP fluorescence changes ($\Delta F/F_0$). Coloured bars indicate application times of the compounds or LED irradiation times; dashed lines indicate baseline fluorescence (BL); dashed boxes indicate time windows used for GCaMP $\Delta F/F_0$ quantification; ***=p<0.001.

Figures created in BioRender.com

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1PE: one-photon excitation; 2PE: two-photon excitation; aCSF: artificial cerebrospinal fluid
MAP2: Microtubule-associated protein 2; pCREB: phosphorylated cAMP response binding protein; GFP: Green fluorescent protein

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