

Radical Caging Strategy for Cholinergic Optopharmacology

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ABSTRACT: Photo-caged methodologies have been indispensable for elucidating the functional mechanisms of pharmacologically active molecules at the cellular level. A photo-triggered removable unit enables control of the photo-induced expression of pharmacologically active molecular function, resulting in a rapid increase in the concentration of the bioactive compound near the target cell. However, caging the target bioactive compound generally requires specific heteroatom-based functional groups, limiting the types of molecular structures that can be caged. We have developed an unprecedented methodology for caging/uncaging on carbon atoms using a unit with a photo-cleavable carbon–boron bond. The caging/uncaging process requires installation of the CH₂–B group on the nitrogen atom that formally assembles an N-methyl group protected with a photoremovable unit. N-methylation proceeds by photoirradiation via carbon-centered radical generation. Using this radical caging strategy to cage previously uncageable bioactive molecules, we have photocaged molecules with no general labelling sites, including acetylcholine, an endogenous neurotransmitter. Caged acetylcholine provides an unconventional tool for optopharmacology to clarify neuronal mechanisms on the basis of photo-regulating acetylcholine localization. We demonstrated the utility of this probe by monitoring uncaging in HEK cells expressing a biosensor to detect ACh on the cell surface, as well as Ca²⁺ imaging in *Drosophila* brain cells (*ex vivo*).

INTRODUCTION

Photolysis-induced activity recovery of physiologically active compounds provides an important tool for controlling the function of native proteins with high spatiotemporal resolution. The photo-triggered activatable probes functionally encapsulate biomolecules with a photoremovable protecting group (PPG) and are called caged compounds. Photoirradiation of these caged compounds releases the bioactive molecules, allowing targeted perturbations of biological processes.^{1–4} Caged chemistry has generated many photoactivatable small molecules and proteins to photo-control their biological behavior in cellular processes (Figure 1a). In particular, the photo-control of ligands for glutamate and GABA receptors has enabled optopharmacological studies on the biophysical action and subcellular location of functional proteins in complex biological environments.^{5–9} Caged chemistry has developed over the decades, and nitrobenzyl and coumarinyl groups remain in general use as PPGs because of their excellent uncaging cross-sections. The development of units that can be uncaged at longer wavelengths has received much attention and include ruthenium complexes, BODIPY, and cyanine dyes, some of which are commercially available.^{10–12} However, the standard caging methodologies using these PPGs generally involve attachment and detachment on nucleophilic heteroatoms (e.g., CO₂H, OH, NH) as the binding site.¹³ There have been few studies of carbon-mediated

caging approaches^{14,15} because uncaging on carbon relies on the high reactivity under physiological conditions of carbanions and carbocations as intermediates generated via activation of the caged probe, thereby hampering recovery of the original bioactive molecules.

We recently designed direct excitable borates in which the carbon–boron bond homolysis under photoirradiation.^{16–20} The photoexcitable borates used in our laboratory undergo a single-electron transfer in the excited state, resulting in the generation of a C(sp³) radical and carbon–boron (C–B) bond cleavage (Figure 1b). We envisioned that the previously developed iodomethyl borate²⁰ would allow formal methylation of the betaine nitrogen atoms, resulting in the construction of an N-methyl caged molecule (caging methylation). *Betaine* is a structural motif frequently found in pharmacologically active compounds that act on the nervous system and are thus important targets for caged chemistry.²¹ The generated C(sp³) radical is instantly protonated to give the N-methylated product. This radical caging process serves as an unusual caging strategy, which attaches and detaches on the carbon atom of the target pharmacological molecule. This C–B bond cleavable retrofit provides the basis for a radical caging strategy enabling the construction of caged betaines such as acetylcholine, which have no 'thin edge of the wedge' can be starting point to be caged.

Acetylcholine (ACh) is a prominent neurotransmitter and was the first neurotransmitter to be discovered and identified over a century ago by Dale and confirmed by Loewi.^{22,23} ACh is the most important neurotransmitter across species and maintaining appropriate ACh concentrations is essential for sustaining vital functions. Disruption of the ACh balance leads to various diseases in humans such as brain dysfunction, including Alzheimer-type dementia, and locomotor disorder, including Parkinson's disease.^{24,25,26} However, controlling the

spatiotemporal dynamics of ACh is currently not possible due to its simple chemical structure,²⁷ yet monitoring the biological response to ACh is indispensable for elucidating the mechanism of neural function. The caged probes described here enabled the photo-control of ACh as a neurotransmitter by direct imaging of extracellular ACh using a biosensor. Furthermore, this probe allowed Ca^{2+} imaging of *Drosophila* brain in an *ex vivo* experiment through stimulation of the ACh receptor.

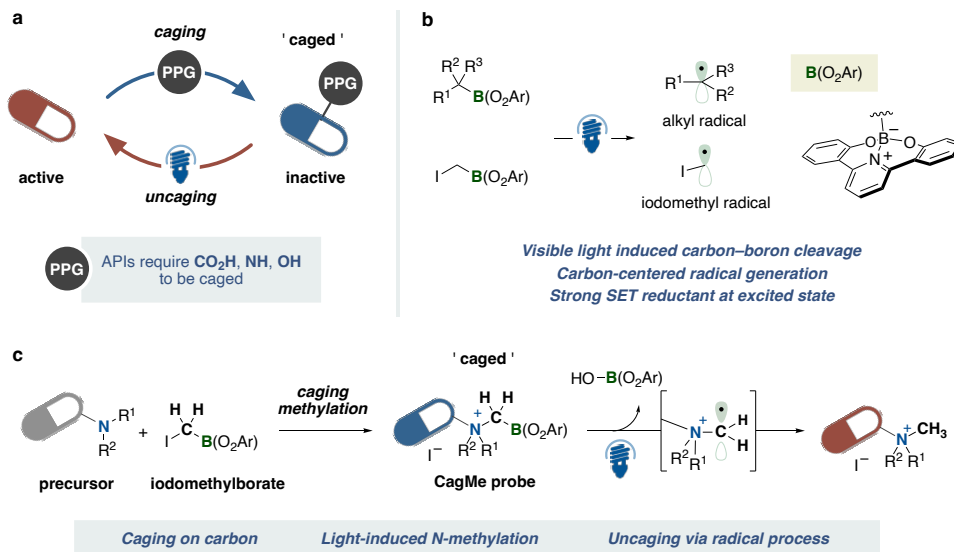


Figure 1. Caged compounds (a) Outline of caged chemistry. (b) Photoexcitable borates: our approach to unusual caged chemistry. (c) Radical caging strategy (this work).

RESULTS AND DISCUSSION

Scope of caging/uncaging sequence The *tertiary* amine or pyridine without the methyl group of a betaine-containing pharmacologically active compound was used as a starting material for preparing the caged compounds. Treatment of these nitrogen-containing precursors with iodomethylborate provokes the Menshutkin reaction ($\text{S}_{\text{N}}2$ reaction) to produce a betaine analogue with a methyl borate group linked to the nitrogen.²⁸ We initially examined the generality of the formal *N*-methylation sequence by caging and photo-uncaging *tertiary* amines (Figure 2). The photoexcitable borate bearing a quaternary ammonium unit **3** was assembled by mixing *tertiary* amine **1** and iodomethyl borate **2**. Homolytic C–B bond cleavage under LED (390 nm) irradiation in deuterated solvent formed quaternary *N*-methylated ammonium compound **4** with deuterium incorporation. This sequence is efficient using simple *tertiary* amines **1a** and **1b**, and the corresponding borate **3b** was unambiguously confirmed by its X-ray crystal structure. Although *N*-methylborylation of benzyldimethylamine (**1c**) efficiently afforded the borate **3c**, photo irradiation gave a substantial amount of dimethylisindolinium derivative **4c'** via intramolecular cyclisation instead of the product **4c**. This result indicated that formation of the ammonium product involves homolytic cleavage to generate the corresponding distonic radical. It was difficult to cage the bulky substrate triethylamine but uncaging of the obtained **3d** proceeded to produce triethylmethyl ammonium salt **4d**. *N*-methylpyridinium is a frequently observed motif in physiologically active compounds and thus it is significant that **4e** and **4f**

could be constructed using pyridines **1e** and **1f** as precursors with this caging/uncaging sequence. We then demonstrated the caging of cholinergic agonists from the corresponding *tertiary* amines and found that caging efficiency was dependent on the nucleophilicity of the precursor. For example, caged-choline **3g** and methacholine **3h** were obtained in moderate yields and methylborylation proceeded quantitatively with caged-clidinium **3i**. Similarly, pyridine-based pharmacologically active pyridostigmine **4j** was obtained from **3j** using this protocol (Figures S24, S29–33, and S40–S42). Although unidentified by-products were observed in trace amount, the main by-product is constantly a demethylated compound.

Photophysical property and uncaging mechanism To verify the feasibility of the radical caging process as an imaging tool, we investigated the property of caged acetylcholine **3k** (CagMe-ACh) (Figure 3 and Figures S6, S29, S30, and S33). CagMe-ACh **3k** was synthesized using the developed protocol (Figure 3a) and was stable in air and towards heat and pH fluctuations in aqueous solution, which were confirmed by NMR monitoring (Figures S8–11). Powdered **3k** was highly stable at room temperature and could be purified using silica-gel column chromatography. Next, we assessed the performance of the caged probes. CagMe probes unexpectedly dissolved readily in water (10 mM in 5% DMSO aqueous solution) despite their carbon-rich structures. The absorption spectra of the caged probes were dominated by the borate skeleton ($\lambda_{\text{max}} = \text{ca. } 370 \text{ nm}$, Figure 3b). The borate **3k** showed a short excitation lifetime and low fluorescence quantum yield, whereas the corresponding converted product, hydroxyborate **5** generated after uncaging,

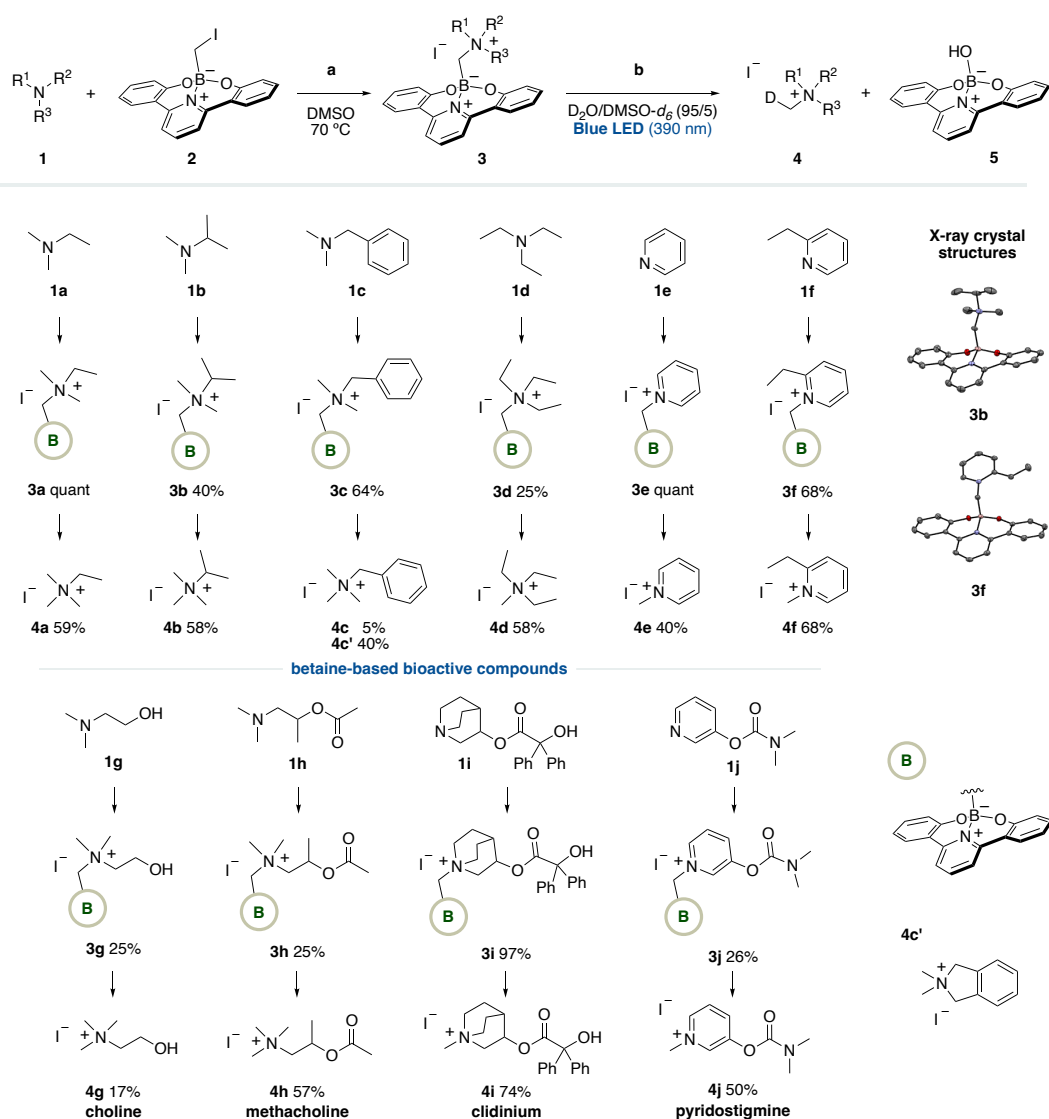


Figure 2. Scope of the radical caging system (a) The 1st reaction was carried out in DMSO (1 M) at 70 °C for 3 hours under a nitrogen atmosphere. (b) The 2nd reaction was carried out in D₂O/DMSO-*d*₆ (0.5 mM) under photo irradiation (390 nm) under a nitrogen atmosphere. All yields of **4** were determined by ¹H NMR.

showed a longer lifetime and higher fluorescence quantum yield (Table S4). The uncaging of CagMe-ACh (**3k**) released acetylcholine within 10 minutes under a nitrogen atmosphere and 390 nm LED irradiation and probe **3k** was remarkably stable under dark conditions. The reaction quantum yields and molar extinction coefficients (ϵ) gave the corresponding uncaging cross-sections, which reflected the overall efficiency of the caged probes.²⁹ The calculated uncaging cross section of **3k** was as well or better than widely used and conventional PPGs (Figure 3b).¹ Intriguingly, the CagMe probes work both under an inert gas atmosphere and under open air conditions (*vide infra*, Figures 3d, 4 and 5). This enables the photocontrol of caged probes in imaging systems under anaerobic conditions, in contrast to conventional caged probes which generally require an active oxygen species.

We proposed mechanisms involving the radical chain pathway or HAT process via the carbon-centered radical-mediated uncaging process based on our previous study on photoexcitable borates (Figure 3c). A typical radical trapping experiment

using TEMPO provided the ESI-mass peak of the corresponding TEMPO-radical adduct, which indicates the mediacy of the radical process (Scheme S1). In the radical chain pathway, both the homolytic cleavage and SET oxidation of photoexcited CagMe-ACh **3k** produces α -ammonium distonic radical **INT-I** as the initiation step. Further reduction of **INT-I** from another excited borate (ca. -1.95 V) as a propagation step can generate the ammonium ylide **INT-II**, which is easily protonated in water, and another **INT-I** is generated. The O-H bond dissociation energy (BDE) of water (110 kcal/mol)³⁰ is higher than that calculated for **INT-I** (105 kcal/mol, Figure S5), suggesting that uncaging process is promoted by additional HAT donor, such as thiol to quench the generated distonic radical **INT-I** (*vide infra*). Electronic perturbations of the framework alter the efficiency of C-B bond homolysis and thus structural modification of the CagMe skeleton improved its performance as a caged probe (Figure 3d). We investigated the changes in physical properties resulting from the introduction of withdrawing groups and confirmed the result by X-ray structural analysis. A compound with

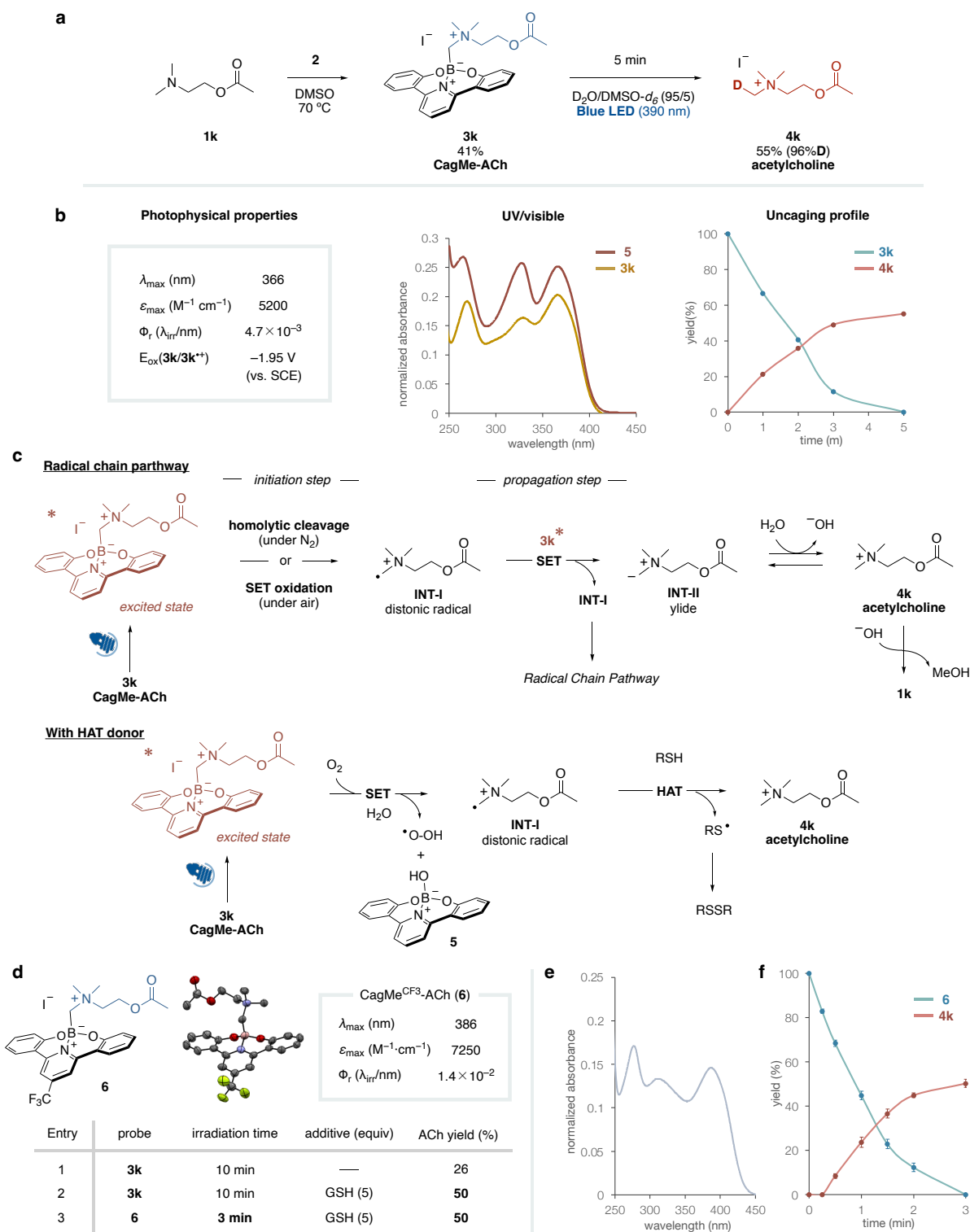


Figure 3. Uncaging study of acetylcholine (a) The uncaging process was carried out at 1 mM in 5% DMSO- d_6 in D_2O using a blue LED lamp (390 nm for 5 minutes) under a nitrogen atmosphere with ^1H NMR monitoring. (b) Photophysical properties of CagMe-ACh (**3k**), UV/visible analyses (red line: **5**, yellow line: **3k**, 100 μM in 5% DMSO aqueous solution), time-course changes in the uncaging process of **3k** based on ^1H NMR yields. (c) Plausible mechanisms for radical chain pathway and HAT process via the radical-mediated uncaging process. (d) Photophysical properties of the CF_3 -installed probe CagMe^{CF3}-ACh (**6**). Effect of the additive and substituent on the caged probe structure and uncaging efficiency. Uncaging was conducted at 1 mM in 5% DMSO- d_6 in D_2O using a blue LED lamp (390 nm for 5 minutes) under air and the yields were determined by ^1H NMR. (e) UV/visible absorption spectrum of **6** measured at 100 μM in 5% DMSO aqueous solution. (f) The time-course changes in the uncaging process of **6** based on ^1H NMR yields.

a strong electron-withdrawing group, CF₃ group-installed CagMe^{CF3}-ACh (**6**), showed a redshift of the absorption wavelength, faster uncaging, stability and solubility comparable to that of **3k** (Figures 3d–f, Figures S6, S17, S19, S30 and S35).

However, the uncaging efficiency was unsatisfactory. Both plausible mechanisms involve the generation of distonic radical **INT-I**. Thiols are common HAT donors and thus we added glutathione (GSH),³¹ a tripeptide containing cysteine that is uneventfully usable under cellular conditions. The addition of GSH significantly improved the uncaging efficiency (Figure 3d, entry 2), with CagMe^{CF3}-ACh (**6**) being uncaged using a shorter irradiation time with comparable efficiency (Figure 3d, entry 3).

Cellular imaging Using CagMe^{CF3}-ACh (**6**) and GSH, we applied this optopharmacological system to the extracellular imaging of ACh (Figure 4a). We used the genetically encoded G-protein-coupled receptor ACh sensor GACH2.0 to directly visualize the uncaging process of caged probe **6**.³² When GACH2.0 was expressed in HEK293T cells, a typical 405 nm confocal laser was focused on a spot near the target cell for 5 seconds in the presence of **6** (Figure 4b). GSH was added to the sample as a HAT donor and scavenger for reactive oxygen species. The fluorescence intensity of GACH2.0 was elevated after laser illumination and then returned to basal level whereas essentially no change in fluorescence was observed in the absence of **6** (Figure 4c). The degree of change in fluorescence intensity depended on the distance between the illuminated spot and ROIs (Figure 4d). We further evaluated the gradient in ACh concentration across the cell (Figure 4e). The increase in fluorescence intensity (F/F_0) after 405 nm laser irradiation was approximately 1.2-fold, indicating that the concentration of ACh closest to the illuminated spot is likely on the order of a few dozen micromolar (based on the calibration curve of GACH2.0 in Figure S44). These results indicate that CagMe^{CF3}-ACh (**6**) releases sufficient native ACh after brief light irradiation, prompting us to conduct further physiological studies. Even at a relatively high probe concentration, the toxicity was not significant for short-term use (Figure S45), particularly in such neurotransmitter applications. As it could also work in DMEM, it would be useful for various applications using mammalian cells (Figure S46).

Ex vivo imaging Finally, we used the CagMe^{CF3}-ACh (**6**) probe for live imaging of *Drosophila* brain (ex vivo). We prepared transgenic *Drosophila* expressing jGCaMP7c as a single fluorescent protein-based Ca²⁺ indicator (Figure 5a).³² Dissected brain was incubated with a solution of CagMe^{CF3}-ACh (**6**) (100 μ M) and GSH. CagMe^{CF3}-ACh (**6**) was uncaged using a 405 nm laser and time-course data were obtained. The uncaging of **6** resulted in a clear increase in Ca²⁺ in the region of the optic lobe (Figures 5b–d). Cells in the region of mushroom body also responded to changes in the local concentration of ACh (Figures 5e–f, Figures S35 and S36). Comparison of the two regions showed that Ca²⁺ was elevated more in the optic lobe than in the mushroom body (Figure 5f) because the nerve system in the mushroom body has a higher firing threshold, resulting in a lower number of cells observed responding to ACh stimulation.³³ Notably, in both regions, elevated Ca²⁺ levels were observed at cells located far from the illumination spot (Figures 5c and 5d). Given the spatial distribution of ACh shown in Figure 4e, the concentration of ACh migrating to distant locations is low, suggesting that stimulated cells near the spot trigger activity through the nervous system and in turn induce the release of Ca²⁺. Indeed, after the treatment with α -bungarotoxin as an inhibitor for ACh receptors, the Ca²⁺ elevation

in the optic lobe was blunted although not significantly in the mushroom body (Figure 5g). Photo-induced uncaging may thus help determine the roles of individual cells and aid investigations of the connecting network in the brain.

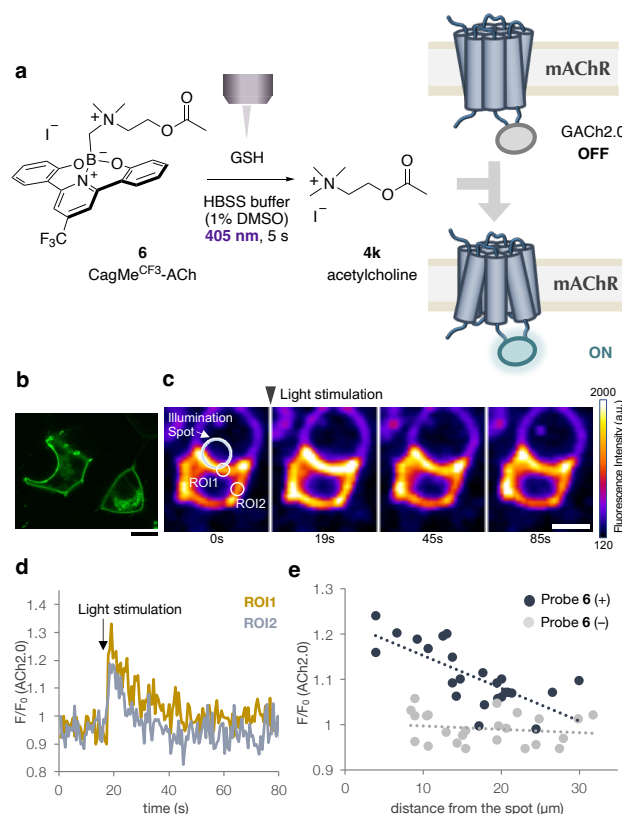


Figure 4. Imaging of the spatial distribution of acetylcholine at the cellular level. (a) Schematic image of ACh uncaging using the GACH2.0 biosensor. Uncaging was conducted using 100 μ M of **6** in HBSS buffer (1% DMSO) and GSH (500 μ M). (b) HEK293T cells expressing the ACh2.0 biosensor. (c)(d) Monitoring the change in acetylcholine concentration near the cellular membrane. The cells were illuminated using a 60 μ W 405 nm laser for 5 sec. The average normalized fluorescence intensity (F/F_0) of ACh2.0 at ROI1 and ROI2 was plotted at each time point. (e) Quantitative analysis of the spatial distribution of acetylcholine. The maximum change of normalized F/F_0 immediately following light illumination was plotted against distance from the illumination spot (black dots: light stimulation with the caged probe, gray dots: only light stimulation as a control). Data sets were obtained using four cells. Scale bars: 10 μ m. The quantitative analysis is presented with larger numbers of cells (Figure S44).

CONCLUSIONS

In summary, we have developed a radical-mediated uncaging system on C(sp³) carbons based on the homolytic cleavage of carbon–boron bonds under visible light irradiation. This uncaging system involves simultaneous caged and formal methylation in the Menschutkin reaction of iodomethylborate and tertiary amines. The proposed protocol enables caging/uncaging on the carbon skeleton without need for the polar functional groups NH, OH, and CO₂H used in conventional methods, making previously uncageable compounds such as acetylcholine cageable. Uncaging can be achieved under confocal laser irradiation, commonly used in cell imaging, for only a few seconds, making this method practical. The uncaging step proceeds without oxygen, making it applicable under anaerobic conditions. Additionally, the boron-based PPG part of caged probes in this

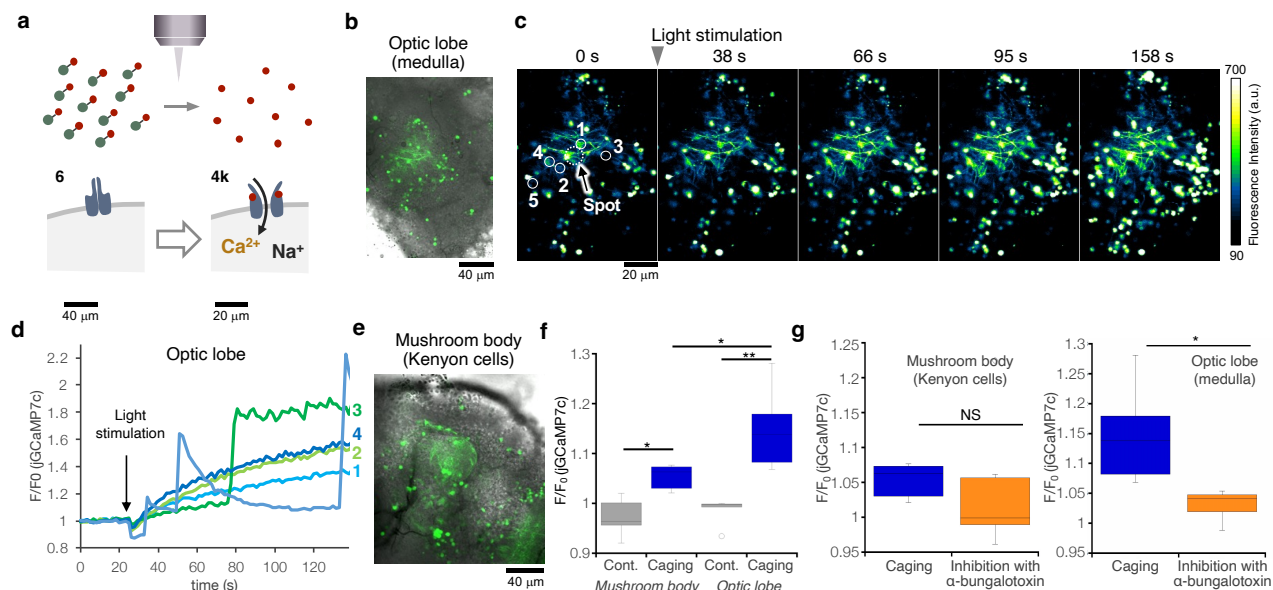


Figure 5. Ca^{2+} imaging in *Drosophila* brain (ex vivo). (a) Schematic illustration of Ca^{2+} imaging using CagMe CF_3 -ACh (6). (b) Confocal images of the optic lobe in *Drosophila* brain expressing jGCaMP7c as a Ca^{2+} indicator (green) merged with the bright field image (gray). Scale bar: 40 μm . (c)(d) Montage of Ca^{2+} imaging in the optic lobe. Light stimulation was performed for 5 sec (405 nm laser, 60 μW). Time courses of the normalized fluorescence intensity of jGCaMP7c were plotted at each ROI1-5 located at different distances from the illumination spot. (e) Confocal images of the mushroom body in *Drosophila* brain expressing jGCaMP7c as a Ca^{2+} indicator (green) merged with the bright field image (gray). (f) Analysis of the light illumination effect on the optic lobe and mushroom body. The normalized fluorescence intensity of jGCaMP7c at the light-stimulated spot was analyzed in the presence or absence (gray) of the caged probe with light illumination (blue). At 60 s after the illumination, the average normalized F/F_0 ($n = 6$, independent brain samples) is plotted as a box-plot and analyzed by Turkey's multiple comparisons. **: $p < 0.0001$ *: $p < 0.05$. The other comparisons were not significant. All experiments were conducted using 100 μM of 6 in the buffer (1% DMSO) and GSH (500 μM). (g) Evaluation of Ca^{2+} dynamics under inhibition of ACh receptors. The normalized fluorescence intensity of jGCaMP7c at the light-stimulated spot was analyzed in the absence (blue) or presence (orange) of α -bungarotoxin (5 μM). At 60 s after the illumination, the normalized F/F_0 ($n = 6-7$, independent brain samples) was plotted as a box-plot and analyzed by unpaired Student t-test. *: $p < 0.05$. Fluorescence intensity in the absence of α -bungarotoxin was the same dataset as Figure 5f.

study involves intramolecular ion pair, which is lipophilic and may show sufficient cell permeability. Thus, this system may be applicable to target intracellular tissue by conjugating the appropriate molecules in the future. We applied the technique to both direct and indirect cell imaging and demonstrated its potential for animal experiments by *ex vivo* imaging using *Drosophila* brain. Further studies for caging on carbon are currently underway.

EXPERIMENTAL/METHOD SECTIONS

Uncaging Experiments with ^1H NMR

Under an ambient atmosphere, a caged compound **3** was dissolved with DMSO- d_6 and D_2O to prepare 1 mM of the solution in 5% DMSO aqueous solution. As an internal standard, 1 mM of the ethylene glycol solution in 5% DMSO aqueous solution was prepared. Then the solution and ethylene glycol solution were mixed in the same amount and dispensed 600 μL into NMR tube. The final concentration of the caged compound was 500 μM in 5% DMSO aqueous solution. The NMR tube was irradiated with 390 nm or 440 nm blue LED at 1 cm away from the light source.

In the case of adding glutathione, 2 mM of the ethylene glycol solution as an internal standard and 10 mM of the glutathione (GSH, reduced form) in 5% DMSO aqueous solution was prepared respectively. The ethylene glycol solution and glutathione solution were mixed in the same amount. Then the caged

solution was diluted with the mixture of ethylene glycol and glutathione and dispensed 600 μL into NMR tube.

When the experiment was conducted under N_2 , the above process was operated in the glovebox and used degassed DMSO- d_6 and D_2O by N_2 bubbling for 5 min.

HEK293T Cell-based Assay of ACh Release Process

CagMe CF_3 -ACh (**6**) stock solution (10 mM in DMSO) and GSH stock solution (50 mM in water) were diluted with HBSS to 100 μM and 500 μM of the final concentration, respectively. HEK293T cells expressing GACH 2.0 were stained with the buffer containing the caged compound and GSH without incubation. Fluorescence images were obtained at timelapse mode through FITC channel (emission filter set: 490–540 nm; excitation laser: 473 nm) by a confocal microscope (FV1200, Olympus) equipped with a 60 $\times$ oil immersion objective lens. During observation, CagMe CF_3 -ACh (**6**) was irradiated with a continuous laser (405 nm, 60 μW . 53.1 W/cm^2) for 5 second for uncaging of CagMe CF_3 -ACh (**6**).

Ex Vivo Assay of ACh Release Process Based on *Drosophila* Brain

Flies (*Drosophila melanogaster*) were maintained on standard fly medium, under normal 12-h light/12-h dark conditions at 25 $^\circ\text{C}$. Through the GAL4/UAS system, jGCaMP7c (BL#79030) was sparsely expressed in *fruitless*-expressing neurons

(BL#66696). Eclosed male flies were collected and maintained in a group (approximately 10–20 flies per vial) until use. In all experiments, flies 6–7 days after eclosion were used.

All imaging experiments were performed in an adult hemolymph like (AHL) saline, containing 108 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 8.2 mM MgCl₂, 4 mM NaHCO₃, 1 mM NaH₂PO₄, 5 mM trehalose, 10 mM sucrose, 5 mM HEPES. The brain was

dissected in Ca²⁺-free AHL saline, and the blood brain barrier was digested with papain (10 U/ml) (Worthington Biochemical Corporation, New Jersey, USA) for 15 min at room temperature. The brain was transferred into a glass bottom dish filled with AHL.

ASSOCIATED CONTENT

Supporting Information

Experimental details and characterization data for all new compounds (PDF). This material is available free of charge via the Internet at <http://pubs.acs.org>.

Additional experimental and computational details and spectroscopic data of all compounds (PDF)

Crystallographic data for **3b** (CIF, CCDC 2217404)

Crystallographic data for **3f** (CIF, CCDC 2217403)

Crystallographic data for **6** (CIF, CCDC 2217405)

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