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A practical guide to the synthesis of dinitroindolinyI-caged neurotransmitters

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Abstract

This protocol describes a method for efficient chemical synthesis of dinitroindolinyI derivatives of glutamate and *gamma*-aminobutyric acid. these caged neurotransmitters are currently the most chemically and photochemically efficient probes for two-photon photolysis in living brain slices. the protocol only requires basic organic synthesis equipment, and no silica gel column chromatography or NMR spectroscopy is needed at any stage. Hplc is used to purify the caged transmitters at the end of the syntheses. thus, the synthesis of dinitroindolinyI-caged neurotransmitters is within the scope of a modestly equipped chemistry laboratory.

Introduction

Two-photon microscopy is a method that uses nonlinear stimulation of chromophores to produce axially confined excitation (i.e., stimulation—and therefore fluorescence—is confined to a single focal plane or even a specific point). It was made practical for the biological sciences in 1990 (ref. 1) when it was envisaged to be particularly useful in two ways. First, the removal of the confocal pinhole would allow fluorescent imaging in biological samples at much greater depths than standard laser-scanning confocal microscopy. Second, it would allow focal photolysis of caged signaling molecules. Subsequent history has amply validated this vision². Importantly, both applications required the development of new enabling technologies. The computer-controlled, solid-state, mode-locked Ti:sapphire lasers that were developed commercially in the past decade have been a vital hardware contribution, as they allow facile access to two-photon excitation wavelengths in the 700–1,100 nm range. Furthermore, the development of probes for imaging³ and uncaging⁴ has been equally important for the wide success of two-photon microscopy. In particular, studies—of transgenetically fluorescent mice⁵ having neocortical neurons sparsely labeled with either enhanced green fluorescent protein (eGFP) or enhanced yellow fluorescent protein (eYFP)—have appeared that can be described as stunning, as the fate of many individual synapses have been followed with time and experience with transcranial imaging^{6–9}. Complementary to these longitudinal structural studies, functional studies of cell activity using two-photon imaging of intracellular calcium in living animals with single-cell resolution have been reported^{10–16}. *In vivo*, two-photon fluorescence microscopy has been reported by many laboratories for several species (e.g., *Drosophila*, *Caenorhabditis elegans*, rats, mice, birds, cats, monkeys and even humans¹⁷), thus it is now a well-established method for modern biomedical research.

When it comes to the second application of two-photon microscopy (focal or three-dimensionally confined uncaging), there are fewer reported studies. The reason for this is, in part, the lack of available chemical probes that undergo effective two-photon uncaging. The development of 4-methoxy-7-nitroindolinyl (MNI)-Glu for two-photon uncaging in 2001 was an important step for the neuroscience community in solving this problem¹⁸. MNI-Glu was shown to be sufficiently sensitive to two-photon excitation at 720 nm at low (<10 mW for 50 μ s) power levels, such that uncaging could be effected at the optical diffraction limit in both lateral and axial dimensions for the first time¹⁸. (The wavelength of light used for microscopy normally places a constraint on the smallest objects that can be clearly resolved; this is defined as the 'diffraction limit'¹⁹.) A prior report of two-photon photolysis of nitrobenzyl-caged carbamoylcholine was not close to the diffraction limit²⁰, and required high power (50 mW for 30–50 ms) and shorter wavelengths (640 nm) in a region that is not accessible to modern Ti:sapphire lasers.

The commercialization of MNI-Glu in 2004 has allowed many laboratories to perform two-photon uncaging studies with this compound^{21–28}. However, even though the synthesis of this small molecule is fairly simple for an organic chemist, it could be considered to be quite challenging for a non-specialist, as silica gel column chromatography is required for four of the five steps in the synthesis. Furthermore, a common complaint among neuroscientists is that the commercially available material is highly variable in quality and often very toxic (e.g., resulting in neuronal hyperexcitability or rapid neuronal death) for neurons in acutely isolated brain slices.

This protocol describes the synthesis of recently developed second-generation nitroindolinyl-caged neurotransmitters, called 4-carboxymethoxy-5,7-dinitroindolinyl (CDNI)-Glu₂₉ and CDNI- γ -aminobutyric acid (GABA)₃₀. The synthesis and two-photon photolysis of a cell-permeant nitroveratryl (NV)-caged IP₃ analog are described in another protocol, which also contains a more detailed discussion of the advantages of the uncaging approach³¹. The caged compounds in these two protocols allow photostimulation of extracellular receptors (CDNI cages) or intracellular receptors (NV-caged IP₃). The former are more important in neurons and the latter in astrocytes. However, mGlu-R also has an important part in astrocytic signaling cascades. Box 1 and Figure 1 show an example of two-photon uncaging of CDNI-Glu in layer 1 of the cortex of a living mouse, using surgical procedures described in another protocol^{14,32}.

As CDNI-Glu is about six times more effective than MNI-Glu₂₉, it has been used at low concentrations for macro two-photon photo-lysis to fire action potentials in hippocampal neurons³³. Furthermore, diffraction-limited excitation of CDNI-Glu at 720 nm can be used to fire action potentials in the context of GABA uncaging at 830 nm³⁴. This protocol includes a complete description of all the necessary steps (usefully simplifying reported procedures used for the initial steps^{35,36}) required for the synthesis and purification of CDNI-Glu and CDNI-GABA from commercially available starting materials. While no caged compound can be called 'perfect', these probes represent the best combination of chemical, photochemical and pharmacological properties of caged neurotransmitters that are currently available for two-photon uncaging, for the following reasons: (i) the quantum yields (ϕ) of release are 0.5–0.6; (ii) the extinction coefficient of the dinitroindolinyl

chromophore is $6,400 \text{ M}^{-1} \text{ cm}^{-1}$; (iii) the probes are soluble in water, physiological buffer or methanol up to 100 mM; (iv) aqueous solutions at pH 2 (i.e., NaH_2PO_4) are stable at -15°C for several months; (v) only mild antagonism toward GABA-A receptors is observed at low concentrations (0.4 mM), but effective two-photon (or UV) uncaging is feasible at this concentration^{30,37}; and (vi) the syntheses of CDNI-Glu and CDNI-GABA (Fig. 2) require no silica gel chromatography. Glu and GABA are the two most important neurotransmitters, but this protocol could be readily applied to other amino-acid neurotransmitters, such as D-Ser³⁸, D-Asp or Gly.

The syntheses of CDNI-Glu (Steps 1–75) and CDNI-GABA (Steps 76–113) are 10 synthetic steps in length. Both share the first five synthetic steps, and only differ in the last five in which amino acid is attached to the caging chromophore (Fig. 2). The scale of the syntheses is in the 1–30 mmol range. As is typical for multi-step syntheses, the first few synthetic steps are carried out on a larger scale, but the overall efficiency is such that hundreds of milligrams of pure CDNI-caged compound can be made in approx. 3 weeks. As several reagents are toxic or harmful, much (but not all) of the work must be carried out in a fume hood using round-bottom flasks with ground glass joints. The synthetic steps used are clean and efficient allowing the by-products for each step to be removed by partitioning into an aqueous phase. No silica gel column chromatography is required for any synthetic step; rather, the organic product is purified by simple filtration or organic phase isolation in a separatory funnel and then concentrated with a rotary evaporator. High-performance liquid chromatography (HPLC) is required for isolation of the caged neurotransmitters and their immediate synthetic precursors. The former is essential because we have found from practice that even minuscule impurities in caged transmitters can be toxic to neurons. The latter is also essential as several attempts to make dinitro-caged compounds from their unpurified mononitro precursors produce intractable, complex mixtures of compounds, with very low yields ($< 1\%$) of the desired compounds.

What are the advantages of CDNI-caged neurotransmitters compared with MNI-caged compounds? MNI-Glu can be used very effectively for uncaging with UV-visible light, as this type of photolysis requires relatively low concentrations of probe (~ 0.1 to 0.3 mM). Furthermore, for many basic two-photon uncaging experiments, MNI-Glu works very well. As MNI-Glu is commercially available and has been effectively used by several laboratories in the past 7 years, what sort of experiments require CDNI-caged compounds? Recently, it has been reported³⁴ that MNI-Glu blocks GABA-A receptors at the concentration level typically used for two-photon uncaging experiments (namely, 10 mM puffer application or 3 mM bath application). The higher photochemical efficiency of CDNI-caged compounds has allowed them to be used at much lower concentrations (0.4 – 1.5 mM) such that GABA-A receptors are only partially inhibited ($\sim 20\%$ at 0.4 mM)³⁴. Furthermore, CNI-GABA (compound **17**) when bath applied at low concentration (0.325 mM) did not inhibit tonic firing of neurons in the deep cerebellar nuclei³⁷, implying this caged transmitter does not block GABA receptors at all at this concentration. Thus, CDNI-caged neurotransmitters should be most useful for the study of synaptic transmission when the functions of GABA receptors are part of the experimental procedure.

Materials

REAGENTS

critical HPLC-grade acetone is important for one synthetic step; otherwise no special care is required for any solvent used in any synthetic step. HPLC-grade acetonitrile, trifluoroacetic acid (TFA) and water must be used for HPLC. Other reagents obtained from Sigma-Aldrich or Acros have been used with equal effectiveness.

caution Chemical safety considerations: Most of the reagents used in the protocol require protective goggles, gloves and lab coats. As several reagents are toxic or harmful, much (but not all) of the work must be carried out in a fume hood using round-bottom flasks with ground glass joints.

4-Hydroxyindole

NaCNBH₃ **caution** It is toxic. **critical** Buy and use fresh NaCNBH₃ for best yields. The solid version of the reducing agent is much more effective than commercial solutions.

Mice (for *in vivo* experiments) **caution** All animal experiments must be conducted in accordance with all relevant institutional and governmental regulations.

Acetic anhydride

Methyl bromoacetate **caution** It is an irritant.

1-(3-dimethylaminopropyl)-3-ethylcarbodiimide-HCl

N-BOC-L-glutamate acid γ -*tert*-butyl ester

N-BOC-aminobutyric acid *tert*-butyl ester

Citric acid

Trifluoroacetic acid, sequenal grade (TFA; Pierce, cat. no. 28903)! TFA must be handled in a fume hood.

NaI

NaNO₃ Acetone (HPLC grade) **caution** It is flammable.

Methylene chloride

Acetonitrile (HPLC grade)! It is flammable.

Methanol **caution** It is flammable.

Chloroform **caution** It is toxic.

Acetone **caution** It is flammable.

Hexane **caution** It is flammable.

Ethylacetate **caution** It is flammable.

DMSO **caution** It is flammable.

HEPES

fluo-4/AM

Silicone oil

NaOH 1 N solution

NaHCO₃

K₂HPO₄

Acetic acid

MgCl₂

NaCl

KOH

Nanopure water

Liquid nitrogen

Dry ice

Pluronic acid, 20% (wt/vol) solution in DMSO (Invitrogen, cat. no. P3000MP) **caution** It is an irritant.

Urethane solution **caution** It is an irritant.

EQUIPMENT

Round-bottom flask Fume hood

Rotary evaporator

Lyophilizer

Filter funnels

Reflux condensers

Separatory funnels

Crystallizing dish (500 ml) for oil bath for heating reactions

Hot plate stirrer

Spatulas

Filter paper

Glass transfer funnels

Teflon-coated magnetic stirrer bars

Thin-layer chromatography (TLC) developing chambers

Thermometer

UV hand-held lamp

Celite

TLC plates (silica gel 60 F254, Fisher, cat. no. 4861-820)

HPLC system with UV-visible absorption detector

HPLC columns. Any analytical C-18 column is suitable for reaction analysis. I recommend Alltech Altima C-18 22 ¥ 250 mm (5 µm particle size) for preparative separations.

Two-photon microscope

Picospritzer (General Valve) Micromanipulator (Sutter)

Plastic Luer lock syringes (10 ml)

Micron nylon syringe filters (0.45 µm)

High-speed drill

Trephine

Pasteur pipette

Yellow-filtered lights (Roscolux 10)

Miscellaneous laboratory glassware (beakers, etc.)

Glass vials with screw caps

Cyanoacrylate glue

REAGENT SETUP

HEPES-buffered saline/artificial cerebrospinal fluid (ACSF), pH 7.4

Prepare HEPES-ACSF solution containing NaCl (125 mM), KCl (5 mM), glucose (10 mM), CaCl₂ (3.1 mM), MgCl₂ (1.3 mM) and HEPES (10 mM). This buffer is used for the uncaging experiment shown in Box 1. A volume of 1 liter consists of NaCl (9.3 g), KCl (0.38 g), glucose (1.8 g), CaCl₂ (0.46 g), MgCl₂ (0.26 g) and HEPES (2.4 g). This is taken to pH 7.4 with KOH. Store at 4 °C for up to 6 months. Check pH at room temperature (RT; 20–25 °C) before using.

EQUIPMENT SETUP

Heating—Reaction mixtures are heated at refluxed temperature by immersion of the reaction flask in a bath of silicone oil in a crystallizing dish, which is warmed on a hot plate stirrer.

PROCEDURE

critical step All experiments are performed in a room with yellow-filtered lights (Roscolux 10) to prevent photolysis of photosensitive compounds. If this is not practical, low levels of normal white light are not very harmful to the probes. Care must be taken to protect the purified probes (compounds **12** and **18**) from white light.

Synthetic step 1: synthesis of 4-hydroxyindoline ● TIMING 2 h

- 1 In a 250-mL round-bottom flask equipped with a stirrer bar, dissolve 4-hydroxyindole **1** (4.25 g, 32 mmol) in glacial acetic acid (60 mL). The solution is dark gray or black.
- 2 Cool the reaction mixture (RM) to 10 °C with water/ice bath.
- 3 Weigh NaCNBH₃ (3.02 g, 47.9 mmol) and add into a 20-mL glass vial with a screw cap.

critical step This must be performed quickly as solid NaCNBH₃ is highly deliquescent.

- 4 Add NaCNBH₃ in portions (~0.5 g) over 30 min. Reseal the vial after removing each portion. The RM gradually becomes lighter during the addition process, finishing as a dark-straw color. The RM is mildly effervescent, even after 1 h.

critical step Maintain temperature of the RM between 9–12 °C.

- 5 After 1 h, add tap water (10 mL).
- 6 Remove most of the acetic acid by rotary evaporation at 65–70 °C *in vacuo*.
- 7 Add ethyl acetate (100 mL) and water (200 mL).
- 8 Transfer the RM to a larger beaker (1 L) and add portions (1–2 g) of solid NaHCO₃ with stirring.

caution Initially this is highly effervescent; hence, use of a large beaker is recommended.

- 9 When no more gas evolves, place the liquids in a separatory funnel and isolate the upper organic phase.

- 10 Dry the organic phase by adding sufficient solid MgSO_4 until some of the drying agent appears to float as a fluffy solid, then remove by filtration and concentrate *in vacuo* on a rotary evaporator at 30–40 °C.
- 11 Isolate indoline **2** ($R_f = 0.15$, ethyl acetate/hexane, 1:2) in 100% yield.
critical step Indoline is not very stable, and oxidizes easily in air. Use in the next step immediately.

Synthetic step 2: synthesis of 4-hydroxyindoline diacetate ● TIMING 7 h

- 12 In a round-bottom flask (100 mL) equipped with a stirred bar, use an oil bath to heat a solution of **2** (1.17 g, 8.77 mmol) in acetic acid (10 mL) and acetic anhydride (10 mL) at reflux temperature for 1 h.
- 13 Add water (3 mL), then remove solvents by rotary evaporation *in vacuo*.
- 14 Add ethyl acetate (50 mL) and saturated NaHCO_3 solution (40 mL) to the RM. Separate aqueous and organic phases in a separatory funnel.
- 15 Dry the organic phase over MgSO_4 , filter and concentrate *in vacuo* on a rotary evaporator at 30–40 °C to give diacetate **3** ($R_f = 0.11$, ethyl acetate/hexane, 1:2) as a white solid. Repeat this step five times. Overall yield 80% (range 50–80%, $n = 5$).

critical step Increasing the scale of the reaction fivefold causes side reactions to occur, necessitating silica gel column chromatography.

pause point The diacetate is stable for several weeks at –20 °C.

Synthetic step 3: synthesis of N-acetyl-4-hydroxyindoline ● TIMING 1 h

16. In 250 mL round-bottom flask equipped with a stirrer bar, dissolve **3** in methanol (120 mL).
17. Add 1 N NaOH (30 mL).
18. After 20 min at room temperature add water (80 mL).
19. Remove the methanol by rotary evaporation *in vacuo*.
20. Add 1 N HCl (30 mL) to give a white precipitate.
21. Filter off the precipitate (the product).
22. Dry the product at high vacuum in a desiccator to give **4** ($R_f = 0.24$, ethyl acetate/hexane, 1:1, with two developments) in 100% yield (range 85–100%, $n = 5$). Phenols tend to oxidize, so this material should be used within a few days for the next step.

Synthetic step 4: synthesis of methyl (1-acetylindolin-4-yl)oxy)acetate ● TIMING 48 h

- 23 In 250-mL round-bottom flask equipped with a stir bar, dissolve **4** (28 mmol) and methyl bromoacetate (10 mL) in HPLC-grade acetone (120 mL). Add NaI (4.5 g, 28 mmol).

caution Methyl bromoacetate is a lachrymator, so the bottle should be handled in a fume hood with gloves.

- 24 Heat the RM at reflux temperature for 2 d.
- 25 Remove the acetone by rotary evaporation *in vacuo*.
- 26 Add water (100 mL) and ethyl acetate (100 mL).
- 27 Dry over MgSO₄, filter off MgSO₄ and concentrate *in vacuo* on a rotary evaporator.
- 28 Transfer the liquids to a separatory funnel and isolate the upper organic phase.
- 29 Dry the organic phase over MgSO₄, filter and concentrate *in vacuo* on a rotary evaporator at 30–40 °C, then at high vacuum (~0.05 Torr) at RT to give ester **5** (*R*_f = 0.31, ethyl acetate/hexane, 1:1 with two developments) a white solid in 79% yield (range 41–100%, *n* = 5).

pause point Compound **5** can be safely stored at –20°C for several months.

Synthetic step 5: synthesis of methyl (indolin-4-yloxy)acetate ● TIMING 5 h

- 30 In a 1000 mL round-bottom flask, dissolve compound **5** (4.66 g, 18.7 mmol) in methanol (500 mL).
- 31 Add water (60 mL) and then concentrated HCl (30 mL) to the RM.
- 32 Heat at reflux temperature for 4 h.
- 33 Add water (200 mL) and then concentrate the RM to ~350 mL *in vacuo* on a rotary evaporator at 30–40°C.
- 34 Add solid NaHCO₃ to the RM until effervescence ceases.
- 35 Add ethyl acetate (250 mL).
- 36 Separate aqueous and organic phases in a separatory funnel.
- 37 Dry the organic phase over MgSO₄, filter and concentrate *in vacuo* on a rotary evaporator at 30–40°C to give indoline **6** (*R*_f = 0.44, ethyl acetate/hexane, 1:1), a brown solid in 78% yield (range 42–78%, *n* = 4). Indolines slowly decompose, so compound **6** should be used for the next step within a few days.

Synthetic step 6: synthesis of methyl{1-[4s-(4-tert-butoxycarbonyl)-4-(tert-butoxycarbonylamino) butanoyl]indolin-4-ylox}acetate **7** ● TIMING 48 h

- 38 In a 125-mL round-bottom flask equipped with a stirrer bar, dissolve compound **6** (4.36 g, 21.0 mmol) and *N*-BOC-L-glutamate acid γ-*tert*-butyl ester (7.32 g, 24.2 mmol) in acetonitrile (60 mL).
- 39 Add 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide as the solid HCl salt (5.15 g, 27 mmol).
- 40 Stir the heterogeneous RM for 40 h.

- 41 Remove most of the acetonitrile by rotary evaporation *in vacuo*, and then add 1 N HCl (45 mL) and ethyl acetate (100 mL) to the RM.
- 42 In a separatory funnel, remove the aqueous layer, and then wash the organic layer with saturated NaHCO₃ (50 mL). The side products from the coupling reaction partition into the acid and base solutions.
- 43 Dry the organic phase over MgSO₄; filter off MgSO₄.
- 44 Concentrate *in vacuo* on a rotary evaporator at 30–40°C.
- 45 Isolate **7** ($R_f = 0.17$, ethyl acetate/hexane, 1:2) as a white solid in yield 94% (range 76–94%, $n = 3$). **pause poInt** Compound **7** is stable for years at –20°C.

Synthetic step 7: synthesis of{1-[4s-(4-*tert*-butoxycarbonyl)-4-(*tert*-butoxycarbonylamino)butanoyl] indolin-4-yllox}acetic acid **8 ● TIMING 4 h**

- 46 In a 125-mL round-bottom flask equipped with a stirrer, mix a solution of **7** (0.846 g, 1.72 mmol) and 1 N NaOH (1.82 mL) in methanol (60 mL) at RT.
- crItIcal step** The methyl ester must be hydrolyzed at this point, as after nitration the indoline bond is base labile.
- 47 After 3.5 h add 1 N citric acid (2.2 mL).
- 48 Remove the methanol by rotary evaporation *in vacuo*.
- 49 Add ethyl acetate (50 mL) and 1 N citric acid (5 mL).
- 50 Separate the organic phase in a separatory funnel.
- 51 Dry the organic phase over MgSO₄; filter off MgSO₄.
- 52 Concentrate *in vacuo* on a rotary evaporator to give **8** ($R_f = 0.11$, ethyl acetate/hexane/acetic acid, 90:9:1) as a yellow gum. Crude yield is ~1.2 g (range 68–100%, $n = 4$).
- pause poInt** Compound **8** can be safely stored at –20 °C for several months.
- caution** On a vacuum pump the product is fluffy and liable to be sucked into the pump. Thus, do not take it to dryness. The material is pure enough for the next steps.

Synthetic step 8: synthesis of{1-[(4s)-(4-amino-4-carboxybutanoyl]-indolin-4-yllox}acetic acid **9 ● TIMING 4 h**

- 53 In a 125-ml round-bottom flask, dissolve compound **8** (0.73 mmol) in TFA (8 mL).
- crItIcal step** Complete removal of the BOC protecting group is essential for high yield in the next synthetic step, as the amine can be nitrated in the protected form, but not when protonated (i.e., deprotected).
- 54 After 4 h, both BOC and *tert*-butyl protecting groups are removed. This material should be used immediately in the next step without isolation.

Synthetic step 9: synthesis of {1-[(4s)-(4-amino-4-carboxybutanoyl]-5-nitroindolin-4-yl}oxy}acetic acid **10 ● TIMING 18 h**

- 55** Add NaNO₃ (0.072 g, 1.2 equivalents) and stirrer bar to the TFA solution from Step 54.

troubleshootIng

- 56** Stir the heterogeneous RM vigorously for 5–10 min.

- 57** Remove the TFA with a gentle stream of nitrogen directed through a Pasteur pipette onto the surface of the RM.

caution This must be performed in a fume hood, as TFA is highly toxic.

- 58** Place the round-bottom flask on a vacuum pump for 18 h.

pause poInt In practice, this is typically left overnight at high vacuum to remove excess TFA.

- 59** Dissolve the solid in water with 0.1% (vol/vol) TFA. The product is fully soluble in this solvent, but there are side products that do not dissolve. These must be removed before HPLC.

critical step All particulates must be removed before HPLC, or the HPLC column will be blocked and ruined.

- 60** Filter the solution in two stages: first, filter it through a short pad of Celite to remove the large particles; second, filter it through a 0.45-μm nylon syringe filter.

critical step If the first stage is not performed, the second-stage filter is rapidly blocked.

pause poInt The mixture of 5- and 7-nitro indolines (compounds **10** and **11**) is stable for several years at –20°C.

troubleshootIng

HPLC purification of mono-nitro compounds ● TIMING 3 d

- 61** Separate the 5- and 7-nitro indolines (compounds **10** and **11**) by HPLC using 80% water, 20% acetonitrile and 0.1% TFA. Carry out isocratic elution at 10 mL/min on an Alltech Altima C-18 column (22 mm × 250 mm). Monitor elution by absorbance at 254 and/or 350 nm. Compound **10** elutes at 15 min and **11** at 11 min. Inject 10 mg of the mixture and collect each isomer by hand in a 500-ml round-bottom flask. Before starting this preparative purification, analysis of the reaction may be performed on an analytical reverse-phase C-18 column with a flow rate of 0.5 mL/min. The retention times of the two products at this flow rate are similar to those seen preparatively.

critical step If compounds **10** and **11** are not purified by HPLC, then the next synthetic step fails. This is the most critical step in the production of dinitroindolinyI-caged transmitters.

pause point At the end of each HPLC session, the flasks may be frozen at -80°C ; alternatively remove the acetonitrile by rotary evaporation before freezing the solution.

caution Take care not to heat the water bath to $> 35^{\circ}\text{C}$.

troubleshooting

- 62 Remove the remaining organic components (TFA and a little acetonitrile) at high vacuum.
- 63 Freeze the aqueous solution with a liquid nitrogen or dry ice/isopropanol bath.
- 64 Lyophilize the samples to dryness to give a combined yield of **10** and **11** of 80% ($n = 4$).

pause point Lyophilization takes 8–48 h depending on the amount of water and the quality of the lyophilizer. Mononitro compounds may be stored in a desiccator at -20°C for several years.

troubleshooting

Synthetic step 10: synthesis of {1-[(4s)-(4-amino-4-carboxybutanoyl)-5,7-dinitroindolin-4-yl]ox} acetic acid **12** ● TIMING 3 d

- 65 In a 100-mL round-bottom flask equipped with a stirrer, dissolve 0.459 g **10** in 15 mL TFA, then add 2.06 g NaNO_3 as a solid.⁶⁶ Stir the reaction for 3 d at RT. The progress of the reaction may be analyzed by reverse-phase HPLC by elution at 1 mL/min on C-18 from 10% to 80% acetonitrile over 30 min (Fig. 3).
- 67 Remove the TFA with a gentle stream of nitrogen directed through a Pasteur pipette onto the surface of the RM. This requires about 30 min, but should be continued until the RM appears like a thick brown syrup.

! caution This must be performed in a fume hood, as TFA is highly toxic.

- 68 Place the round-bottom flask on a vacuum pump for 18 h.

pause point In practice, this is left overnight at high vacuum to remove excess TFA.

- 69 Dissolve the solid in water with 0.1% (vol/vol) TFA. The product is fully soluble in this solvent, but there are side products that do not dissolve. These must be removed before HPLC.

critical step All particulates must be removed before HPLC, or the HPLC column will be blocked and ruined.

- 70 Filter the solution in two stages: first, filter it through a short pad of Celite to remove the large particles; second, filter it through a $0.45\text{-}\mu\text{m}$ nylon syringe filter.

If the first stage is not performed, the second-stage filter is rapidly blocked.

troubleshooting

- 71** Separate the 5- and 5,7-dinitro indolines (compounds **10** and **12**) by HPLC using 75% water, 25% acetonitrile and 0.1% TFA. Carry out isocratic elution at 10 ml min⁻¹ on an Alltech Altima C₁₈ column (22 × 250 mm). Compound **10** elutes at ~10 min and **12** ~12.5 min (Fig. 4). Inject 10 mg of the mixture and collect each isomer by hand in 500-mL round-bottom flask. Before starting this preparative purification, analysis of the reaction may be performed on an analytical reverse-phase C₁₈ column with a flow rate of 0.5 mL/min. The retention times of the two products at this flow rate are similar to those seen preparatively.

pause point At the end of each HPLC session, the flasks may be frozen at –80°C; alternatively the acetonitrile can be removed by rotary evaporation before freezing the solution.

caution Take care not to heat the water bath to > 35°C.

troubleshooting

- 72** Remove the remaining organic components (TFA and a little acetonitrile) at high vacuum.
- 73** Freeze the aqueous solution with a liquid nitrogen or dry ice/isopropanol bath. This solution is sufficiently acidic to preserve the integrity of the dinitro product.
- 74** Lyophilize the sample to dryness.

pause point Lyophilization takes 8–48 h depending on the amount of water and the quality of the lyophilizer.

troubleshooting

- 75** Isolate CDNI-Glu **12** as a fluffy, light orange solid in a yield of 25–29% (*n* = 10).

Synthesis of CDNI-GABA ● TIMING 13 d

- 76** The first five synthetic steps for CDNI-GABA are the same as CDNI-Glu above (Steps 1–37 of PROCEDURE).
- 77** In a 125-mL round-bottom flask equipped with a stirrer bar, dissolve compound **6** (0.84 g, 4.06 mmol) in acetonitrile (30 mL).
- 78** Add *N*-BOC-4-aminobutyric acid (0.914 g, 4.5 mmol).
- 79** Add 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide as the solid HCl salt (1.5 g, 6.0 mmol).
- 80** Stir the heterogeneous RM at RT for 40 h.
- 81** Remove the acetonitrile by rotary evaporation at 30–40°C *in vacuo*. Add 1 N HCl (45 mL) and ethyl acetate (100 mL) to the RM.
- 82** In a separatory funnel, remove the aqueous layer, then wash the organic layer with saturated NaHCO₃ (50 mL). The side products from the coupling reaction partition into the acid and base solutions.

- 83 Dry the organic phase over MgSO_4 , filter off MgSO_4 and concentrate *in vacuo* on a rotary evaporator at 30–40°C.
- 84 Isolate **13** ($R_f = 0.34$, ethyl acetate/hexane, 1:1) as a white solid in a yield of 90%. **pause point** Compound **13** is stable for years at –20°C.
- 85 In a 125-mL round-bottom flask equipped with a stirrer, mix a solution of **13** (0.99 g, 2.40 mmol) and 1 N NaOH (3.6 mL) in methanol (50 mL) at RT.
- 86 After 3.5 h, add 1 N citric acid (3.6 mL).
- 87 Remove the methanol by rotary evaporation at 30–40°C *in vacuo*.
- 88 Add ethyl acetate (50 mL) and separate the aqueous and organic layers.
- 89 Dry the organic phase over MgSO_4 , filter off MgSO_4 .
- 90 Concentrate *in vacuo* on a rotary evaporator at 30–40°C to give **14** ($R_f = 0.22$, ethyl acetate/hexane/acetic acid, 90:9:1) as a light yellow solid. Crude yield is ~0.78 g (77%).
- caution** On a vacuum pump the product is fluffy and liable to be sucked into the pump. Thus, do not take it to dryness. The material is pure enough for the next steps.
- critical step** Complete removal of the BOC protecting group is essential for high yield in the next step, as the amine can be nitrated in the protected form, but not when protonated (i.e., deprotected).
- 91 In a 125-mL round-bottom flask, dissolve compound **14** (1.85 mmol) in TFA (8 mL).
- 92 After 3 h, both BOC and *tert*-butyl protecting groups are removed. The product (**15**) can be used in the next step without isolation.
- 93 Add NaNO_3 (0.22 g, 1.4 equivalents) and stirrer bar to the TFA solution from Step 91.
- troubleshooting
- 94 Stir the heterogeneous RM vigorously for 5–10 min.
- 95 Remove the TFA with a gentle stream of nitrogen directed through a Pasteur pipette onto the surface of the RM.
- caution** This must be performed in a fume hood, as TFA is highly toxic.
- 96 Place the round-bottom flask on a vacuum pump for 18 h.
- pause point** In practice, this is left overnight at high vacuum to remove excess TFA.
- 97 Dissolve the solid in water with 0.1% (vol/vol) TFA. The product is fully soluble in this solvent, but there are side products that do not dissolve. These must be removed before HPLC.

critical step All particulates must be removed before HPLC, or the HPLC column will be blocked and ruined.

- 98** Filter the solution in two stages: first, filter it through a short pad of Celite to remove the large particles; second, filter it through a 0.45- μ m nylon syringe filter.

critical step If the first stage is not performed, the second-stage filter is rapidly blocked.

pause point The mixture of 5- and 7-nitro indolines is stable for several months at -20°C .

troubleshooting

HPLC purification of mono-nitro compounds

- 99** Separate the 5- and 7-nitro indolines (compounds **16** and **17**) by HPLC using 75% water, 20% acetonitrile and 0.1% TFA. Carry out isocratic elution at 10 mL/min on an Alltech Altima C-18 column (22 $\times$ 250 mm). Compound **16** elutes at $\sim$ 12.3 min and **17** at $\sim$ 14.7 min. Inject 10 mg of the mixture and collect each isomer by hand in a 500-ml round-bottom flask. Before starting this preparative purification, analysis of the reaction may be performed on an analytical reverse-phase C₁₈ column with a flow rate of 0.5 mL/min. The retention times of the two products at this flow rate are similar to those seen preparatively.

critical step If compounds **16** and **17** are not purified by HPLC, then the next synthetic step fails. This is the most critical step in the production of dinitroindoliny- caged transmitters.

pause point At the end of each HPLC session, the flasks may be frozen at -80°C ; alternatively, the acetonitrile can be removed by rotary evaporation before freezing the solution.

caution Take care not to heat the water bath to $> 35^{\circ}\text{C}$.

troubleshooting

- 100** Remove the remaining organic components (TFA and a little acetonitrile) at high vacuum.
- 101** Freeze the aqueous solution with a liquid nitrogen or dry ice/isopropanol bath.
- 102** Lyophilize the sample to dryness.

pause point Lyophilization takes 8–48 h depending on the amount of water and the quality of the lyophilizer.

troubleshooting

- 103** In a 100 mL round-bottom flask equipped with a stirrer, dissolve 0.351 g **16** in 10 mL TFA, then add 2.6 g NaNO₃ as a solid.

- 104** Stir the reaction for 3 d at RT.
- 105** Remove the TFA with a gentle stream of nitrogen directed through a Pasteur pipette onto the surface of the RM.
- caution** This must be performed in a fume hood, as TFA is highly toxic.
- 106** Place the round-bottom flask on a vacuum pump for 18 h.
- pause point** In practice, this is left overnight at high vacuum to remove excess TFA.
- 107** Dissolve the solid in water with 0.1% (vol/vol) TFA. The product is fully soluble in this solvent, but there are side products that do not dissolve. These must be removed before HPLC.
- critical step** All particulates must be removed before HPLC, or the HPLC column will be blocked and ruined.
- 108** Filter the solution in two stages: first, filter it through a short pad of Celite to remove the large particles; second, filter it through a 0.45- μ m nylon syringe filter.
- critical step** If the first stage is not performed, the second-stage filter is rapidly blocked.
- troubleshooting
- 109** Separate the 5- and 5,7-dinitro indolines (compounds **16** and **18**) by HPLC using 75% water, 25% acetonitrile and 0.1% TFA. Carry out isocratic elution at 10 ml min⁻¹ on an Alltech Altima C-18 column (22 $\times$ 250 mm). Compound **16** elutes at ~10.7 min and **18** at ~12.5 min (Fig. 5). Inject 10 mg of the mixture and collect each isomer by hand in a 500-mL round-bottom flask. Before starting this preparative purification, analysis of the reaction may be performed on an analytical reverse-phase C₁₈ column with a flow rate of 0.5 mL/min. The retention times of the two products at this flow rate are similar to those seen preparatively.
- pause point** At the end of each HPLC session, the flasks may be frozen at –80°C; alternatively, the acetonitrile can be removed by rotary evaporation before freezing the solution.
- caution** Take care not to heat the water bath to > 35 °C.
- troubleshooting
- 110** Remove the remaining organic components (TFA and a little acetonitrile) at high vacuum.
- 111** Freeze the aqueous solution with a liquid nitrogen or dry ice/isopropanol bath.
- 112** Lyophilize the sample to dryness.
- pause point** Lyophilization takes 8–48 h depending on the amount of water and quality of lyophilizer.

troubleshooting

- 113** Isolate CDNI-GABA **18** as a fluffy light orange solid in a yield of 25–29%. CDNI-GABA can safely be stored in a desiccator at –20°C for more than 24 months.

Troubleshooting

Troubleshooting advice can be found in Table 1.

● **TIMING**—Steps 1–11: 2 h

Steps 12–15: 7 h

Steps 16–22: 1 h

Steps 23–29: 48 h

Steps 30–37: 5 h

Steps 38–45: 48 h

Steps 46–52: 4 h

Steps 53 and 54: 4 h

Steps 55–60: 18 h

Steps 61–64: 3 d

Steps 65–75: 3 d

Steps 76–113: 13 d

Anticipated results

Analytical data

Compound **2**: ¹H NMR (300 MHz, CDCl₃) δ: 7.42 (s, 1H), 6.83 (dd, *J* = 8.1, 7.8 Hz, 1H), 6.73 (d, *J* = 8.1 Hz, 1H), (d, *J* = 7.8 Hz, 1H), 6.20 (t, *J* = 8.5 Hz, 2H), 2.98 (t, *J* = 8.5 Hz, 2H), 2.66 (br s, 1H).

Compound **3**: ¹H NMR (300 MHz, CDCl₃) δ: 8.09 (d, *J* = 8.2 Hz, 1H), 7.23 (t, *J* = 8.2 Hz, 1H) 6.72 (d, *J* = 8.2 Hz, 1H), 4.07 (t, *J* = 8.0 Hz, 2H), 3.06 (t, *J* = 8 Hz, 2H) 2.29 (s, 3H), 2.21 (s, 3H).

Compound **4**: ¹H NMR (300 MHz, CDCl₃) δ: 8.95 (s, 1H), 7.62 (d, *J* = 8.2 Hz, 1H), 6.97 (t, *J* = 8.2 Hz, 1H), 6.51 (d, *J* = 8.2 Hz, 1H), 4.07 (t, *J* = 9.1 Hz, 2H), 3.10 (t, *J* = 9.1 Hz, 2H), 2.21 (s, 3H).

Compound **5**: ^1H NMR (300 MHz, CDCl_3) δ : 7.86 (d, J = 8.5 Hz, 1H), 7.14 (t, J = 8.5 Hz, 1H), 6.43 (d, J = 8.2 Hz, 1H), 4.66 (s, 2H), 4.08 (t, J = 8.9 Hz, 2H), 3.78 (s, 3H), 3.23 (t, J = 8.9 Hz, 2H), 2.21 (s, 3H).

Compound **6**: ^1H NMR (300 MHz, CDCl_3) δ : 6.95 (t, J = 8.0 Hz, 1H), 6.34 (d, J = 8.0 Hz, 1H), 6.14 (d, J = 8.0 Hz, 1H), 4.64 (s, 2H), 3.79 (s, 3H), 3.58 (t, J = 8.3 Hz, 2H), 3.06 (t, J = 8.3 Hz, 2H).

Compound **7**: ^1H NMR (300 MHz, CDCl_3) δ : 7.87 (d, J = 8.3 Hz, 1H), 7.13 (t, J = 8.3 Hz, 1H), 6.42 (d, J = 8.3 Hz, 1H), 5.21 (br d, J = 7.8 Hz, 1H), 4.66 (s, 2H), 4.05 (t, J = 7.7 Hz, 2H), 3.79 (s, 3H), 3.19 (t, J = 7.7 Hz, 2H), 2.54–2.49 (m, 1H), 2.29–2.21 (m, 2H), 2.05–1.99 (m, 1H), 1.47 (s, 9H), 1.42 (s, 9H).

Compound **9**: ^1H NMR (300 MHz, CD_3OD) δ : 7.39 (d, J = 8.2 Hz, 1H), 6.94 (t, J = 8.2 Hz, 1H), 6.37 (d, J = 8.2 Hz, 1H), 4.62 (s, 2H), 3.96 (t, J = 6.5 Hz, 1H), 3.69 (t, J = 8.4 Hz, 2H), 2.76 (t, J = 8.4 Hz, 2H), 2.46–2.39 (m, 2H), 2.08–2.00 (m, 2H).

Compound **10**: ^1H NMR (300 MHz, CD_3OD) δ : 8.04 (d, J = 8.9 Hz, 1H), 7.88 (d, J = 8.9 Hz, 1H), 4.69 (s, 2H), 4.22 (t, J = 8.4 Hz, 2H), 4.11 (t, J = 6.5 Hz, 1H), 3.34 (t, J = 8.4 Hz, 2H), 2.81 (t, J = 6.5 Hz, 2H), 2.41–1.99 (m, 2H).

Compound **12 (CDNI-Glu)**: ^1H NMR (300 MHz, D_2O) δ : 8.53 (s, 1H), 4.81 (s, 2H), 4.43 (t, J = 8.2 Hz, 2H), 3.96 (t, J = 7.1 Hz, 1H), 3.39 (t, J = 8.2 Hz, 2H), 2.88 (t, J = 7.1 Hz, 2H), 2.25 (q, J = 7.1 Hz, 2H). ϵ_{350} = 6,400 $\text{M}^{-1} \text{cm}^{-1}$.

Compound **13**: ^1H NMR (300 MHz, CDCl_3) δ : 7.88 (d, J = 8.0 Hz, 1H), 7.14 (t, J = 8.0 Hz, 1H), 6.43 (d, J = 8.0 Hz, 1H), 4.8 (br s, 1H), 4.67 (s, 2H), 4.08 (t, J = 8.5 Hz, 2H), 3.80 (s, 3H), 3.26–3.17 (m, 4H), 2.47 (t, J = 7.0 Hz, 2H), 1.93 (m, 2H), 1.46 (s, 9H). ^{13}C NMR (70 MHz, CDCl_3): 170.97, 169.53, 156.34, 154.20, 144.90, 129.24, 119.45, 111.33, 107.09, 79.48, 65.71, 52.62, 48.86, 40.65 (2C not resolved), 33.68, 28.82, 25.19.

Compound **14**: ^1H NMR (some peaks appear as rotamers) (300 MHz, CDCl_3) δ : 7.86 (d, J = 8.0 Hz, 1H), 7.13 (t, J = 8.0 Hz, 1H), 6.46 (d, J = 8.0 Hz, 1H), 4.84 (br s, 1H), 4.65/4.61 (s, 2H), 4.01 (br s, 2H), 3.22 (br s, 2H), 3.14/3.07 (br t, 2H), 2.6–2.4 (m, 2H), 2.0–1.8 (m, 2H), 1.49/1.42 (s, 9H).

Compound **16**: ^1H NMR (300 MHz, CD_3OD) δ : 7.90 (d, J = 8.9 Hz, 1H), 7.80 (d, J = 8.9 Hz, 1H), 4.66 (s, 2H), 4.19 (t, J = 8.5 Hz, 2H), 3.28 (t, J = 8.5 Hz, 2H), 3.08 (t, J = 6.4 Hz, 2H), 2.68 (t, J = 6.4 Hz, 2H), 2.03 (m, 2H).

CDNI-GABA 18: ^1H NMR (300 MHz, CD_3OD) δ : 7.80 (s, 1H), 4.81 (s, 2H), 4.29 (t, J = 7.1 Hz, 2H), 3.37 (t, J = 7.1 Hz, 2H), 3.00 (t, J = 6.8 Hz, 2H), 2.71 (t, J = 6.3 Hz, 2H), 2.03–1.98 (m, 2H). ^{13}C NMR (70 MHz, CD_3OD): 173.26, 172.03, 153.08, 141.95, 140.09, 137.20, 133.72, 124.12, 71.47, 52.25, 40.90, 33.96, 28.67, 24.41. ϵ_{350} = 6,400 $\text{M}^{-1} \text{cm}^{-1}$.

HPLC: The preparative chromatograms (Figs. 4 and 5) are representative of single separations. It is important to note that peak retention times will often vary slightly from run to run, for this reason it is advisable to always collect each eluted fraction by hand.

Expected results from uncaging experiments

Local uncaging of glutamate on an astrocyte will rapidly activate metabotropic glutamate receptors, which are coupled to the IP₃ signaling pathway, thus increasing intracellular calcium concentration (Fig. 1). The calcium signal should return to baseline within about 60 s of uncaging, and the stimulation may be repeated several times. The method described in Box 1 is an adaptation of that developed for two-photon uncaging of glutamate in brain slices₁₈ and the dye injection protocol described in reference 32.

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Box 1**Two-photon uncaging of CDNI-Glu in vivo. ● TIMING 5 hours**

1. Anesthetize a mouse with urethane solution (20% solution in nanopure water) injected intraperitoneally (2 mg/g).
2. Remove skin from the skull of a mouse and glue (with cyanoacrylate glue) a head plate to allow access to the somatosensory cortex.
3. Perform a craniotomy with a high-speed drill and trephine (2 mm).
4. Remove dura then apply solution of calcium dye (fluo-4/AM; 25 μ g initially dissolved in 20% Pluronic/DMSO solution 5 μ L, then mixed into 45 μ L HEPES, pH 7.4). After 1 h, irrigate with ACSF, then mount mouse on two-photon microscope (Fig. 1, upper left panel).
5. Dissolve CDNI-Glu at 15 mM in ACSF, fill a patch pipette (resistance $\sim$ 2 M Ω) and mount in a picospritzer.
6. Position pipette near an astrocyte in the mouse by imaging with two-photon microscope (Fig. 1, upper left panel).
7. Select a region of interest for uncaging (e.g., Fig. 1, red box, second panel of first row).
8. Puff solution of CDNI-Glu onto astrocyte.
9. Photolyze with 720-nm light (10 mW, 8×2 ms) in the region of interest.
10. Monitor intracellular calcium signal with fluorescent dye.

Figure 1.
Two-photon uncaging of CDNI-Glu as described in Box 1.

Figure 2.

Scheme for the synthesis of CDNI-Glu and -GABA. (a–j) Reagents and conditions. (a) NaCNBH_3 , (b) $(\text{AcO})_2\text{O}$, (c) NaOH /methanol, (d) methyl bromoacetate, (e) HCl /methanol, (f) 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide, (g) NaOH , (h) TFA , (i) NaNO_3 (1.4 equivalents)/ TFA for 10 min, then HPLC, (j) NaNO_3 (20 equivalents)/ TFA for 3 d.

Figure 3.

Analytical HPLC of CDNI-Glu synthesis. The progression of the dinitration reaction can be followed with time over 3 d. Pink trace is compound **10** ($t = 0$) and green trace in the reaction mixture showing one major product peak at $t = 3$ d (compound **12**). Elution on C-18 analytical reverse-phase column (4.6 mm $\times$ 250 mm) with water and increasing percentage of acetonitrile (MeCN) with 0.1% (vol/vol) TFA. Flow rate 1 mL/min. HPLC absorption chromatogram monitored at 254 nm.

Figure 4.

Preparative HPLC purification of CDNI-Glu synthesis. Separation of compounds **10** (peak at 9.90 min) and **12** (peak at 12.47 min) on C-18 preparative reverse-phase column (20 × 250 mm) with 25% (vol/vol) acetonitrile (MeCN) with 0.1% (vol/vol) TFA. Flow rate 10 mL/min. HPLC absorption chromatogram monitored at 254 nm.

Figure 5.

Preparative HPLC purification of CDNI-GABA synthesis. Separation of compounds **16** (peak at 10.70 min) and **18** (peak at 12.45 min) on C-18 preparative reverse-phase column (20 × 250 mm) with 25% (vol/vol) acetonitrile (MeCN) with 0.1% (vol/vol) TFA. Flow rate 10 mL/min. HPLC absorption chromatogram monitored at 254 nm.

Step	Problem	Solution
55 and 93	Mononitration does not go to completion	Add more NaNO_3 (0.2 equivalent) and stir for 10 min
60, 70, 98, 108	Syringe filter is blocked	Replace 0.45- μm filter and continue filtration
61, 71, 99, 109	Peaks poorly separated on HPLC	Column may too old; replace with a new HPLC column. Alternatively, column may be overloaded with sample, so reduce loading by 50%
64, 74, 102, 112	Lyophilization not complete in 48 h	Check vacuum quality (seal small leaks or change vacuum pump oil). Check that vacuum trap is not full