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Improved Synthesis of Caged Glutamate and Caging Each Functional Group

Charitha Guruge[1], Yannick P. Ouedraogo[1], Richard L. Comitz[1], Jingxuan Ma[1], Attila Losonczy[2], Nasri Nesnas[1,*]

1 Department of Chemistry, Florida Institute of Technology, Melbourne FL 32901, United States

2 Department of Neuroscience, Columbia University, New York, NY 10032, United States

* Corresponding Author
Professor Nasri Nesnas, Ph.D.
Department of Chemistry
Florida Institute of Technology
150 West University Blvd., Melbourne, FL 32901, United States
E-mail: nesnas@fit.edu

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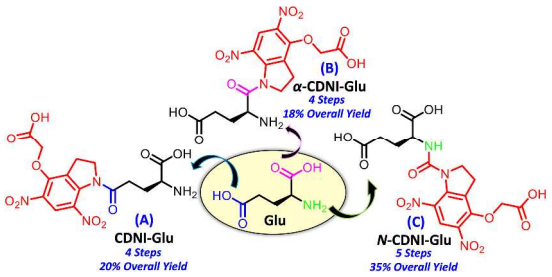
Abstract

Glutamate is an excitatory neurotransmitter that controls numerous pathways in the brain. Neuroscientists make use of photoremovable protecting groups, also known as cages, to release glutamate with precise spatial and temporal control. Various cage designs have been developed and amongst the most effective has been the nitroindolinyl caging of glutamate. We, hereby, report an improved synthesis of one of the current leading molecules of caged glutamate, 4-carboxymethoxy-5,7-dinitroindolinyl glutamate (CDNI-Glu), which possesses efficiencies with the highest reported quantum yield of at least 0.5. We present the shortest route, to date, for the synthesis of CDNI-Glu in 4 steps, with a total reaction time of 40 h and an overall yield of 20%. We also caged glutamate at the other two functional groups, thereby, introducing two new cage designs: α-CDNI-Glu and *N*-CDNI-Glu. We included a study of their photocleavage properties using UV-vis, NMR, as well as a physiology experiment of a two-photon uncaging of CDNI-Glu in acute hippocampal brain slices. The newly introduced cage designs may have the potential to minimize the interference that CDNI-Glu has with the $GABA_A$ receptor. We are broadly disseminating this to enable neuroscientists to use these photoactivatable tools.

Graphical abstract



Introduction

The brain is the most complex animal organ. It is the core of the nervous system and as a result controls the entire organism. The average human brain contains at least 85 billion neurons interconnected with a range of 1000 to 10,000 synapses.[1–3] Neurons communicate with one another using chemical messengers, known as neurotransmitters, which are released by the presynaptic neuron across synaptic junctions. Postsynaptic neurons have receptors that bind the neurotransmitters thereby activating pathways that lead to signal transmission. While there are a handful of neurotransmitters, glutamate (Glu) and gamma aminobutyric acid (GABA) are amongst the most common as they are the excitatory and inhibitory neurotransmitters, respectively.[4–8]

The immense complexity of the brain's network renders brain mapping a colossal challenge. The development of molecular tools that can be activated upon demand at specific synaptic sites has become of apparent value. Neuroscientists have made use of photo responsive protecting groups that can be released upon demand at very specific synapses.[9] These are known as cages, since their principal role is to completely inactivate the agonists, analogous to incapacitating a caged lion. However, they have to respond to light in an efficient manner to release the active agonist using specific wavelengths of laser.[10,11] This process is known as uncaging.

Several cage designs have been developed over the years.[12–15] They vary in their photo properties, which include: 1) wavelength 2) extinction coefficient and 3) quantum yield. Amongst the most efficient cage designs are those developed by Corrie[16-18] and Ellis-Davies[19–22]:

4-methoxy-7-nitroindolinyl glutamate (MNI-Glu) and 4-carboxymethoxy-5,7-dinitroindolinyl glutamate (CDNI-Glu), respectively.

We describe an improved synthesis of CDNI-Glu and introduce two new cage designs. Glutamate has three functional groups: an α-amino, an α-carboxy, and an ω-carboxy. Ellis-Davies and coworkers reported caging the latter,[19] naming it CDNI-Glu in 2007, and subsequently published a more detailed protocol in 2011.[20] We are presenting the first synthesis of caged Glu at the α-carboxy and at the amine groups, naming them: α-CDNI-Glu and *N*-CDNI-Glu, respectively. One of the advantages of exploring these new caged agonists is their potential at minimizing the observed inhibition of $GABA_A$ receptors when CDNI-Glu was used.[10,21] The structures of the three variants of caged Glu (A, B, and C), attached at each of the three functional groups, are shown in Figure 1.

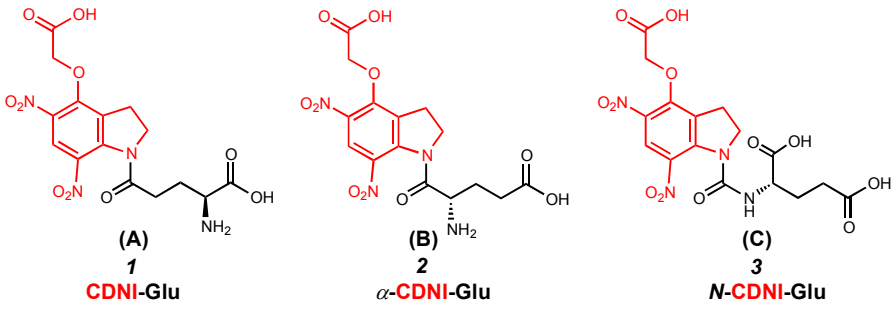


Figure 1. The three types of caged-Glu (A, B, and C); B and C have not been previously reported; caging units are shown in red for clarity.

CDNI has become an essential photoprotecting tool amongst neuroscientists due to its superior photochemical properties compared to other caging systems. Our earlier studies included a

comparison with MNI, which has one nitro group instead of two. Using NMR techniques with photolysis, we showed that the additional nitro group in the indole ring of CDNI increases the relative rate of photocleavage by a factor of 5.8.[23] The more recent protocol for the preparation of CDNI-Glu and CDNI-GABA presents details of a 10-step synthesis carried out over 200 h of reaction time, with overall yields of 7-11%.[20] The immense significance that CDNI-Glu has had in the field of optical neuroscience, especially due to its responsiveness to two-photon irradiation,[24] prompted us to develop a faster and more efficient route that we are currently employing in other systems. Our improved synthesis employs 4 steps over a reaction time of 40 h, with an overall yield of 20%.

Furthermore, CDNI-Glu causes off-target effects with the $GABA_A$ receptor at high concentrations (3-12 mM).[10,21] The proximity of the zwitterionic functions of COO^- and NH_3^+ in CDNI-Glu mimics the curved conformation of GABA when binding the $GABA_A$ receptor. This leads to interference between CDNI-Glu and the inhibitory $GABA_A$ receptor, effectively mitigating the excitatory signals of photolyzed CDNI-Glu. There has been several attempts to minimize or eliminate this interference via elaborate designs of different photocaged glutamates.[10] These designs are mainly based on bulking up the caged molecules[25] thereby minimizing their competitive interaction with the receptors.

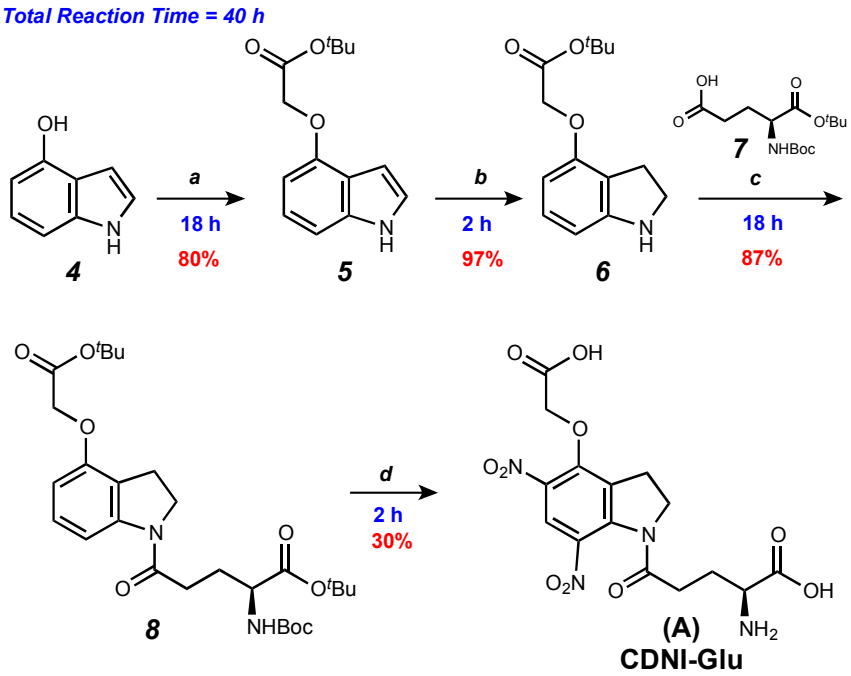
Herein, we present an improved synthesis of a critical photo responsive molecular tool, CDNI-Glu, via a 4-step synthesis and overall yields of 20%. We also synthesized two new caged Glu (B and C) by protecting the other two functional groups of the agonist. This may lead to minimized interference with the $GABA_A$ receptor. We also deliver the photo properties of all

three caged Glu molecules using UV-vis and NMR, per our previous work, as well as an in vitro two-photon uncaging experiment in acute mouse brain slice. Future studies will involve the evaluation of the new designs (cages B and C) in brain slices. We are receptive to collaboration with neuroscience labs that are conducting brain slice experiments with two-photon laser, and as such, we will gladly provide them with all three caged Glu A, B, and C.

Results and Discussion

Synthetic Procedures

The improved procedures focus on two predominant factors: direct access to the indolinyl system and direct deprotection and nitration pathways. Our nitration step takes advantage of an old protocol, published in 1956 by G. A. Olah,[26–29] that uses nitronium tetrafluoroborate, $NO_2^+ BF_4^-$, to directly introduce the nitronium ion for electrophilic aromatic substitution. We found this to be a rapid and smooth technique to nitrate the indolinyl moiety directly to the dinitro system. The use of freshly purchased reagent under anhydrous conditions led to a near quantitative conversion to the dinitroindolinyl system, as observed by TLC. Our isolated yields of 30% for that step suffered during the HPLC purification and lyophilization of the final product. Scheme 1 outlines the 4 steps for the synthesis of CDNI-Glu along with their corresponding reaction times and yields for each step.

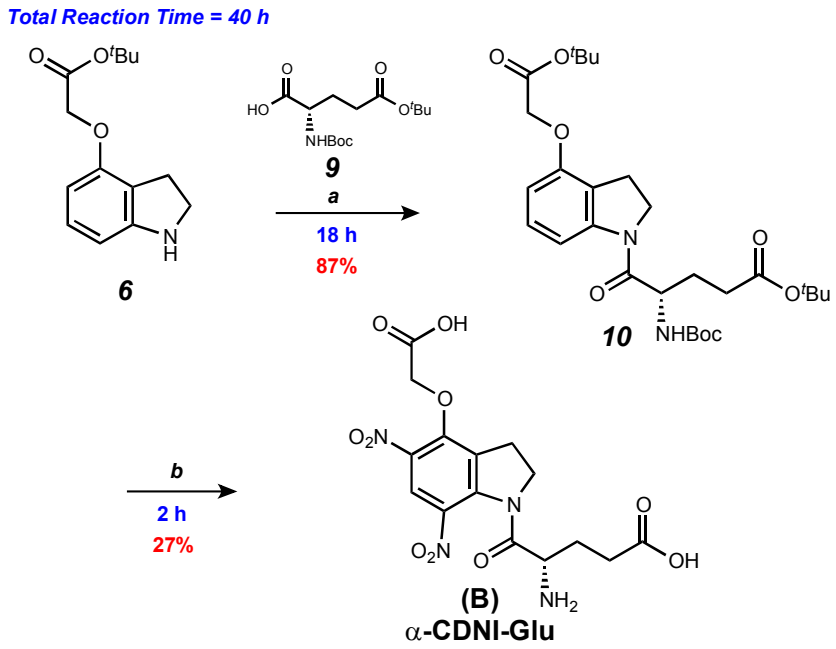


Scheme 1. Synthesis of CDNI-Glu (A); a) *tert*-butyl bromoacetate, K_2CO_3, acetone, RT; b) $NaBH_3CN$, AcOH, RT; c) 1-(3-dimethylaminopropyl)-3-ethylcarbodimide, DMAP, Boc-Glu-O*t*-Bu **7**, CH_2Cl_2, RT; d) TFA, nitronium tetrafluoroborate (4 equiv), RT.[26–29]

Previous protocols[20] make use of acetyl protecting groups to control the selectivity of the functionalization of the N and O of the reduced hydroxyindole, i.e. indolinyl. Once the hydroxyindole ring is reduced, the nucleophilic reactivity of the O and N of the indolinyl system become comparable, thus necessitating the use of protecting groups. However, in the presence of the 2,3-olefin in **4**, the nucleophilic potency of the N is somewhat subdued by to the partial aromaticity of the pyrrole ring. This enabled us to selectively functionalize the phenolic O with the ester group under basic conditions of K_2CO_3 over 18 h, leading to **5** with 80% yield. Our choice for a *t*-butyl ester instead of a methyl ester was mainly to enable a one-step deprotection under TFA conditions needed for the subsequent nitration steps. Reduction of

the indole double bond to form **6** proceeded with a 97% yield, in 2 h under acidic reductive conditions of $NaBH_3CN$ in acetic acid. The attachment of the glutamate chain was accomplished using standard EDC coupling with protected Glu **7**. The reaction was carried out for 18 h and afforded compound **8** in an 87% yield, which was purified through standard normal phase chromatography. The final step was carried out in TFA, which served the dual purpose of an instant deprotection of both *tert*-butyl groups of **8** and an acidic medium for the subsequent nitration. The direct introduction of two nitro groups on the indolinyl ring was accomplished using nitronium tetrafluoroborate in TFA, affording target compound, CDNI-Glu (A), in 30% yield after HPLC purification and lyophilization.

We employed this protocol to generate the other caged Glu designs (Figure 1). Caging the α-carboxy group of Glu is unprecedented and may possess improved electrophysiological properties; therefore, it is worthwhile to make it available to neuroscientists. Scheme 2 highlights the steps for an efficient synthesis of α-CDNI-Glu (B) in similar yields to our synthesis of CDNI-Glu (A).

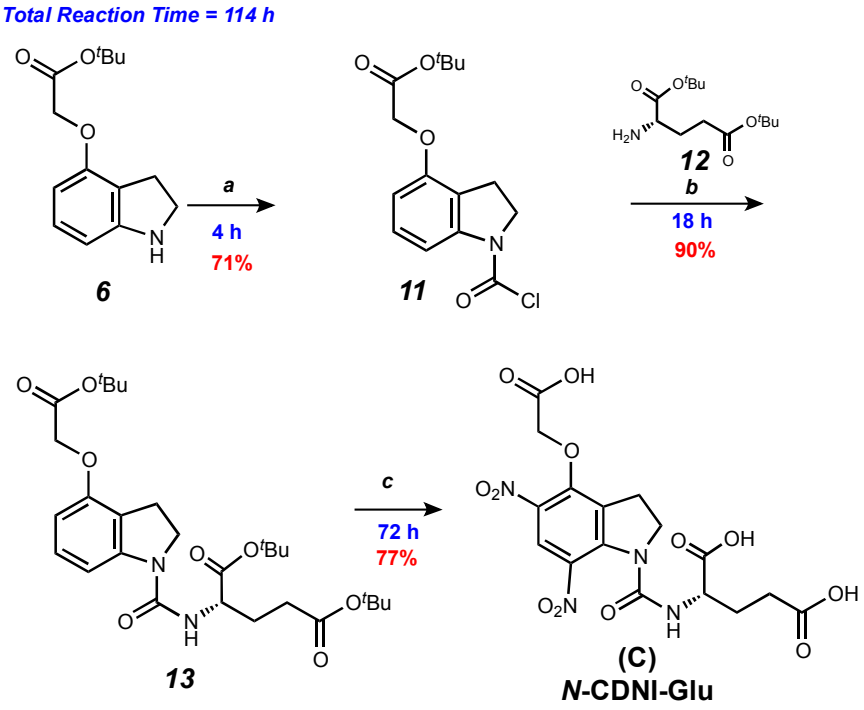


Scheme 2. Synthesis of α-CDNI-Glu (B); a) 1-(3-dimethylaminopropyl)-3-ethycarbodimide, DMAP, Boc-D-Glu(O*t*Bu)-OH **9**, CH_2Cl_2, RT; b) TFA, nitronium tetrafluoroborate, RT.

Starting from the common intermediate **6** (from Scheme 1), an EDC coupling reaction was carried out with commercially available protected Glu **9** leading to the pre-nitration protected intermediate **10** in 87% yield. The deprotection and nitration steps were carried out in TFA, followed by the addition of nitronium tetrafluoroborate, similar to the procedure in Scheme 1. The combined yield of the isolated and HPLC purified and lyophilized target α-CDNI-Glu (B), was 27%. The overall yield for the synthesis of caged Glu (B) from **4** was 18%.

The dinitroindolinyl caging system has only previously been used to cage carboxy groups apart from one system on material assembly.[30,31] We investigated the possibility of using the CDNI system to protect the amine functionality of Glu, leading to a new form of a caged Glu, which we hereby refer to as: *N*-CDNI-Glu (C). Clearly, the assembling route differed from the

protocols used to cage the α- and ω-carboxy functionalities. We were able to access the newly caged design with only one additional step (see Scheme 3). The overall reaction time was 114 h.



Scheme 3. Synthesis of *N*-CDNI-Glu (C); a) triphosgene, pyridine, CH_2Cl_2, 0°C to RT; b) di-*tert*-butyl glutamate **12**, Et_3N, CH_2Cl_2, 0°C to RT c) $NaNO_3$, TFA, RT.

Also starting with common intermediate **6**, triphosgene was added to form chlorocarbonyl indolinyl **11**. Then, it was coupled with di-*tert*-butyl ester protected Glu **12**, under basic conditions of triethylamine, to form pre-nitrated intermediate **13**, which is easily purified. Finally, the aromatic ring was nitrated with $NaNO_3$ in TFA to afford *N*-CDNI-Glu (C) in 77% yield, and overall yield of 35% from **4**.

The latter nitration of **13** using nitronium tetrafluoroborate consistently led to the cleavage of the Glu moiety, irrespective of our numerous attempts to use lower temperatures and milder

conditions. It is likely that the more electron rich carbonyl of the urea unit of **13** interacts with the electrophilic NO_2^+ leading to an activated labile carbonyl system, which may subsequently undergo an intramolecular decomposition via the α-carboxy group. This was not observed in the less electron-enhanced carbonyl of the amide systems of **8** and **10**, shown in Schemes 1 and 2, respectively. Therefore, we resorted to the alternative milder nitration pathway of $NaNO_3$ in TFA and achieved a yield of 77% after 3 days of reaction time.

It is important to note that the easily accessible common intermediate **6** can be subjected to only 2 additional steps to make α-CDNI-Glu (B), or just 3 additional steps to make *N*-CDNI-Glu (C).

Photocleavage Studies

We also studied the photo properties of the three caged Glu designs and compared their efficiency of uncaging under identical conditions. Although the CDNI cage has been extremely valued for its two-photon uncaging properties, we focused our studies on a Rayonet photochemical reactor (RPR 100) as the light source to compare the three designs. Each molecule was irradiated with longer wavelength UV light at 350 nm. Our instrument has a calcd light flux (light intensity) of 24 Wm^{-2}.

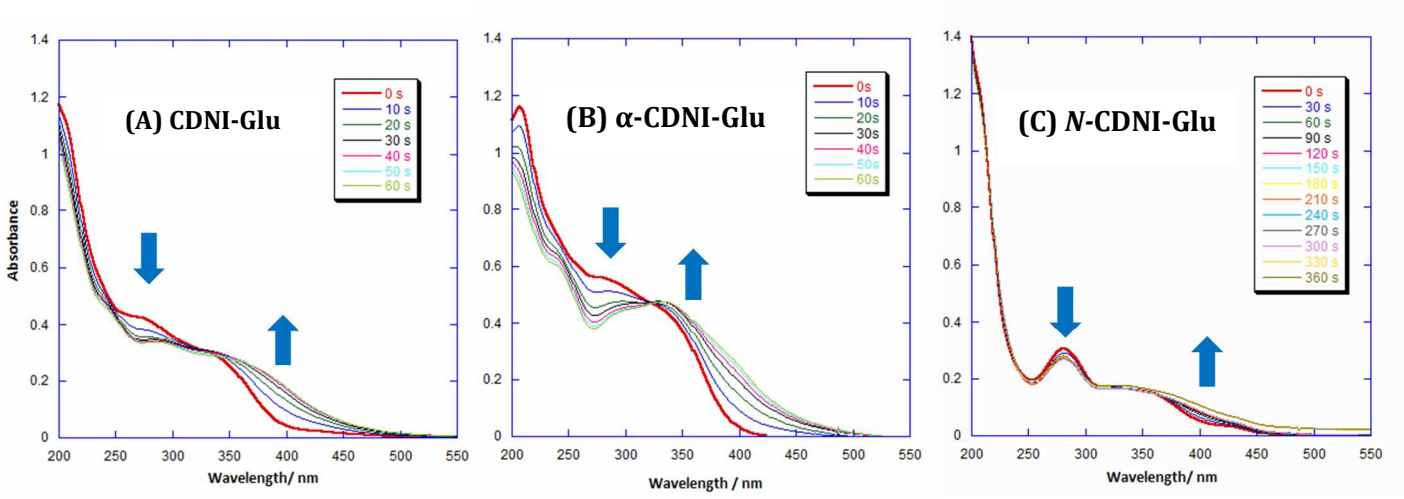


Figure 2. UV spectra for progressive photolysis of (A) CDNI-Glu (4.70×10^{-5} M), (B) α-CDNI-Glu (7.94×10^{-5} M) and (C) *N*-CDNI-Glu (2.00×10^{-5} M) at a wavelength of 350 nm in DI water at pH 7.

Irradiation took place in the photo reactor at room temperature at specified intervals of 10s, for CDNI-Glu (A) and α-CDNI-Glu (B), and for 30s for *N*-CDNI-Glu (C). It necessary to irradiate the

latter for longer time, due to it lower quantum yield relative for the former two. The absorbance changes, resulting from UV irradiation experiments on both CDNI-Glu and α-CDNI-Glu, reflect a peak decrease at 272 nm and an increase at 350 nm (Figure 2 a and b). Conversely, the *N*-CDNI-Glu (C) photolysis was relatively slow at UV light of 350 nm and the absorption peaks increased around 400 nm (Figure 2c).

Relative photolysis rates of each molecule were also measured using ^{1}H NMR. A known amount of sample molecule and maleic acid (internal standard reference) were dissolved in a specific amount of deuterium oxide (0.5 mL), as NMR solvent, and transferred into a transparent NMR tube. The NMR tube was then placed in the photochemical reactor (24 Wm^{-2}) and irradiated at 350 nm for 30s time intervals. After each irradiation, the ^{1}H NMR was collected using a Bruker 400 MHz NMR. The depletion of a selected peak (the aromatic H_a) was measured relative to the internal standard peak, maleic acid. Based on peak integrals, the concentration of the molecule was calcd (Figure 3).

The 7-nitro group, which is *ortho* to the labile amide, is key in the photolysis that leads to Glu release. It effectively delivers an O to the amide leading to Glu release, and is subsequently converted to a nitroso group,[14] leading to an upfield shift in the aromatic proton H_a as it becomes H_b. This process also leads to the aromatization of the indolinyl ring to indole, giving rise to H_c and H_d with ^{1}H NMR peaks at the 7.2 and 6.8 ppm, respectively (Figure 3).

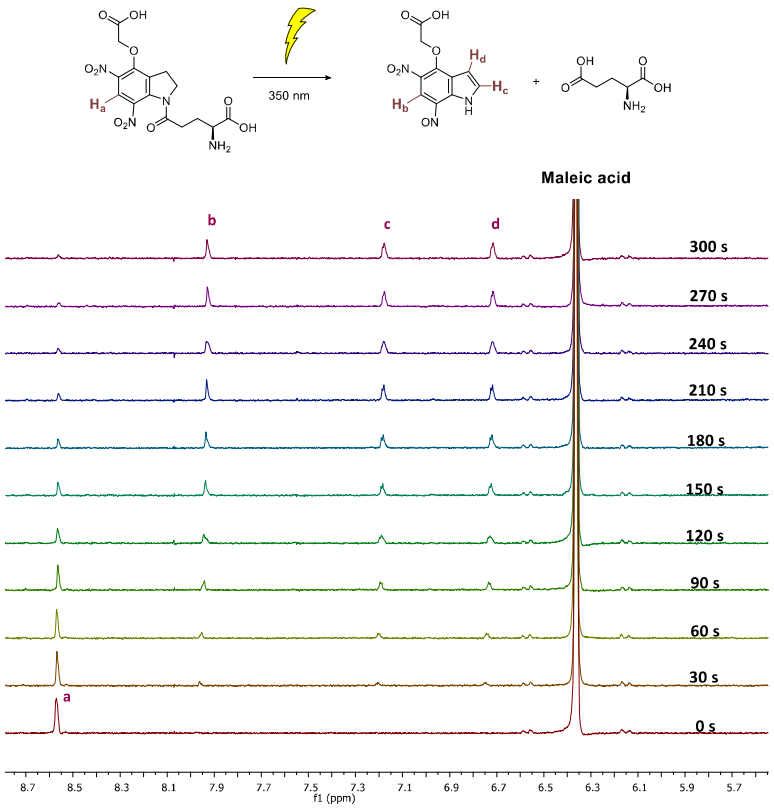


Figure 3. ^{1}H NMR (400 MHz, D_2O) spectra for progressive photolysis of CDNI-Glu (A) at a concentration of 1.47 mM. The progressive photolysis spectra for α-CDNI-Glu (B) and *N*-CDNI-Glu (C) are shown in SI.

The concentrations of these molecules were calcd based on the peak integrals of specific protons (see SI for the calculations). The photolysis reactions for these molecules obey the first order rate law (Figure 4). Of the three molecules, CDNI-Glu showed the highest rate of photolysis, while *N*-CDNI-Glu showed the lowest. The time taken to completely photolyze CDNI-Glu or α-CDNI-Glu was 300s, whereas it took *N*-CDNI-Glu more than 4000s to completely release Glu. It is important to note that during neurological studies, it is not necessary for these chemical tools to be completely photolyzed, as long as photolysis leads to the release of Glu

and the caged molecule does not interfere in other competing signaling pathways. Therefore, from the neuroscientists' perspective, α-CDNI-Glu (B) and *N*-CDNI-Glu (C) may resolve the GABA inhibition issue at relatively higher concentrations than CDNI-Glu (A).

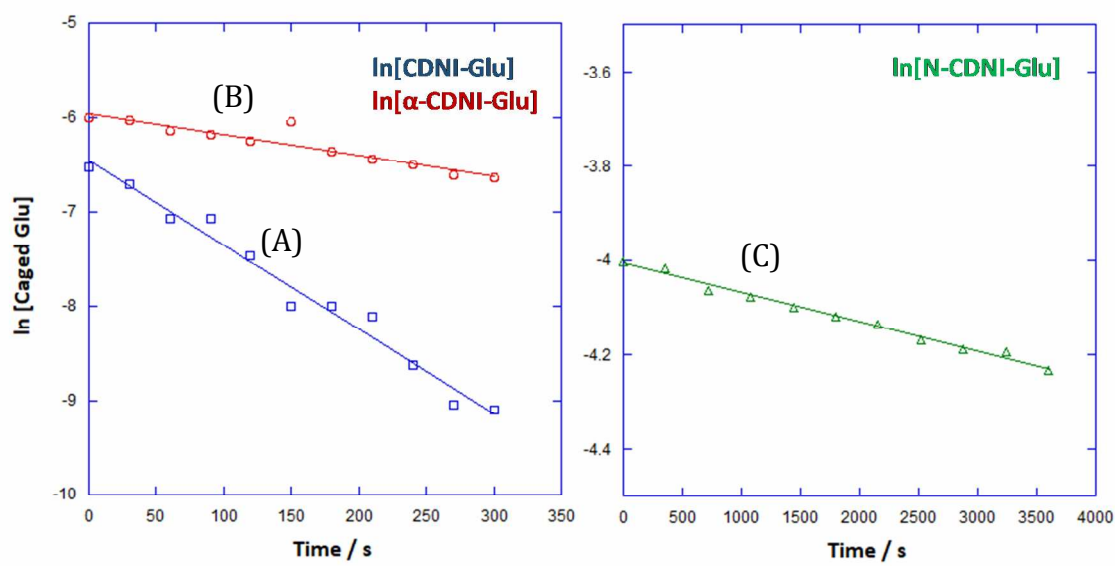


Figure 4. Rate of photolysis for CDNI-Glu (A), α-CDNI-Glu (B) and *N*-CDNI-Glu (C).

The rate constant for the reactions (*k*), maximum absorbance wavelength (λ_{max}), quantum yields (Φ) and photo cross sections (ε.Φ) were calcd using the data retrieved from Figures 2 and 4. Based on our results, the CDNI-Glu shows the most efficient photolytic properties, with a higher quantum yield (0.5) and a photo cross section (3200 $M^{-1}cm^{-1}$); Table 1. The photolysis reactions mentioned above were conducted in aqueous media.

Table 1. Photochemical properties of CDNI based glutamate cages.

Caged Glu	k / s^{-1}	λ_{max} / nm	ε (λ_{max}) / $M^{-1}cm^{-1}$	Φ	ε.Φ / $M^{-1}cm^{-1}$
CDNI-Glu	8.9×10^{-3}	330	6400	0.5	3200
α-CDNI-Glu	2.2×10^{-3}	330	6400	0.13	832.0

***N*-CDNI-Glu**	6.2×10^{-5}	359	7478	0.003	22.43

Two-photon uncaging CDNI-Glu across the synapse in hippocampal brain slices

We have confirmed the responsiveness of CDNI-Glu for two-photon uncaging in acute mouse hippocampal slices (Figure 5). Simultaneous two-photon uncaging of CDNI-Glu (2mM) on multiple dendritic spines of CA1 pyramidal cells evoked large amplitude membrane potential depolarizations recorded at the cell body in whole-cell current-clamp configuration. The CDNI-Glu uncaging evoked responses were completely blocked by antagonists of ionotropic glutamate receptors. These experiments indicate that CDNI-Glu can be used to effectively activate glutamate receptors on postsynaptic spines *in vitro*.

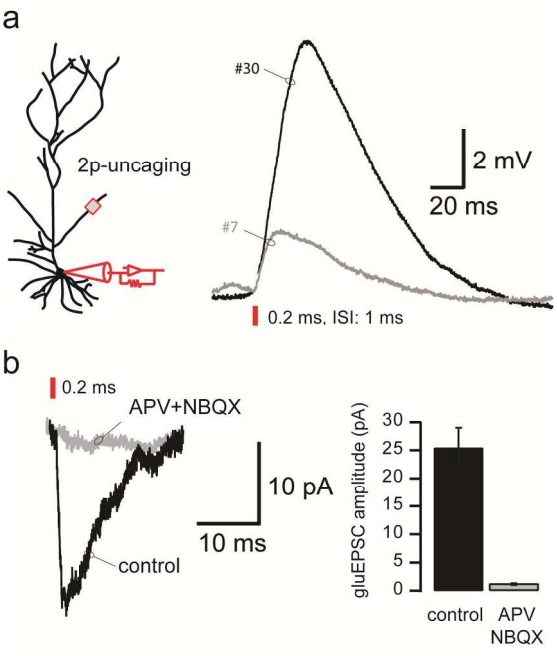


Figure 5. Two-photon uncaging of CDNI-Glu (A) in acute hippocampal brain slices. a) *Left*: schematics of the experimental configuration; CA1 pyramidal neurons were recorded in in

whole-cell patch clamp mode in acute mouse hippocampal slices and individual spines on radial obliques dendritic region (red box) were selected for two-photon uncaging. The resulting responses are recorded at the soma. *Right*: CDNI-Glu (2mM applied locally with a pipette) uncaging evoked excitatory postsynaptic potentials (gluEPSPs) recorded from a CA1 pyramidal cells by uncaging on 7 (grey trace) and 30 (black trace) spines on a dendritic segment using with 0.2 ms uncaging laser duration (D) and 1 ms interstimulus interval (ISI) at 720 nm. **b)** *Left*: CDNI-Glu uncaging-evoked excitatory currents (gluEPSCs) are mediated by ionotropic glutamate receptors. *Left*: CDNI-Glu uncaging evoked excitatory postsynaptic current traces (gluEPSC) at a single spine in control (black) and in the presence of ionotropic glutamate receptor antagonists (grey, NBQX: 2 μM, DL-AP5: 50 μM). *Right*: summary of the CDNI-Glu evoked single-spine gluEPSC amplitude in control and in the presence of ionotropic glutamate receptor antagonists (mean ± s.e.m, n=5 recordings).

CONCLUSION

We have improved the synthesis of CDNI-Glu, an important light triggered molecular tool that is critical for neurological studies. Relative to published methods, our synthesis uses half the number of steps, one fifth of the reaction time, and affords nearly double the yields. We have also introduced two novel caged Glu, from the common intermediate **6** (used in CDNI-Glu synthesis) with 2 or 3 additional steps to generate α-CDNI-Glu and *N*-CDNI-Glu, respectively. We reported the photophysical properties of all three caged-Glu variants and compared them using both UV-vis and NMR. We have also shown the electrophysiological response of CDNI-

Glu in a brain slice. The quantum efficiency of CDNI-Glu is best amongst the three reported. The two newly described caged Glu may have the potential to minimize interference with $GABA_A$ receptor, and we wish to present these types of caged Glu here to enable further investigation by neuroscientists. We have successfully employed our synthetic protocol to generate other photo cleavable agonists that will be published in due course.

METHODS

The starting material for all three caged Glu molecules (Scheme 1, 2 and 3) presented was the commercially available 4-hydroxyindole. Unless otherwise mentioned, chemicals were purchased from Fisher or Aldrich in highly purified form and used without further purification. The solvents used: dichloromethane (CH_2Cl_2), hexanes, and ethyl acetate (EtOAc) were purchased from VWR. The anhydrous solvent CH_2Cl_2 was obtained from a solvent purification system (MBRAUN, MB-SPS), which uses an activated alumina column. The reactions were conducted under N_2 or Ar using standard Schlenk line techniques. The reaction vessels were flame dried before conducting reactions and charged with N_2/Ar (The flame dry vacuum/N_2 charge was repeated three times). Reaction progress was monitored using TLC (EMD Millipore TLC Silica Gel 60 F254) and observed under short and long wavelength ultraviolet light or stains (*p*-anisaldehyde). The products of each step in the schemes were purified using column chromatography with 60 Å silica gel (Fisher scientific). All final products of caged Glu **A**, **B**, and **C**, which are quite polar, were purified using reverse phase HPLC: Waters 2695 separation module with Waters 996 photodiode array detector. The separations were conducted using an XSELECT C18 OBD prep column (10 mm × 25 mm). HPLC grade solvents acetonitrile (ACN),

methanol (MeOH) and Millipore water were used as the mobile phases. After purification, compounds were freeze-dried (lyophilized) via LABCONCO Freezone 4.5. The nitration steps and their corresponding purification were conducted in the dark, under red light. The final products were stored in the dark (covered in Al foil) at -20 °C until further needed. Characterization of each product was performed via ^{1}H NMR and ^{13}C NMR, using a Bruker 400 MHz NMR spectrometer. NMR solvents ($CDCl_3$, D_2O) were purchased from Cambridge Isotope Laboratories and Acros. Chemical shifts were calcd relative to the solvent residual peaks ($CDCl_3$ = 7.26 ppm, D_2O = 4.79 ppm). The UV-vis spectra were obtained with an Agilent 8453 diode array UV-vis. High-resolution mass spectra (HRMS) were measured using a JEOL DART-AccuTOF mass spectrometer, while low-resolution mass analyses were conducted on an Agilent LC-MS with ES+ and 6120 quadrupole. The caged molecules were irradiated during photochemical studies in a Rayonet photochemical reactor (RPR 100) with an intensity of 24 Wm^{-2}.

tert-Butyl 2-((1H-indol-4-yl)oxy)acetate ***(5)****.* A 50 mL round-bottomed flask was charged with 4-hydroxyindole (0.659 g, 4.96 mmol), K_2CO_3 (3.424 g, 24.78 mmol), freshly distilled acetone (10 mL), and freshly activated molecular sieves. The reaction was allowed to stir for 15 min at 25 °C under an inert atmosphere (N_2). After 15 min *tert*-butyl bromoacetate (1.29 mL, 8.42 mmol) was added resulting in a purple colored solution. The reaction mixture was left to stir overnight. After 18 h, the reaction mixture was filtered through a Büchner funnel. The solvent was removed under vacuum resulting in the formation of purple oil. The crude product was purified by column chromatography (3:1 hexane/ethyl acetate) to yield white crystals at 25 °C (0.912 g, 3.67 mmol, 80% yield). ^{1}H NMR: (400 MHz, $CDCl_3$) δ 8.16 (s, 1H), 7.13 (t, *J* = 4.0 Hz, 1H), 7.10-7.04 (m, 2H), 6.73 (t, *J* = 4.0 Hz, 1H), 6.41 (d, *J* = 4.0 Hz, 1H), 4.67 (s, 2H), 1.50 (s, 9H). ^{13}C NMR:

(101 MHz, $CDCl_3$) δ 168.36, 151.77, 137.46, 122.79, 122.51, 118.86, 105.18, 100.84, 100.20, 82.09, 66.09, 28.09. HRMS (m/z): calcd for $C_{14}H_{17}NO_3$ $[M+H]^+$ 248.1242, found 248.1191.

tert-Butyl 2-(indolin-4-yloxy)acetate ***(6).*** $NaBH_3CN$ (0.525 g, 8.36 mmol) was stirred with compound **5** (0.762 g, 3.083 mmol) in a 25 mL round-bottomed flask charged with freshly activated molecular sieves. Then, 7 mL of acetic acid was added, and the reaction mixture allowed to stir for 2 h at 25 °C. After 2 h, vacuum was used to remove the acetic acid. Ethyl acetate was added to dilute the mixture, and the mixture was neutralized using $NaHCO_3$ solution. The organic layer was separated and dried over anhydrous Na_2SO_4. The solvent was removed under vacuum, and the crude product was purified through column chromatography (3:2 hexane/ethyl acetate) to afford product **6** (0.746 g, 2.991 mmol, 97% yield). ^{1}H NMR: (400 MHz, $CDCl_3$) δ 6.94 (t, *J* = 8.0 Hz, 1H), 6.32 (d, *J* = 7.7 Hz, 1H), 6.13 (d, *J* = 8.0 Hz, 1H), 4.51 (s, 2H), 3.56 (t, *J* = 8.0 Hz, 2H), 3.06 (t, *J* = 8.0 Hz, 2H), 1.48 (s, 9H). ^{13}C NMR: (101 MHz, $CDCl_3$) δ 168.34, 154.89, 153.67, 128.46, 116.45, 103.73, 102.37, 82.08, 65.77, 47.49, 28.07, 26.91. HRMS (*m/z*): calcd for $C_{15}H_{18}N_2O_4$ $[M+H]^+$ 250.1398, found 250.1541.

tert-Butyl (S)-5-(4-(2-(tert-butoxy)-2-oxoethoxy)indolin-1-yl)-2-((tert-butoxycarbonyl) amino-5-oxopentanoate ***(8).*** A 25 mL flame dried round-bottomed flask was charged with compound **6** (0.272 g, 1.092 mmol), EDC (0.290 g, 1.9 mmol), DMAP (0.230 g, 1.9 mmol), and 5 mL of dry CH_2Cl_2. The reaction mixture was allowed to stir for 15 min until complete solubility for all reaction mixture components was achieved. Then Boc-Glu-O*t*-Bu (**7**) was dissolved in 5 mL of dry CH_2Cl_2 and added dropwise to the reaction mixture. The reaction was allowed to stir overnight (18 h) at 23 °C under Ar. After 18 h, the organic layer was extracted with CH_2Cl_2,

neutralized with $NaHCO_3$, and dried over anhydrous Na_2SO_4. The crude product was purified by column chromatography (3:2 hexane/ethyl acetate) to afford compound **8** as brown oil, (0.51 g, 0.95 mmol, 87% yield). ^{1}H NMR: (400 MHz, $CDCl_3$) δ 7.86 (d, *J* = 8.0 Hz, 1H), 7.12 (t, *J* = 8.0 Hz, 1H), 6.42 (d, *J* = 8.0 Hz, 1H), 5.22 (d, *J* = 8.0 Hz, 1H), 4.53 (s, 1H), 4.24–4.21 (m, 1H), 4.04 (t, *J* = 8.0 Hz, 2H), 3.18 (t, *J*= 8.0 Hz, 2H), 2.58–2.42 (m, 2H), 2.30-2.25(m, 1H), 2.04-1.97 (m, 1H), 1.50-1.46 (m, 27H). ^{13}C NMR: (101 MHz, $CDCl_3$) δ 173.54, 171.54, 170.38, 170.27, 167.96, 154.11, 144.54, 128.89, 119.01, 110.78, 106.79, 83.32, 82.27, 65.72, 53.77, 48.40, 32.22, 31.15, 28.05, 25.09. HRMS (m/z): calcd for $C_{28}H_{43}N_2O_8$ $[M+H]^+$ 535.3014, found 535.2967.

(S)-2-amino-5-(4-(carboxymethoxy)-5,7-dinitroindolin-1-yl)-5-oxopentanoic acid ***(1), Caged Glu (A) .*** **Step 1:** A two dram amber vial was charged with compound **8** (0.1104 g, 0.2 mmol) and trifluoroacetic acid, TFA, (1 mL). The reaction mixture was allowed to stir for 30 min at 23 °C. After 30 min, a TLC was taken (2:1 hexane/ethyl acetate) to verify a single spot for the formation of the deprotected intermediate, then a nitrogen stream was applied to remove the TFA to give deprotected compound (0.064 g, 0.2 mmol, quantitative yield). ^{1}H NMR: (400 MHz, D_2O) δ 7.31 (d, *J* = 8.0 Hz, 1H), 6.85 (t, *J* = 8.0 Hz, 1H), 6.25 (d, *J* = 8.0 Hz, 1H), 4.33 (s, 2H), 3.92 (t, *J* = 8.0 Hz, 1H), 3.52 (t, *J* = 8.0 Hz, 2H), 2.61 (t, *J* = 8.0 Hz, 2H), 2.32-2.29 (m, 2H), 2.01-1.97 (m, 2H). ^{13}C NMR: (101 MHz, D_2O) δ 172.68, 171.28, 170.98, 153.48, 143.24, 128.66, 117.35, 110.29, 107.82, 64.68, 55.76, 48.07, 30.98, 29.47, 24.15. HRMS (m/z): calcd for methylated $C_{17}H_{24}N_2O_6$ $[M+H]^+$ 351.1551, found 351.1586.

Step 2: Following the evaporation of TFA, 2 mL of fresh TFA was added. The reaction mixture was cooled to 0 °C, and nitronium tetrafluoroborate (0.1056 g, 0.8 mmol) was added and

stirred for 1.5 h, in the dark, while allowing it to reach room temperature. After TFA removal with a nitrogen stream, the crude product was dissolved in 1.5 mL of H_2O: acetonitrile (75%: 25%) and filtered using a 0.45 micron filter. The product was purified using reverse phase HPLC chromatography with an isocratic mixture of 60% water, 40 % acetonitrile and 0.1% TFA with an elution rate of 2 mL min^{-1}. Product **1**, caged Glu (A), was eluted at 6 min, collected, and lyophilized to dryness. (0.025 g, 0.06 mmol, 30% yield). ^{1}H NMR: (400 MHz, D_2O) δ 8.57 (s, 1H), 4.86 (s, 2H), 4.77 – 4.71 (m, 1H), 4.59 (td, *J* = 9.8, 2.8 Hz, 1H), 4.42 (q, *J* = 9.7 Hz, 1H), 3.49 (td, *J* = 18.4, 9.3 Hz, 1H), 3.38 (ddd, *J* = 12.4, 9.2, 2.7 Hz, 1H), 2.70 (t, *J* = 7.2 Hz, 2H), 2.41 – 2.22 (m, 2H). ^{13}C NMR: (101 MHz, D_2O) δ 175.97, 172.31, 168.79, 156.07, 155.74, 151.20, 138.73, 132.33, 122.95, 69.74, 51.93, 51.09, 28.39, 26.79, 24.96. Compound **1** is not stable under DART ionization conditions. Dinitro containing fragments, and methylated intermediates (due to CH_3OH solvent used) can be observed with DART MS (fragments **a** and **b** shown in S3, SI). HRMS (ESI) showed characteristic fragmentation peaks: *m/z* calcd for methylated aqueous byproduct ($C_{11}H_9N_3O_6^{\bullet+}$): 279.0491, found 279.0482; for free glutamate ($C_5H_9NO_4^{\bullet+}$): 147.0531, found 147.0570; and for the organic byproducts: for a ($C_{11}H_{12}N_3O_7^+$): 298.0675, found 298.0617; for b ($C_{11}H_{12}N_2O_5^{\bullet+}$): 252.0746, found 252.0764, for ($C_8H_8N_3O_5^+$): 226.0464, found 226.0508, and for ($C_8H_7N_2O_2^+$): 163.0507, found 163.0487. However, the intact caged Glu **(A)** was observed with ES+ MS (m/z) calcd for $C_{15}H_{17}N_4O_{10}$ $[M+H]^+$ 413.09, found 413.1.

tert-Butyl(S)-5-(4-(2-(tert-butoxy)-2-oxoethoxy)indolin-1-yl)-4-((tert-butoxycarbonyl)amino)-5-oxopentanoate ***(10).*** A 25 mL flame dried round-bottomed flask was charged with *tert*-butyl 2-

(indolin-4-yloxy)acetate (0.36 g, 1.44 mmol), EDC (0.47 g, 2.45 mmol), DMAP (0.30 g, 2.45 mmol), and 7.5 mL of dry CH_2Cl_2. The reaction mixture was allowed to stir for 15 min until complete solubility for all reaction mixture components was achieved. Then Boc-D-Glu(O*t*-Bu)-OH (**9**) (0.43 g, 1.44 mmol) was dissolved in 5 mL of dry CH_2Cl_2 and added dropwise to the reaction mixture. The reaction was allowed to stir overnight (18 h) at 23 °C under Ar. The organic layer was subsequently extracted with CH_2Cl_2, neutralized with $NaHCO_3$, and dried over anhydrous Na_2SO_4. The crude product was purified by column chromatography (3:2 hexane/ethyl acetate) to get compound **10** as brown oil (0.50 g, 0.95 mmol, 87% yield). ^{1}H NMR: (400 MHz, $CDCl_3$) δ 7.78 (d, *J* = 8.0 Hz, 1H), 7.07 (t, *J* = 8.0 Hz, 1H), 6.40 (d, *J* = 8.0 Hz, 1H), 5.40 (d, *J* = 8.0 Hz, 1H), 4.57 – 4.53 (m. 1H), 4.48 (s, 1H), 4.27-4.19 (m, 2H), 3.15 (t, *J* = 8.0 Hz, 2H), 2.35 – 2.28 (m, 2H), 2.13 – 2.05 (m, 1H), 1.85 – 1.76 (m, 1H), 1.45 – 1.43 (m, 27H). ^{13}C NMR: (101 MHz, $CDCl_3$) δ 170.81, 169.18, 166.68, 165.33, 153.25, 143.08, 127.99, 118.82, 109.88, 105.99, 83.82, 81.35, 79.31, 64.84, 50.79, 47.13, 30.42, 29.41, 26.98, 23.72, 21.25. HRMS (m/z): calcd for $C_{28}H_{43}N_2O_8$ $[M+H]^+$ 535.3014, found 535.2965.

(S)-4-Amino-5-(4-(carboxymethoxy)-5,7-dinitroindolin-1-yl)-5-oxopentanoic acid ***(2), Caged Glu (B)***. A two dram amber vial was charged with compound **10** (0.115 g, 0.22 mmol) and TFA (1 mL). The reaction mixture was allowed to stir for 30 min at 23 °C. After 30 min, a nitrogen stream was applied to remove the TFA. Then 2 mL of fresh TFA was added and the reaction was cooled to 0 °C. Then nitronium tetrafluoroborate (0.12 g, 0.9 mmol) was added and the reaction mixture was stirred for 1.5 h, in the dark, while allowing it to reach room temperature.

The TFA was removed using a nitrogen stream, and the residue was dissolved in 1.5 mL of H_2O:acetonitrile (75%: 25%) and filtered using a 0.45 micron filter. The crude product was purified by reverse phase HPLC chromatography using 60% water, 40 % acetonitrile, and 0.1% TFA with an isocratic elution at 2 mL min^{-1}. Caged Glu (B), compound **2**, was eluted at 6 min, collected, and then lyophilized to dryness (0.027 g, 0.066 mmol, 27% yield). ^{1}H NMR: (400 MHz, D_2O) δ 8.55 (s, 1H), 4.83 (s, 2H), 4.59-4.56 (m, 1H), 4.40 (q, *J* = 10.0 Hz, 1H), 3.49-3.35 (m, 1H), 3.33-3.25 (m, 1H). ^{13}C NMR: (101 MHz, D_2O) δ 175.98, 172.54, 168.77, 162.79, 151.26, 139.47, 138.69, 134.91, 132.19, 122.91, 69.86, 51.91, 51.07, 28.39, 26.80, 24.96. LCMS (m/z) calcd for $C_{15}H_{17}N_4O_{10}$ $[M+H]^+$ 413.09, found 413.0.

tert-Butyl 2-((1-(chlorocarbonyl)indolin-4-yl)oxy)acetate ***(11)***. To a flame dried round-bottom flask triphosgene (0.46 equiv, 0.08 g, 0.27 mmol) was added. Then 3 mL of CH_2Cl_2 was added, and the reaction mixture was cooled to 0° C. Afterwards, pyridine (1 equiv) 0.09 mL was added and the reaction mixture was allowed to stir for 5 min. Then compound **6** (0.13 g, 0.52 mmol) was dissolved in 1 mL of CH_2Cl_2 and added dropwise to the reaction mixture. The reaction mixture was stirred for 3 h while allowing warm up to room temperature. After 3 h it was quenched with HCl (1 M) and extracted using CH_2Cl_2. The organic layer was washed with saturated $NaHCO_3$ and dried with anhydrous Na_2SO_4. The product was then purified using column chromatography (3:2 hexane: ethyl acetate) (0.115 g, 0.37 mmol, 71% yield). ^{1}H NMR: (400 MHz, $CDCl_3$) δ 7.48(d, *J* = 8.0 Hz, 1H), 7.14(t, *J* = 8.0 Hz, 1H), 6.48(d, *J* = 8.0 Hz, 1H), 4.53(s, 2H), 4.23(t, *J* = 8.0 Hz, 2H), 3.15(t, *J* = 8.0 Hz, 2H), 1.47(s, 9H). ^{13}C NMR: (101 MHz, $CDCl_3$) δ

167.69, 154.37, 144.89, 142.65, 129.08, 128.78, 120.23, 109.74, 108.05, 82.46, 65.71, 52.11, 27.67, 24.20. HRMS (m/z): calcd for $C_{15}H_{19}ClNO_4$ $[M+H]^+$ is 312.0997, found 312.0994.

di-tert-Butyl (4-(2-(tert-butoxy)-2-oxoethoxy)indoline-1-carbonyl)glutamate ***(13)****.* Compound **12** and triethylamine (0.1 mL) were transferred to a flame dried round-bottomed flask and 5 mL of CH_2Cl_2 was added. The reaction mixture was stirred at 0 °C. Then compound **11** (0.10 g, 0.32 mmol) was dissolved in 1 mL of CH_2Cl_2 and added dropwise. The reaction mixture was allowed to stir and reach room temperature overnight. Then the reaction mixture was washed with saturated NH_4Cl solution and brine. Afterwards, it was dried with anhydrous Na_2SO_4. Finally, the product was purified using column chromatography (3:2 hexane: ethyl acetate) (0.15 g, 0.29 mmol, 90% yield). ^{1}H NMR: (400 MHz, $CDCl_3$) δ 7.52(d, *J* = 8.0 Hz, 1H), 7.06(t, *J* = 8.0 Hz, 1H), 6.30(d, *J* = 8.0 Hz, 1H), 5.45(d, *J* = 8.0 Hz, 1H), 4.50(s, 2H), 4.46(t, *J* = 8.0 Hz, 1H), 3.97(t, *J* = 8.0 Hz, 2H), 3.15(t, *J* = 8.0 Hz, 2H), 1.45-1.42 (m, 27H). ^{13}C NMR: (101 MHz, $CDCl_3$) δ 172.63, 172.09, 168.00, 154.38, 145.13, 128.80, 118.07, 108.68, 105.22, 82.15, 82.09, 80.60, 65.69, 53.30, 47.41, 31.67, 27.99, 24.84. HRMS (m/z): calcd for $C_{28}H_{23}N_2O_8$ $[M+H]^+$ is 535.3014, found 535.2236.

(4-(Carboxymethoxy)-5,7-dinitroindoline-1-carbonyl)glutamic acid ***(3), Caged Glu (C)****.* A 2 dram vial was charged with compound **13** (0.04 g, 0.064 mmol) and TFA and stirred for 30 min at room temperature. After completion, a nitrogen stream was applied to remove the TFA. Then 1 mL of fresh TFA and sodium nitrate (0.04 g, 0.44 mmol) were added and the reaction mixture was stirred for 3 days, in the dark. The TFA was removed using a nitrogen stream. Then, the

compound was dissolved in 1.5 mL of H_2O:acetonitrile (75%: 25%) and filtered using a 0.45 micron filter. The product was purified by reverse phase HPLC chromatography using 60% water, 40 % acetonitrile and 0.1% TFA with an isocratic elution at 2 mL min^{-1}. Compound **3** was eluted at 6 min, collected, and then lyophilized to dryness (0.0225 g, 0.049 mmol, 77% yield). ^{1}H NMR: (400 MHz, D_2O) δ 8.45(s, 1H), 4.75(s, 1H), 4.31-4.29(m, 3H), 3.35(t, *J* = 8.0 Hz, 2H), 2.53(t, *J* = 8.0 Hz, 2H), 2.26-2.22(m, 1H), 2.04-2.01(m, 1H). ^{13}C NMR: (101 MHz, D_2O) δ 174.52, 172.44, 170.07, 153.60, 148.39, 140.12, 133.41, 129.85, 128.04, 120.85, 67.06, 50.52, 49.21, 27.55, 23.43, 22.63. LCMS (m/z) calcd for $C_{16}H_{16}N_4NaO_{12}$ $[M+Na]^+$ 479.1, found 479.1.

Two-photon CDNI-Glu uncaging in acute hippocampal slices. Whole-cell patch clamp recordings were performed from somata of hippocampal CA1 pyramidal neurons in acute hippocampal slices prepared from C57BL/6 mice as described previously.[32] Briefly, slices were perfused with artificial cerebrospinal fluid at 32–33 °C and containing: NaCl 125 mM, $NaHCO_3$ 25 mM, KCl 2.5 mM, NaH_2PO_4 1.25 mM, $MgCl_2$ 1.0 mM, $CaCl_2$ 2.0 mM, glucose 22.5 mM, Na-pyruvate 3.0 mM, ascorbate 1.0 mM, and saturated with 95% O_2 and 5% CO_2. Slices were visualized with a Zeiss Examiner.Z1 with a 63x objective (NA 1.0) and a two-photon scanning microscope (Bruker). Somatic whole-cell patch clamp recordings were performed from visually-identified CA1 pyramidal neurons with a Dagan BVC-700A amplifier (filtered at 1–10 kHz and digitized at 50 kHz). Recording pipettes were pulled from borosilicate glass to tip resistances of ~4–8 MΩ and contained: K-gluconate 130 mM, KCl 8.0 mM, NaCl 4.0 mM, HEPES 10 mM, Mg_2ATP 4.0 mM, $Tris_2GTP$ 0.3 mM, phosphocreatine 14 mM, Alexa 594 0.05 mM, pH 7.25. Two-photon Glu uncaging was performed as described previously.[33] Briefly, a dual galvanometer based scanning system was used to photo-release glutamate at multiple dendritic spines. Ultra-

fast, pulsed laser light (Chameleon Ultra II; Coherent) at 920–930 nm was used to excite Alexa 594, while 720 nm was used to photolyze CDNI-Glu (2 mM applied via broken pipette above slice). The uncaging laser power was kept below 8 mW at slice surface.

ASSOCIATED CONTENT

The supporting Information is available free of charge on the ACS Publication website at DOI: 10.1021/acschemneuro.7b0xxxx

NMR spectra of caged Glu **A**, **B**, and **C**, and all intermediates leading up to these target structures; Kinetic data; LC MS data, and Direct Analysis in Real Time (DART)-MS data.

AUTHOR INFORMATION

Corresponding Author

*E-mail: nesnas@fit.edu

ORCID

Nasri Nesnas: 0000-0003-3511-940X

Attila Losonczy: 0000-0002-7064-0252

Charitha Guruge: 0000-0003-4405-2905

Author Contributions

C.G. performed the synthesis of all compounds reported; Y.P.O. and N.N. developed the

improved synthesis of CDNI-Glu, and R.L.C. and Y.P.O. developed the NMR technique as an unambiguous means to determine quantum efficiencies. C.G. and N.N. conceived the development of α-CDNI-Glu, and C.G. originated all work on *N*-CDNI-Glu independently. J.M. and C.G. performed the photophysical and NMR studies. A.L. is our neuroscience collaborator and conducted brain slice electrophysiological studies reported. All authors contributed to and approved the final manuscript.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

CDNI-Glu, 4-carboxymethoxy-5,7-dinitroindolinyl glutamate; MNI-Glu, 4-methoxy-7-nitroindolinylglutamate; GABA, gamma aminobutyric acid; RT, room temperature; h, hour(s); min, minute(s); s, seconds; DMAP, 4-dimethylaminopyridine; Boc, *tert*-butyloxycarbonyl; Boc-Glu-OtBu, Boc-L-glutamic acid 1-*tert*-butyl ester; NMR, nuclear magnetic resonance; TFA, trifluoroacetic acid; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; AcOH, acetic acid; Boc-D-Glu(O*t*Bu)-OH, Boc-D-glutamic acid 5-*tert*-butyl ester; UV, ultraviolet; vis, visible; ppm, parts per million; s, seconds; TLC, thin layer chromatography; HPLC, high performance (pressure) liquid chromatography; DART, Direct Analysis in Real Time; LCMS, liquid chromatography–mass spectrometry; HRMS, High Resolution Mass Spectrometry; ESI, electron

spray ionization; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); ATP, adenosine triphosphate; GTP, guanosine-5'-triphosphate.

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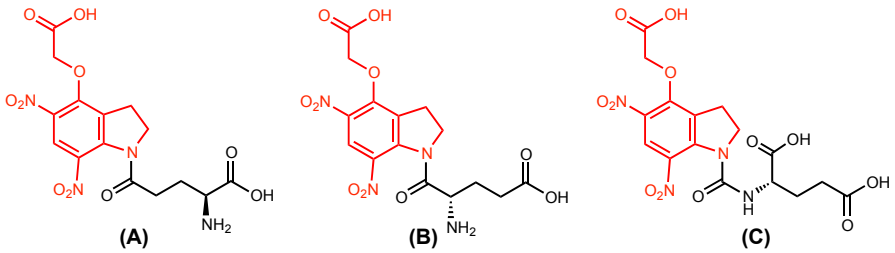
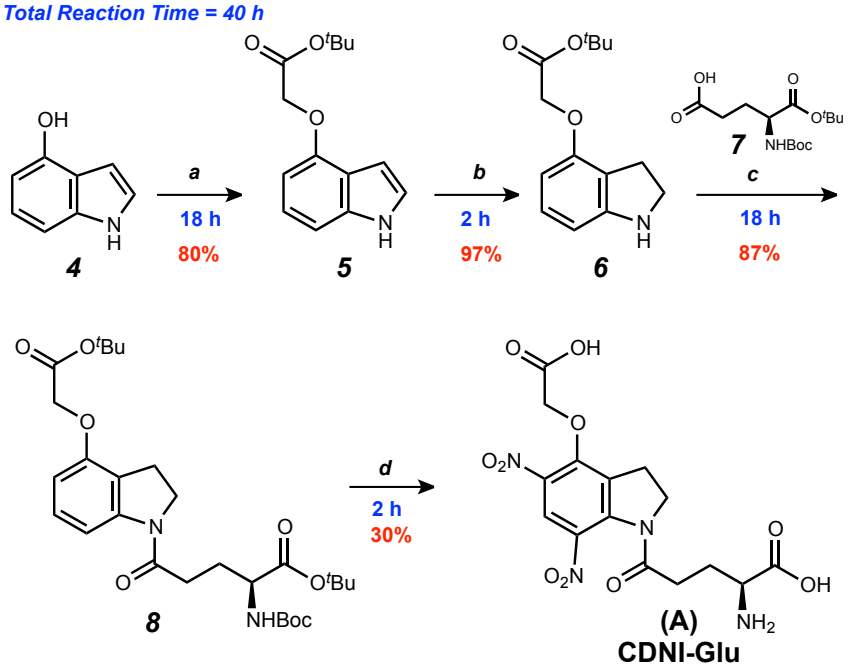
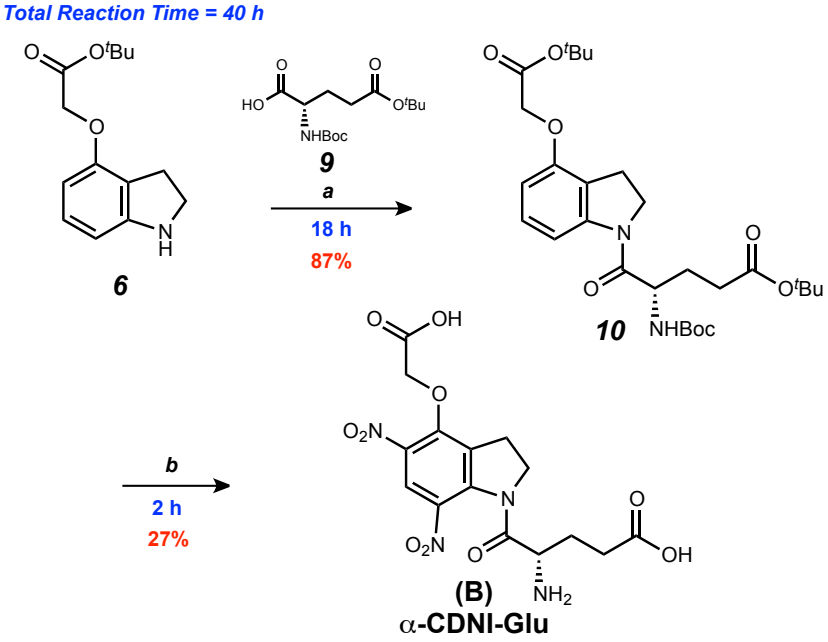
Figure 1.



Scheme 1.


Total Reaction Time = 40 h
OH
4
a
18 h
80%
O
O
tBu
O
N
H
5
b
2 h
97%
6
OH
O
O
tBu
7
NHBoc
c
18 h
87%
8
d
2 h
30%
O2N
NO2
(A)
CDNI-Glu
NH2

Scheme 2



Scheme 3.

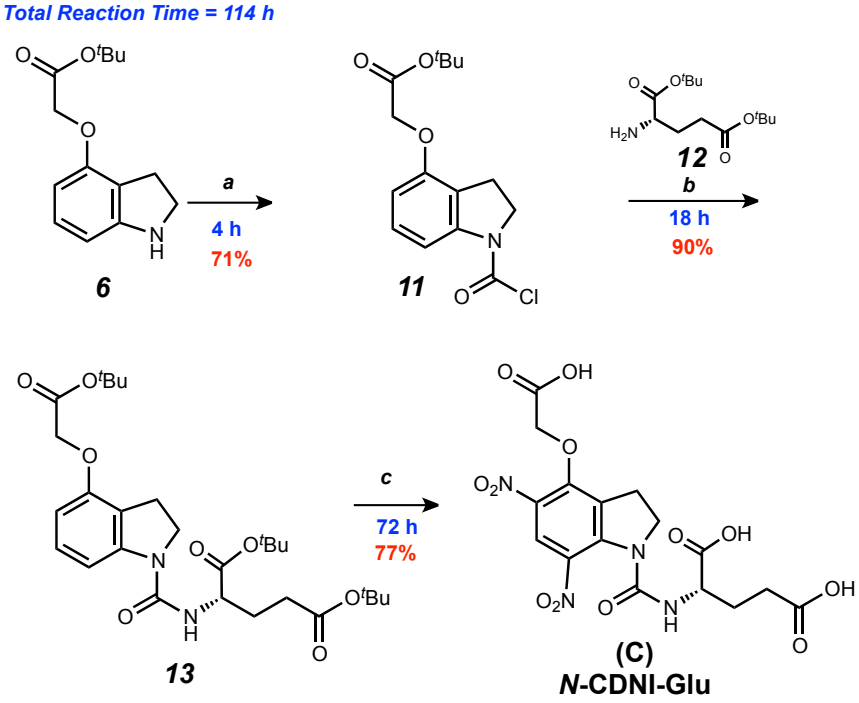

Total Reaction Time = 114 h
6
a
4 h
71%
11
12
b
18 h
90%
13
c
72 h
77%
(C)
N-CDNI-Glu

Figure 2.

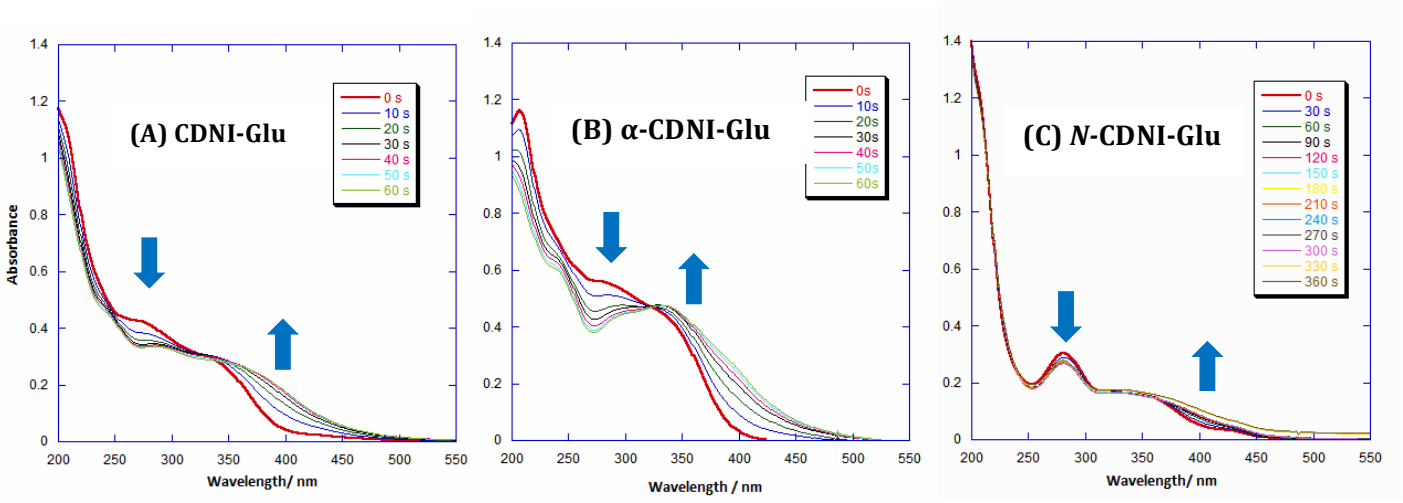

(A) CDNI-Glu
0 s
10 s
20 s
30 s
40 s
50 s
60 s
Absorbance
Wavelength/ nm
(B) α-CDNI-Glu
0s
10s
20s
30s
40s
50s
60s
Wavelength / nm
(C) N-CDNI-Glu
0 s
30 s
60 s
90 s
120 s
150 s
180 s
210 s
240 s
270 s
300 s
330 s
360 s
Wavelength/ nm

Figure 3.

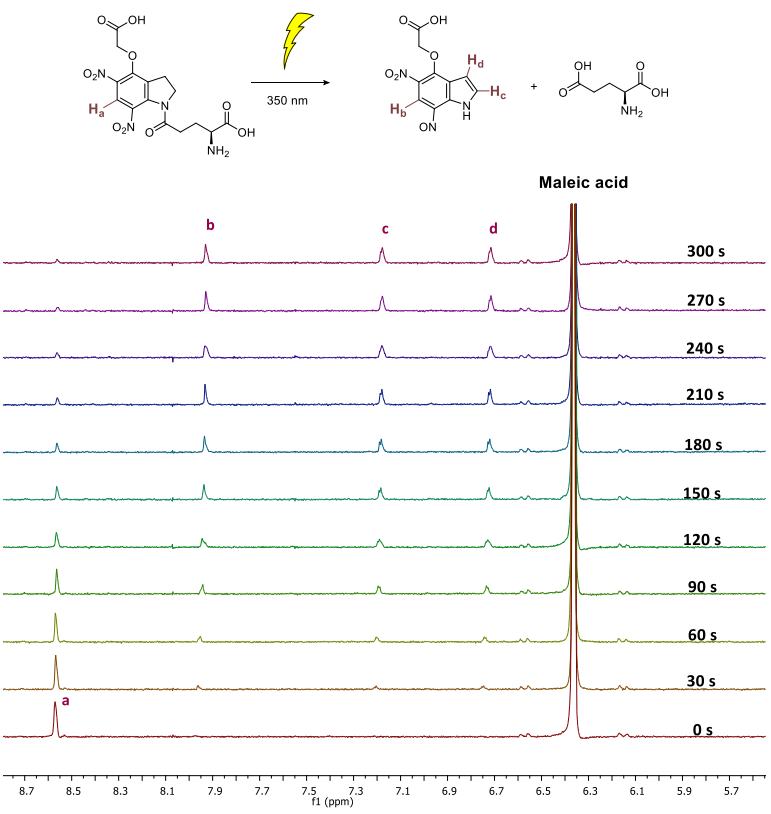

350 nm
Maleic acid
b
c
d
a
300 s
270 s
240 s
210 s
180 s
150 s
120 s
90 s
60 s
30 s
0 s
f1 (ppm)

Figure 4.

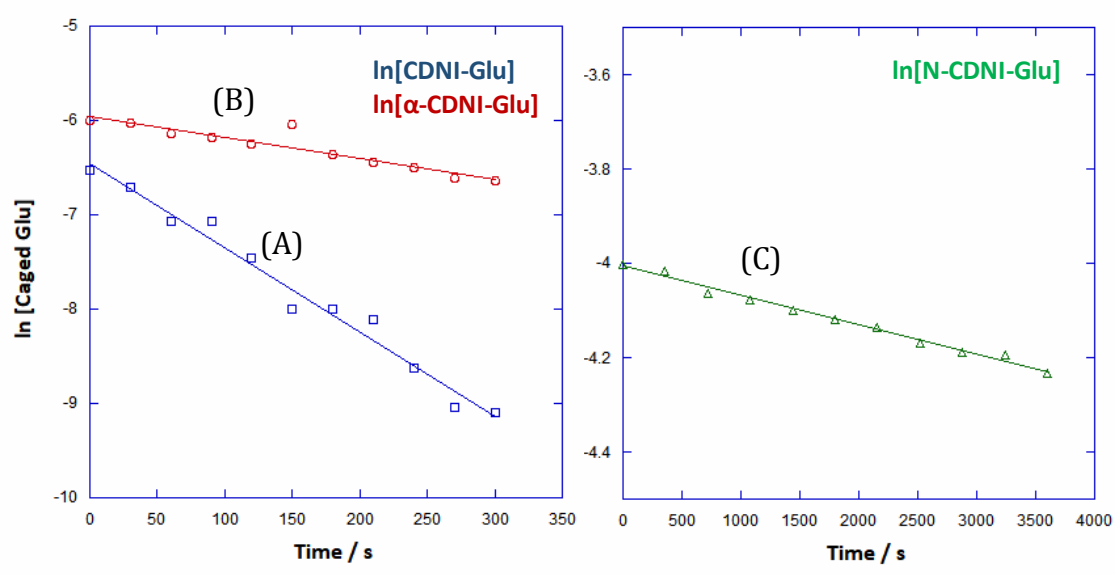
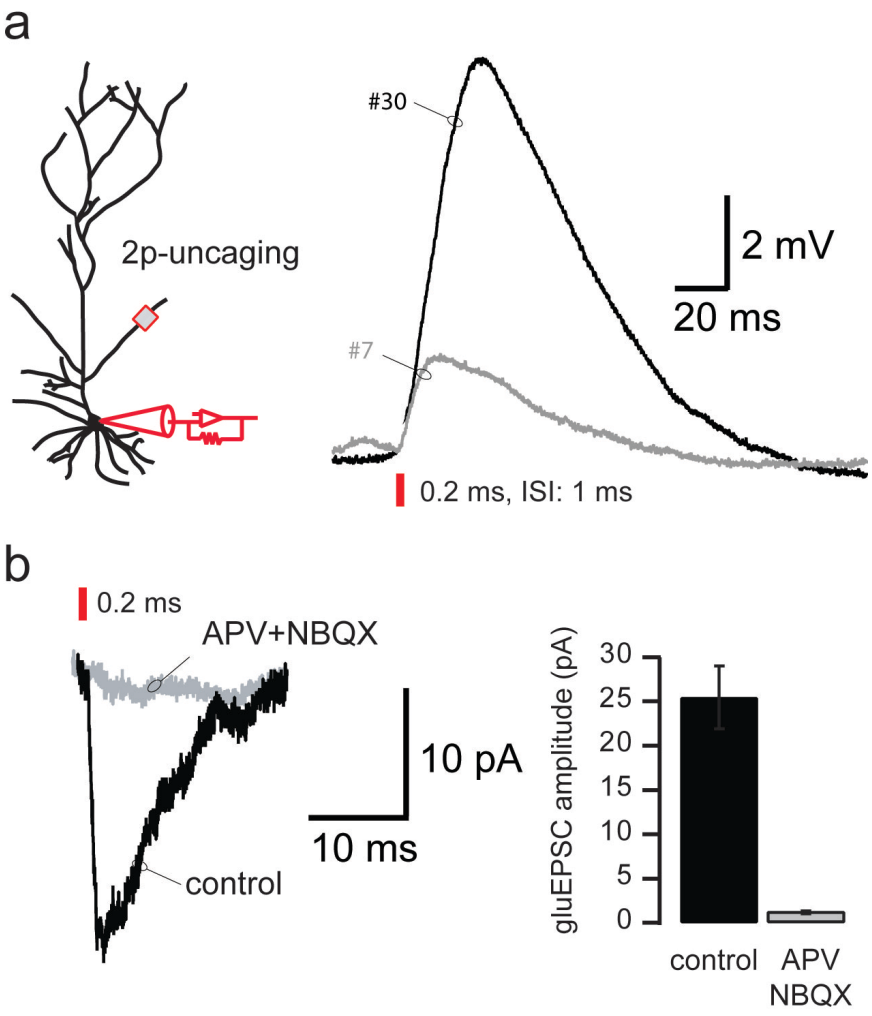

ln[CDNI-Glu]
ln[α-CDNI-Glu]
ln[N-CDNI-Glu]
(A)
(B)
(C)
ln [Caged Glu]
Time / s
Time / s

Table 1.

Caged Glu	k / s^{-1}	λ_{max} / nm	$\varepsilon(\lambda_{max})$ / $M^{-1}cm^{-1}$	Φ	$\varepsilon.\Phi$ / $M^{-1}cm^{-1}$
CDNI-Glu	8.9×10^{-3}	330	6400	0.5	3200
α-CDNI-Glu	2.2×10^{-3}	330	6400	0.13	832.0
***N*-CDNI-Glu**	6.2×10^{-5}	359	7478	0.003	22.43

Figure 5.


a
2p-uncaging
#30
#7
2 mV
20 ms
0.2 ms, ISI: 1 ms
b
0.2 ms
APV+NBQX
control
10 pA
10 ms
gluEPSC amplitude (pA)
30
25
20
15
10
5
0
control
APV
NBQX

Graphical abstract