Optical control of NMDA receptors with a diffusible photoswitch

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N-methyl-D-aspartate receptors (NMDARs) play a central role in synaptic plasticity, learning and memory, and are implicated in various neuronal disorders. We synthesized a diffusible photochromic glutamate analogue, azobenzene-triazole-glutamate (ATG), which is specific for NMDARs and functions as a photoswitchable agonist. ATG is inactive in its dark-adapted trans-isoform, but can be converted into its active cis-isoform using one-photon (near UV) or two-photon (740 nm) excitation. Irradiation with violet light photo-inactivates ATG within milliseconds, allowing agonist removal on the timescale of NMDAR deactivation. ATG is compatible with Ca^2+ imaging and can be used to optically mimic synaptic coincidence detection protocols. Thus, ATG can be used like traditional caged glutamate compounds, but with the added advantages of NMDAR specificity, low antagonism of GABAR-mediated currents, and precise temporal control of agonist delivery.

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Onotropic glutamate receptors mediate fast excitatory synaptic transmission and are ubiquitously expressed in the central nervous system. They can be separated into three major classes: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs), kainate receptors and N-methyl-d-aspartate receptors (NMDARs). The latter are involved in the induction of synaptic plasticity, the cellular correlate of learning and memory. NMDARs, of which a functional tetrameric receptor structure has been reported lately,2,3, have also been implicated in a variety of neurological diseases and dysfunctions including ischaemia-related cell death, Alzheimer’s, Huntington’s and Parkinson’s Diseases, as well as schizophrenia and autism spectrum disorders. NMDARs are heterotetramers whose subunit composition varies based upon brain region, maturation and synaptic activity. Subunit differences result in variations in receptor kinetics, which confer different computational properties on the receptors.4,5 Such variations in kinetic behaviour of NMDAR subunits have been examined on extrasynaptic and recombinant receptors using outside-out patch clamp recordings and fast application of agonists5,6.

The study of glutamate receptors in their native environment has been facilitated by the development of optical tools, namely light-sensitive caged agonists and antagonists, which take advantage of the temporal and spatial precision that light provides. Compounds including caged glutamate, caged NMDA and caged MK-801 have proven very useful for finely tuned, non-invasive studies of NMDARs7–11. For example, MNI-glutamate uncaging in a diffraction-limited laser illumination volume enables synaptic-like activation of both AMPARs and NMDARs.12–15. Larger illumination volumes can be used to quantify activation and desensitization kinetics of synaptic receptors.15 However, the study of deactivation of receptors must be done following rapid removal of agonist, a feat not possible with caged glutamate because of slow clearance by diffusion or uptake.15 This is particularly challenging in functional networks within brain tissue.

Molecular photoswitches provide an interesting alternative to caged compounds since they can be rapidly and repeatedly switched on and off and do not generate photochemical byproducts. Over the past decade, several photoswitches have been developed including both photoswitchable tethered ligands and freely diffusible photochromic ligands (PCL) that allow for the optical control of transmembrane receptors and, by extension, neural systems. GluAzo is a photochromic version of glutamate that functions as a PCL for the kainate receptors GluK1 and GluK2, and ATA-3 is a PCL selective for AMPARs.16 (Fig. 1a). Both GluAzo and ATA-3 are active in the thermally relaxed, dark-adapted state, thus their use with networks of neurons is challenging as the whole preparation must be illuminated to prevent tonic activity.

We now report a unique photochromic agonist that targets a different family of glutamate receptors. This compound, azobenzene-triazole-glutamate (ATG), complements ATA-3 and GluAzo since it selectively activates NMDARs.17,18 (Fig. 1b). In addition, it possesses an important functional advantage that distinguishes it from our previously developed PCLs: it is inactive in the dark-adapted trans-isomer, but quickly converts into its active cis-isomer when irradiated with ultra-violet (UV) light (370 nm). As such, it is not excitotoxic when applied to neural networks in the absence of light, and it becomes an agonist of NMDARs with millisecond precision when irradiated. In addition, we show that ATG is the first diffusible PCL that can be precisely controlled with two-photon excitation (740 nm).

Results
Synthesis and Photophysical Characterization of ATG. ATG was synthesized in a few steps from the known glutamate derivative 4(R)-propargyl glutamate (1) using click chemistry (Fig. 1c). In brief, 1 was treated with azobenzene azide 2 in the presence of a copper catalyst to afford triazole 3. Global deprotection then yielded ATG. A thermally stable stilbene analogue of cis-ATG, termed cis-STG, was synthesized in a similar fashion by treating 1 with 4 and deprotecting the resultant triazole 5 (Fig. 1d). Details of the synthesis and full characterization can be found in Supplementary Fig. 10, Supplementary Note 1 and in the Supplementary Methods.

ATG behaves as a regular azobenzene that can be converted to its cis-isomer with UV-A light. Conversion into the thermodynamically favourable trans-isomer requires irradiation with violet light (Fig. 1 and Supplementary Fig. 1a). Thermal relaxation into the trans-isomer is very slow in physiological buffer solution in accordance with the ‘regular azobenzene’ nature of ATG (Supplementary Fig. 1b).

Photopharmacology of ATG. To evaluate ATG as a photo-switchable agonist in neurons, we performed electrophysiological recordings in mouse layer 2/3 cortical neurons in acute coronal slices, while continuously perfusing 200 μM ATG in artificial cerebrospinal fluid (ACSF) (Fig. 2, Supplementary Fig. 2a,b). Using the whole-cell voltage-clamp configuration, we examined the spectral sensitivity of cis-ATG-evoked currents from 350 to 410 nm (Fig. 2a, Supplementary Fig. 2a). Maximal ATG-elicted currents were observed in response to 360 nm light, (3.75 mM s−2; Supplementary Fig. 2c), whereas above 390 nm cis-ATG-mediated currents were negligible (< −10 pA). This action spectrum corresponds to the maximal conversion to cis-ATG after 365 nm irradiation, as determined by UV–VIS spectroscopy (Supplementary Fig. 1a). Switching back to trans-ATG was fastest using 425 nm light (τ½ = 0.17 ± 0.03; n = 16, Fig. 2b and Supplementary Fig. 2b). According to these data, ATG is cis-active, which is in sharp contrast to other photoswitchable glutamate receptor agonists previously developed17,18. Using our illumination system, the dose–response curve of ATG indicated an EC50 value of 185 μM under 370 nm light (Fig. 2c). In whole cell current-clamp recordings of mouse cortical layer 2/3 neurons, ATG triggered action potential (AP) firing under 370 nm light (Fig. 2d). Using 420 nm light, AP-firing could be quickly silenced. Thus, ATG photoswitching can be used to control neural activity with light.

We examined cis-ATG-mediated currents in the presence of various antagonists of ionotropic glutamate receptors (iGluRs) to identify the molecular targets of ATG. Application of NBQX (25 μM), an AMPAR-selective antagonist, had no effect on light-evoked currents (Supplementary Fig. 3). By contrast, D-AP5 (40 μM), a competitive NMDAR-selective antagonist, and MK-801 (50 μM), a use-dependent pore blocker that preferentially acts on NMDARs, completely abolished cis-ATG-mediated AP firing and currents, respectively (Fig. 2e and Supplementary Fig. 3).19

Using heterologous expression strategies we next examined whether cis-ATG can activate NMDA receptors containing different isomers. Cis-ATG-mediated currents were not detectable in HEK cells expressing different subunit combinations. In Xenopus oocytes, however, the high level of receptor expression facilitated the detection of cis-ATG-mediated currents (Supplementary Fig. 4). To normalize for different expression levels we compared the steady-state amplitude of cis-ATG-mediated currents in oocytes expressing recombinant diheteromeric NMDARs with those evoked by saturating NMDA concentrations (1 mM, Supplementary Fig. 4). We found that photoactivation of ATG is able to activate all subunit combinations between GluN1-1a and either GluN2A, B, C or D. The observation that cis-ATG-mediated currents were smaller...
than those elicited by superfusion of NMDA (<5%), may be because of increased light absorption resulting in incomplete photoconversion in the oocyte setup.

To further demonstrate the selectivity of ATG for NMDARs, we recorded current–voltage (I–V) relationships comparing NMDA (200 μM) puff application with ATG photoswitching (Fig. 2f). Because of their magnesium sensitivity, NMDARs are partially blocked at resting membrane potentials, imparting a J-shaped I–V relationship20 (Fig. 2f, right, black), which we observed for both NMDA application and cis-ATG-mediated currents (Fig. 2f). As expected for non-selective cation channels, the reversal potential was close to 0 mV. In the absence of external Mg2+ the I–V relationship was found to be linear, as expected for NMDARs (Fig. 2f, right, blue).

Figure 1 | Design and synthesis of ATG. (a) Structures of GluAzo, a photochromic agonist of kainate receptors, and ATA, a photochromic agonist of AMPA receptors in their respective trans isomeric forms. (b) Structure and photophysical properties of ATG. The molecule consists of a photoswitchable azobenzene, a triazole and a glutamate moiety. The trans- and cis-configuration of ATG are shown. (c) Synthesis of the azobenzene ATG using click chemistry. (d) Synthesis of the stilbene cis-STG using click chemistry.
Figure 2 | Photopharmacology of ATG. (a) Action spectrum of ATG recorded in layer 2/3 cortical neurons in an acute slice preparation in presence of 200 μM ATG in ACSF. Current amplitude was measured after 5 s light stimulation with the respective wavelength and normalized to the maximal current amplitude at 360 nm. (b) Wavelength screening for τ_{off} kinetics of ATG-mediated currents between 400 and 560 nm light. Best τ_{off} kinetics were achieved at 400-450 nm light. (c) Dose–response relationship of ATG-mediated currents in cortical slice preparations. Concentrations from 1 to 500 μM were tested. The EC_{50} is 185 μM (black dashed line) and was calculated using the Hill-equation. (d) Current-clamp recording of a layer 2/3 cortical neuron. Irradiation with 370 nm light (purple) induces robust action potential firing that is terminated by irradiation with 420 nm light (blue). (e) Washing in D-AP-5 (40 μM), an NMDA-specific antagonist, blocks the ATG-mediated light-dependent action potential firing. (f) Current–voltage relationships indicative of NMDARs as targets for ATG. Black; current–voltage relationship of puff-applied NMDA (200 μM) currents (n = 12 cells). Red; current–voltage relationship of ATG-mediated currents under 370 nm light (n = 10 cells). Blue; current–voltage relationship of ATG-mediated currents in the absence of Mg^{2+} ions (n = 10 cells). Error bars indicate s.e.m.
We further examined whether the thermally stable cis-ATG analogue (cis-STG, Fig. 1d), which does not photoswitch, has similar pharmacology and specificity for NMDARs. When puff applied, the stilbene cis-STG indeed elicited APs in mouse layer 2/3 cortical neurons (Supplementary Fig. 5a). The J-shaped I-V relationship (Supplementary Fig. 5b) indicates that cis-STG also targets NMDARs. As such, cis-STG represents a new structural class of agonist for these receptors.

One drawback of traditional caged glutamate compounds is that they are often antagonists of GABA<sub>A</sub>R-mediated synaptic currents<sup>21</sup>. We therefore tested the effect of trans-ATG on GABA<sub>A</sub>R-mediated inhibitory postsynaptic currents (IPSCs) in hippocampal CA1 pyramidal neurons. Using 400 µM bath application of ATG, we observed no detectable alteration of spontaneous IPSCs, but observed a 38 ± 6% (<i>n</i> = 10 cells) block of evoked IPSCs and an increase in coefficient of variation, consistent with a presynaptic target (Supplementary Fig. 6). Nevertheless, this is less than the 50% block by RuBi-glutamate (300 µM<sup>21</sup>), the 55% block by CDNI-glutamate (400 µM<sup>22</sup>) and the 83% block by the commonly used MNI-glutamate (300 µM<sup>21</sup>).

ATG-mediated photoswitching of NMDAR gating. We next considered the possibility that ATG photoswitching could be used to perform temporally precise agonism of NMDARs. We used fast digitally controlled diode lasers at 375 nm to switch to cis-ATG and 405 nm to preferentially switch to trans-ATG (Fig. 3a). The 375 nm laser light was focused over a larger volume, eliciting a double exponential decay function. Nevertheless, this is less than the 50% block by RuBi-glutamate (300 µM<sup>21</sup>), the 55% block by CDNI-glutamate (400 µM<sup>22</sup>) and the 83% block by the commonly used MNI-glutamate (300 µM<sup>21</sup>).
Additionally, local application of caged compound MNI-glutamate at similar concentrations resulted in an enhanced fractional reduction of cis-ATG-mediated currents upon 405 nm light illumination (Fig. 3c and Supplementary Fig. 9b), up to 77 ± 3% reduction, 100 μM (n = 9 cells). Finally, and most importantly, local application of ATG resulted in faster decay of NMDAR currents following 405 nm illumination (ATGoff): the half-decay of 33 ± 4 ms (n = 9 cells; Fig. 3d) was over 12 times faster than NMDAR current decays recorded in response to MNI-glutamate uncaging (422 ± 72 ms, n = 5 cells, P = 0.01). Interestingly, the weighted decay time constant of ATGoff (τweighted of 102 ± 42 ms; n = 9 cells; see example fit in Fig. 3c inset) was intermediate to the decay values for recombinant receptors containing either GluN1/GluN2A (τweighted = 29 ms) and GluN1/GluN2B (τweighted = 193 ms)24.

Figure 4 | Comparison of ATG photoswitching responses between wild-type and GluN2A KO animals. (a) Population averages of light-evoked currents from WT CA1 pyramidal cells in response to 375 nm (100 ms) only, 375 nm followed by 405 nm (50 ms), and 405 nm only when locally applying ATG (100 μM) with a patch pipette (n = 9 cells). (b) Population averages of photoswitching currents from GluN2A KO animals under same conditions as (a) (n = 5 cells). (c) (left) Normalized currents in response to 375–405 nm photoswitching from (a) and (b) and population averages of NMDAR EPSCs in wild-type (n = 13 cells) and KO animals (n = 10 cells). Traces were aligned on their peaks and electrical artifacts from presynaptic stimulation have been blanked. Right: Bar graph of half-decays. Error bars indicate s.e.m. *P < 0.05 and NS indicates comparisons that are not significantly different (Steel Dwass all pairs nonparametric multiple comparison test).

Figure 5 | Localized two-photon activation of ATG. (a) Cis-ATG-mediated current evoked by two-photon illumination (1 ms, 740 nm) in a CA1 pyramidal cell while bath applying 400 μM ATG. (b) 2P-evoked cis-ATG-mediated currents with illumination spot parked at 0.5, 2 and 4 μm away from spine head. Illumination duration was 1 ms, and the wavelength set at 740 nm. (inset) Enlarged view of distance-dependent ATG evoked responses. Box over traces illustrates the time window over which spatial dependence was estimated for isochronal amplitude plots in (c) (Scale bar 2 μm). This was chosen to correspond to the time point at which the largest current reached 75% of its amplitude. (c) Normalized isochronal plots for six cells, with the average in black (half-width half-maximum = 2.0 μm, red dotted lines). Error bars indicate s.e.m.
We therefore considered the possibility that the rapid ATG$_{\text{off}}$ decay could be used to estimate relative contributions of GluN2A and GluN2B to dendritic NMDAR activation.

In mature CA1 pyramidal neurons (>P21), NMDARs are mostly comprised of tri-heteromers of GluN1, GluN2A and GluN2B$^{4,25}$ whose kinetics are dominated by the rapid deactivation of GluN2A$^{26,27}$. We therefore compared the decay of ATG$_{\text{off}}$ in WT and GluN2A KO mice. To get a better estimate of the ATG$_{\text{off}}$ kinetics without contamination of 405 nm-induced cis-ATG-mediated currents, we subtracted a scaled curve fit to the 405 nm-induced current of each individual cell (Fig. 4a,b). The half-decay of subtracted ATG$_{\text{off}}$ currents was $30 \pm 4\text{ ms} (n = 9\text{ cells})$ similar to the decay of NMDAR EPSCs recorded at the same age ($40 \pm 3\text{ ms}, n = 13\text{ cells}; P = 0.18$; Steel Dwass all pairs nonparametric multiple comparison test); but the evoked synaptic responses were nearly three times slower (KO half-decay: $31 \pm 5\text{ ms}, n = 5, P = 0.99$ Steel Dwass all pairs nonparametric multiple comparison test), confirming the kinetic influence of GluN2A expression. Thus, the ATG$_{\text{off}}$ decay is insensitive to subunit composition of NMDARs that are known to alter channel deactivation$^{28}$.

Two-photon activation of ATG. One strategy to achieve very localized photoactivation is to use two-photon (2P) excitation.

We found that, during bath application of 400 μM ATG, femtosecond pulsed-laser illumination at 725–740 nm evoked detectable cis-ATG-mediated currents when using illumination durations as brief as 250 μs, which is much more efficient than one-photon activation of ATG (Fig. 5a), and similar to illumination durations required to evoke NMDAR currents using MNI-glutamate$^{29–32}$. One millisecond duration pulses (740 nm) produced an average cis-ATG-mediated current of $–48 \pm 7\text{ pA}$ (rise time (10–90%) = $44 \pm 4\text{ ms}, n = 16\text{ spines}$), larger than the published values for single spine activation using caged-glutamate$^{29–31}$. Nevertheless, spatial dependence of the amplitude of 2P-evoked cis-ATG-mediated currents indicated a local activation within 2 μm of the spine head (Fig. 5b,c).

Combining ATG photoactivation with Ca$^{2+}$ imaging. The calcium permeability of NMDARs links their activity to postsynaptic biochemical alterations, such as spine morphology and glutamate receptor expression, which are associated with synaptic plasticity$^{20}$. Since ATG specifically acts on NMDARs, we examined the possibility of imaging its effect with a Ca$^{2+}$-sensitive fluorescent dye (Fig. 6). To this end, we incubated hippocampal slices with Quest Fluo-8-AM, a membrane-permeable Ca$^{2+}$ indicator that is compatible with the activation wavelength of ATG because of its excitation wavelength of 490 nm (Fig. 6b). We applied tetrodotoxin citrate (TTX) (1 μM) and felodipine (40 μM), which prevents opening of voltage-gated Ca$^{2+}$ channels to limit the response to NMDAR-mediated calcium entry (Fig. 6 right half). Indeed, upon irradiation with 370 nm in the presence of ATG,
we observed light-evoked cis-ATG-mediated Ca\(^{2+}\) transients (Fig. 6b; \(\Delta F/F = 20 \pm 3\%\), \(n = 18\)) which decreased in amplitude in the presence of blockers (\(\Delta F/F = 13 \pm 3\%\), \(n = 10\)). We attributed the remaining Ca\(^{2+}\) transient to the influx through ATG-mediated NMDAR opening.

**Mimicking synaptic coincidence detection with ATG.** One of the most intriguing properties of NMDARs is their voltage-sensitive Mg\(^{2+}\) block at resting membrane potential. Accordingly, the presence of a neurotransmitter and concomitant postsynaptic depolarization are required to activate the ion channel, rendering NMDARs coincidence detectors of pre- and postsynaptic activity\(^{33}\). To test whether cis-ATG-mediated NMDAR activation can replace the synaptic stimulation necessary for coincidence detection, we designed a stimulus protocol, which couples antidromic stimulation (electrical stimulus of the axon hillock; Fig. 7, black line) of the postsynaptic cell with light activation (Fig. 7, purple line) of the presynaptic cell 10 ms before, during and 10 ms after the light stimulation (Fig. 6b; 2P activation) and quickly deactivated with 405–460 nm light. Using rapid laser illumination, we demonstrated the unique photoswitching between a cis-active and trans-inactive compound, which enabled activation and deactivation of NMDARs on the timescale of their intrinsic gating properties. We also demonstrated that ATG is amenable to combination with other optical techniques, namely Ca\(^{2+}\) imaging. Finally, we showed that ATG could be used to mimic synaptic activation in coincidence detection protocols.

In the absence of detailed structural data and molecular dynamics calculations, it is difficult to explain why cis-ATG is the active form and why it is selective for NMDARs. Structure-activity relationship studies indicate that the pharmacological space available for ATG is relatively narrow, which makes it a challenge to develop red-shifted derivatives (D. Trauner, unpublished results). As the physiological activity of the stilbene analogue cis-STG indicates, the diazene unit (N=N bond) is not essential for ligand binding and is probably not engaged in hydrogen-bonding interactions to the ligand-binding domain. This is also apparent in a recent X-ray structure of GluAzo bound to the GluK2 ligand binding domain\(^{34}\). Attempts to red-shift the action spectrum of ATG by substituting it with strongly electron-donating substituents, such as a diethylamino group, in the 4’-position have so far yielded inactive compounds, indicating that substituents in this position clash with the ligand-binding domain in its closed state. Other ways to red-shift, for example, with heterocyclic azobenzenes, can be imagined and are under active investigation.

Finally, cis-ATG and cis-STG represent a new class of agonists (and potentially antagonists) for ionotropic glutamate receptors that can be rapidly assembled using click-chemistry. Herein, we show that ATG photoswitching and light-dependent NMDAR activation can be achieved with a monochromator, LEDs or laser light source. In principle, simple LEDs or standard light sources used in fluorescence imaging could also

**Figure 7 | Coincidence detection using ATG in layer 2/3 cortical neurons.** Coincidence detection of cis-ATG mediated current (200 \(\mu\)M) paired with antidromic stimulation. (a) Antidromic stimulation (black bars) of the postsynaptic cell 10 ms before, during and 10 ms after the light stimulation (purple trace). (b) As in (a), but with 50 ms intervals. (c) As in (a), but with 100 ms intervals. (d) Quantification of coincidence detection. Relative number of spikes compared with condition ZERO, when both stimuli were applied together (\(n = 11\) cells). Statistics were calculated using the Wilcoxon rank-sum test (*\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\)).
be used. It should be taken into account, however, that the kinetics of photoswitching are strongly dependent on the applied light intensities. ATG exhibits a high molar extinction coefficient (48,778 cm$^{-1}$M$^{-1}$ at 330 nm). Although such a feature enables efficient light absorption, it also reduces the light intensity at the focal point as a consequence of Lambert-Beer’s law. We circumvented this limitation by performing local perfusion of ATG to limit the optical path length in which photoswitching occurred. Two-photon excitation also provides a solution to the problem of out-of-focus light absorption while in parallel improving depth penetration in scattering tissue and providing intrinsic localization, properties well-suited for activation of single spines with a PCL. Deactivation at longer wavelengths can still be performed by large 1P illumination areas, which is advantageous for switching any molecules that have diffused out of the diffraction-limited illumination volume. Although the 2P cross-section has not been estimated, the ability of brief laser illumination durations (<1 ms) to evoke synaptic-like NMDAR current amplitudes could render ATG a useful complement to MNI-glutamate. Very recently, MAG$_{29}$, a derivative of glutamate that can be activated with its absorption, has been introduced. This compound, however, is covalently attached to the receptor and not freely diffusible like ATG.

Because diffusion-limited illumination volumes are much larger than a single synaptic vesicle fusion event, the photolysis of caged neurotransmitter in diffraction-limited spots (by one- or two-photon excitation) is unlikely to mimic the rapid decay of neurotransmitter clearance. PCL compounds, however, are ideally suited to overcome this diffusion-limited problem because photoisomerization of azobenzenes can occur on a picosecond timescale, sufficient for photo-inactivation of an agonist before it diffuses away. It remains to be determined if the switching speed is similar when cis-ATG is bound to the receptor. Nevertheless, we were able to demonstrate that ATG photoswitching can accelerate NMDAR current decays which, when using classical caged agonists, would have been limited by diffusional clearance. This requires that the inactive trans-ATG have an affinity low enough to be effectively a non-agonist. Consistent with the relative low-affinity of trans-ATG, the ATG$_{off}$ decays are nearly thirty times faster than the decay of cis-ATG currents. Trans-ATG also appears to unbind NMDARs more rapidly than glutamate, as the half-decay is over twelve times faster than that achieved by glutamate uncaging (Fig. 3). We demonstrate, for the first time, the fundamental ability to switch ‘off’ channel agonism, thereby speeding the decay of NMDAR currents.

We were surprised that the ATG$_{off}$ decay was not altered in GluN2A KO mice, especially since faster unbinding rates of glutamate are thought to underlie the faster deactivation rates of GluN2A-containing channels. One possible interpretation of these results is that the affinity of trans-ATG is so low that photoconversion reveals a ligand-independent closing transition. To confirm this, future experiments would be required, especially to confirm rapid switching of cis-ATG when bound to the NMDAR.

Our results show that ATG is a powerful tool that permits precise temporal control of NMDAR gating not otherwise achieved with state-of-the-art caged compounds. They extend the reach of photopharmacology to an important subtype of glutamate receptors and demonstrate that photoswitchable neurotransmitters that are inactive in the dark can be synthesized and used to precisely control receptor activation in their native environment.

**Methods**

**UV–Vis spectra.** ATG was dissolved to a concentration of 50 μM in buffer containing (in mM) 138 NaCl, 1.5 KCl, 2.5 CaCl$_2$, 1.2 MgCl$_2$, 10 Glucose and 5 HEPES, adjusted to pH 7.4. UV–VIS spectra were taken in a 100 μl cuvette with the switching light (monochromator) introduced through a glass fibre from the top of a 1 μm-diameter glass capillary (to avoid a back light path of the spectrometer close to the sample). The kinetics of the trans- to cis- conversion were recorded at the maximal absorption wavelength of trans-ATG (330 nm). To achieve fast switching rates, we used high power LEDs at 365 and 460 nm (Prizmatix) for trans-cis and cis-trans isomerization, respectively.

**Cortical slice preparation and external solutions.** Cortical coronal slices were prepared from C57Bl6/J mice (postnatal day 10–15, both male and female animals were used without known experimenter bias). Following decapitation, the brain was rapidly removed and transferred to an ice-cold saline solution composed of (in mM) 2.5 KCl, 1.25 Na$_2$HPO$_4$, 25 NaHCO$_3$, 0.5 CaCl$_2$, 7 MgCl$_2$, 25 glucose, 75 sucrose, and 2 D-serine (Abcam Biochemicals). In some experiments, slices were saturated with carbogen (95% O$_2$/5% CO$_2$). After incubation, slices were stored at room temperature from 30 min to five hours before being recorded. Experiments were carried out at room temperature. Unless stated otherwise, ATG was added from a 200 mM dimethyl sulfoxide stock to the ACSF to yield a final concentration of 200 μM. The ACSF was heated to 40°C to improve the solubility of the ATG stock. The solution was not filtered because ATG adheres to filter materials.

For the identification of target receptors and calcium imaging experiments, the iGlut antagonists NBQX (25 μM) and D-AP-5 (40 μM), and the channel blockers TTX (1 μM) and felodipine (40 μM) were bath-applied, whereas MK-801 (50 μM) (all from Abcam) was loaded into the patch pipette. NMDA (1 mM, Sigma-Alrich) and cis-STG (200 μM) were pumped through a glass pipette using a pressure ejection system (PDES, NPI Electronic). For voltage-clamp recordings, TTX was added to the ACSF.

**Patch clamp recordings of cortical layer 2/3 neurons.** Pyramidal neurons were patched using fire-polished glass electrodes with a resistance of 6–9 MΩ. Current-clamp recordings were carried out using the following intracellular solution (in mM): 140 K-glucuronate, 10 HEPES, 4 Mg-ATP, 0.4 Na$_2$-GTP. For whole-cell voltage-clamp recordings, we used (in mM) 110 Cs-glucuronate, 15 NaCl, 10 HEPES, 5 TEA, 0.16 EGTA, 4 Mg-ATP, 0.4 Na$_2$-GTP. Recordings were made with an EPC 10 USB amplifier, controlled by the PatchMaster software (HEKA). Data was filtered at 2.9–10 kHz and digitized at 50 kHz. Holding potential was corrected for a 14 mV liquid junction potential.

Cells were rejected if leak currents were > 200 pA or series resistance > 25 MΩ. Data was analysed using the Patch’s Power Tools (MPI Göttingen) and routines written in IgorPro (Wavemetrics).

For antidromic stimulation, glass electrodes (5 MΩ) filled with ACSF were placed within 20 μm of the axon hillock and the stimulus pulse was applied through an isolated stimulation unit (A-M Systems). The stimulation intensity was set to be subthreshold. The temporal pattern of the antidromic and ATG light stimuli were controlled through the Patchmaster software (HEKA).

**Hippocampal slice preparation and external solutions.** Hippocampal coronal slices were prepared from C57Bl6/J mice (Janvier Labs) (postnatal day 15–35, both male and female animals were used without known experimenter bias) following dorsalization and rapid removal of the brain. A Leica VT1200S vibratome was used to make 250 μm thick slices while the brain was immersed in an ice-cold saline solution composed of (in mM) 2.5 KCl, 1.25 Na$_2$HPO$_4$, 25 NaHCO$_3$, 0.5 CaCl$_2$, 8 MgCl$_2$, 25 glucose, 230 sucrose and 0.5 ascorbic acid saturated with 95% O$_2$/5% CO$_2$. After 30 min incubation at 33°C in solution composed of (in mM) 125 NaCl, 2.5 KCl, 1.25 Na$_2$HPO$_4$, 25 NaHCO$_3$, 2 CaCl$_2$, 1 MgCl$_2$, 25 glucose and 0.5 ascorbic acid saturated with 95% O$_2$/5% CO$_2$ slices were stored at room temperature from 30 min to 5 h in the same solution before being recorded. NMDAR currents were recorded in the presence of (in μM) 10 SR95531 (Abcam Biochemicals) to block GABARs, 0.3 strychnine (Sigma-Alrich) to block glycine receptors, 5 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo-quinoxaline-7-sulfonamide (NBQX) (Abcam Biochemicals) to block AMPARs, and 1 TTX (Abcam Biochemicals) to minimize spontaneous activity. 50 μM d-serine (Sigma-Alrich) was also included in the bath solution to saturate the co-agonist binding site of NMDAR GABAR currents were isolated through the addition of 0.3 M D-serine, 5 NBQX, 10 L-2-Amino-5-phosphono pentanoic acid (Abcam Biochemicals) and 20 7-chlorokynurenic acid (Abcam Biochemicals). ATG or 4-methoxy-7-nitroindol-caged-i-glutamate (MNI-glutamate; Tocris Bioscience) was perfused locally using a 3–6 μm tip diameter patch pipette. The ATG and MNI-glutamate perfusion solutions contained (in mM) 110 NaCl, 2.5 KCl, 1.25 Na$_2$HPO$_4$, 30 HEPS, 10 Glucose, 2 CaCl$_2$, 1 MgCl$_2$, 0.05 Alexa Fluor 488 (Life Technologies), 0.01 SR95531, 0.0003 strychnine, 0.005 NBQX, 0.001 TTX, 0.05 d-serine, and where noted, 0.05–0.1 μl, L-threo-β- Benzylxystospartic acid (Tocris Bioscience, Bristol, UK). Alexa Fluor 488 was used to visualize the perfusion and ensure its regularity over the course of the experiment. The pH of the final perfusion solution was adjusted to 7.3 after dilution. As ATG is not readily soluble in water, a stock solution (10 mM) was prepared in 0.1M NaOH.
Patch clamp recordings in hippocampal CA1 pyramidal neurons. Whole-cell voltage-clamp was performed from visually identified hippocampal CA1 pyramidal cells at room temperature using a patch clamp 700B, Molecular Devices. Current eliciting pulses were 500 ms duration with 50 ms intervals. For light stimulation, the recording chamber was illuminated with a 405 nm diode laser (Model PhoxX 405-120, Omicron Laserage) and a 375 nm pulsed Ti:Sapphire laser (Chameleon Ultra II, Coherent) tuned to 488 nm. One-photon illumination spots were typically 40 μm in diameter and 4–10 kHz. Holding potentials were corrected for a DC leak and a voltage shift due to the capacitive current of the seal (www.neuromatic.thinkrandom.com/). Offline, traces were filtered between 100 kHz (NI PCI-6052E, National Instruments) using the software Neuromatic in Barth’s solution supplemented with 100 μM glycine.

In vitro transcription and preparation of cRNA. For expression in Xenopus laevis oocytes, clones (derived from Rattus norvegicus) of GluN1-1a (genbank accession number: U08261), GluN2A (AF001423), GluN2B (U08259.1) and GluN2D (U08260.1), each in the PIP5, HEKA) and filled with 3 M KCl.

Statistical analyses requiring multiple comparisons were first examined with a two-sample t-test (independently). The coefficients of variation of the GluN2A and GluN2B IPSCs were compared using an independent samples t-test (independently). The coefficient of variation of the GluN2A IPSCs was compared using an independent samples t-test (independently). The coefficient of variation of the GluN2A IPSCs was compared using an independent samples t-test (independently).

Analysis of variance (ANOVA) was used to determine the significance of the differences between the groups. Statistical analysis was performed using the statistical software package GraphPad Prism (GraphPad Software). The data are presented as mean ± s.e.m. unless otherwise stated. Results were considered significant at *p < 0.05, **p < 0.01, and ***p < 0.001. Four independent experiments were performed. The data were analyzed using a two-tailed Student’s t-test.

The fits of each individual cell were scaled by eye to maximize the overlap of the decay component of 405 nm only and 375/405 nm-induced currents. We used such a scaling because the population traces overlapped (Fig. 4a,b). The scaled fits of each individual cell were scaled by eye to maximally overlay the slow component with a spin-column kit (Clean & Concentrator 25, Zymo) and cRNA integrity was determined photometrically with a NanoPhotometer (Implen) and the concentration of cRNA was estimated from a 200 μm window centered at the time point at which the on-spike response had reached 75% of its peak value.

To better estimate the NMDAR current decay following 405 nm illumination without contamination from partial cis-activation of ATG, we performed a subtraction protocol. In vitro 405 nm-induced currents were fit to an empirical function that describes the rising phase and dual exponential decay38.

$\frac{I(t)}{I_{peak}} = A_t \left( 1 - \exp\left(-\frac{t}{t_{rise}} \right) \right) \times A_s \exp\left(-\frac{t}{t_{decay}} \right) + (1 - A_t) \exp\left(-\frac{t}{t_{decay}} \right)$

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Additional information

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