Optofluidic control of rodent learning using cloaked caged glutamate

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Glutamate is the major excitatory neurotransmitter in the brain, and photochemical release of glutamate (or uncaging) is a chemical technique widely used by biologists to interrogate its physiology. A basic prerequisite of these optical probes is bio-inertness before photolysis. However, all caged glutamates are known to have strong antagonism toward receptors of γ-aminobutyric acid, the major inhibitory transmitter. We have developed a caged glutamate probe that is inert toward these receptors at concentrations that are effective for photolysis with violet light. Pharmacological tests in vitro revealed that attachment of a fifth-generation (G5) dendrimer (i.e., cloaking) to the widely used 4-methoxy-7-nitro-indolinyl(MNI)-Glu probe prevented such off-target effects while not changing the photochemical properties of MNI-Glu significantly. G5-MNI-Glu was used with optofluidic delivery to stimulate dopamine neurons of the ventral tegmental area of freely moving mice in a conditioned place-preference protocol so as to mediate Pavlovian conditioning.

optofluidics | GABA-A antagonism | biologically inert | caged glutamate | conditioned place-preference

Photochemical release of chemically protected biomolecules (i.e., uncaging) has been very widely used for control of cell signaling in vitro for more than 40 y (1–7). The reason for this is that optical and chemical technologies form a powerful pair to enable biological experimentation. Well-defined structure–activity relationships of biomolecules allow synthetic organic chemists to create biologically inert probes that respond to light delivery (1). Optical technology can deliver light to broad areas to envelope many cells or to focus and direct illumination to subcellular locations. Thus, molecules such as ATP, cAMP, IP_3, and Ca^{2+} have all been released inside cells, as well as neurotransmitters on cells, to enable many hundreds of studies of cellular physiology and biochemistry in cultured cells (1, 8).

In contrast, the delivery of light with chemical probes in vivo has proved challenging, thus limiting the use of photochemically responsive organic molecules in freely moving animals. Examples of uncaging ATP in drosophila (9) or photoswitching glutamate receptors in zebrafish (10) take advantage of the optical transparency of such species. However, applications of this approach to rodents has been restricted mainly to head-fixed animals (11–13). Recently, we have developed an optofluidic approach for control of rodent behavior with a synthetic photoswitch that circumvents many such limitations (14). Furthermore, other studies have described fabrication of novel head-mounted (15) or tethered optofluidic (16) systems for drug and light delivery in freely moving mice.

Glutamate uncaging has been used by neurophysiologists since it was first realized in complex brain tissue in 1993 (17). Since the first set of caged glutamate probes was developed by biochemists (18, 19), the idea that such molecules might have off-target interactions with other receptors such as those for γ-aminobutyric acid (GABA)-A has not been examined. Indeed, the first examples of second-generation nitroindolinyl-caged glutamate probes were reported to be nonantagonistic toward GABA-A receptors (20). Thus, it came as a real surprise that probes such as 4-methoxy-7-nitro-indolinyl(MNI)-Glu had such off-target antagonism (21). But since caged glutamates do not antagonize ionotropic glutamate receptors (20, 22), the off-target effects do not limit the use of such probes for the study of these receptors in vitro (23). However, when MNI-Glu was used in vivo, we found that coapplication of the Na channel blocker tetrodotoxin was required to block undesirable side effects from GABA-A receptor antagonism (13). While the idea of reducing antagonism of caged neurotransmitters by decoration with a high density of negative charge seems very reasonable (24), in practice it has been found the improvements are, at best, modest (25). Indeed, one negative charge is as (in)effective as four (26–28). These data suggested to us that a completely novel means of reducing GABA-A antagonism was required. So in 2017 we advanced the idea of enveloping the caged neurotransmitter with a dendrimer cloak to prevent probe binding to GABA-A receptors. A cloaked caged GABA was found to have ∼90-fold lower antagonism than its noncloaked analog (29). Here we describe the application of cloaking technology to caged glutamate. We show that at concentrations that are very effective for one-photon (1P) photolysis, a cloaked caged glutamate (**1**) is essentially inert and can be used for photorelease of glutamate in vitro and in vivo. Importantly, we show that the probe can be used with an optofluidic device (14) to control rodent learning in a conditioned place preference protocol.

Significance

Caged glutamates are photolabile compounds that are widely used by neuroscientists. However, these probes have off-target pharmacological side effects, in that they block inhibitory neurotransmitter receptors. We have developed a caged glutamate molecule decorated with a large dendrimer cloak that prevents such blockade. This photoprobe is an example of a caged glutamate that is fully biologically inert. We combine this compound with the newly developed technique of optofluidics, which allows us deliver the probe with simultaneous photolysis in freely moving mice. Uncaging in the brain region involved in the reward pathway mediated Pavlovian conditioning during a behavioral test. This work forms a useful paradigm for future experiments involving real-time phasic manipulation of the behavior of higher-order animals

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Results

The synthesis of **1** started with **2** (30), using the pendant carboxylate to attach an azide functionality via condensation aminopropylchloride to give **3**, followed by halogen replacement by reaction with sodium azide, to give **4** (Fig. 1). The nitro functionality was installed by reaction of **4** with Claycop to give fully protected caged glutamate **5**. This intermediate was then conjugated with the dendrimer. A fifth-generation (G5) polyester dendron with an alkyne focal point and a neutral hydroxyl periphery was used for copper(I)-catalyzed azide-alkyne click cycloaddition to give fully protected G5-MNI-Glu **6**, which could be purified using flash chromatography on silica gel. Brief treatment of **6** with trifluoacetic acid yielded the target cloaked caged glutamate compound, **1**, in essentially quantitative yield. Importantly, and unlike MNI-Glu, which requires HPLC for use with brain slices (22), this compound can be applied to slices for hours without any apparent toxic effects toward neurons.

Photolysis of 7-nitroindolinyl-protected acids has been well characterized, and is known to give the uncaged acid and nitrosoindole in essentially quantitative yields (31–34). The change in the UV-visible absorption spectrum of the aromatic chromophore correlates quantitatively with acid release (32) and can be used to follow the uncaging reaction, and thus measure the quantum yield of photolysis. Therefore, when individual solutions of either MNI-Glu or G5-MNI-Glu were irradiated at the reaction isosbestic point, using 365-nm light, the time-course of the change of the latter occurred at a rate that was 87% of the former (Fig. 1, box), corresponding to a quantum yield of photolysis of 0.057 [using the MNI-Glu quantum yield of 0.065 (35)]. Encouraged by these results, we tested the physiological efficacy of G5-MNI-Glu in vitro on neurons in acutely isolated brain slices.

Pyramidal neurons from the CA1 region of the hippocampus were filled with a fluorescent dye via a patch pipette, and imaged using 2-photon microscopy (Fig. 2*A*). This allowed us to direct a violet laser (410 nm) to the edge of the cell body, using galvanometer control. Local perfusion of a solution of G5-MNI-Glu (1 mM) from a pipette position just above the surface of the brain slice delivered the caged compound to the selected cell. Irradiation produced robust inward currents from three points that were highly reproducible in size (Fig. 2*B*). These currents are similar in size to those evoked by photolysis of MNI-Glu (bath applied at 0.66 mM) on the same microscope in a different study (36). Similar results were detected when voltages were recorded (Fig. 2*C*). Next we tested the lateral resolution from 1P laser uncaging on cells. When the laser was moved in 1-µm increments away from the cell body, similar to previous reports (21), the signals on single trails were barely detectable above noise at a distance of 4 to 5 µm (Fig. 2 *D* and *E*). Cellular responses were found to be graded with laser power in a linear manner, consistent with 1P uncaging (36, 37) (Fig. 2 *F* and *G*). In a final set of experiments using G5-MNI-Glu with CA1 neurons, we established uncaging at single spines performed in a similar manner to MNI-Glu (*SI Appendix*, Fig. S1). To prepare for use of G5-MNI-Glu in vivo, we also tested uncaging on midbrain dopamine neurons using full illumination with light from a 385-nm LED. For these experiments, the patch-clamped cell was

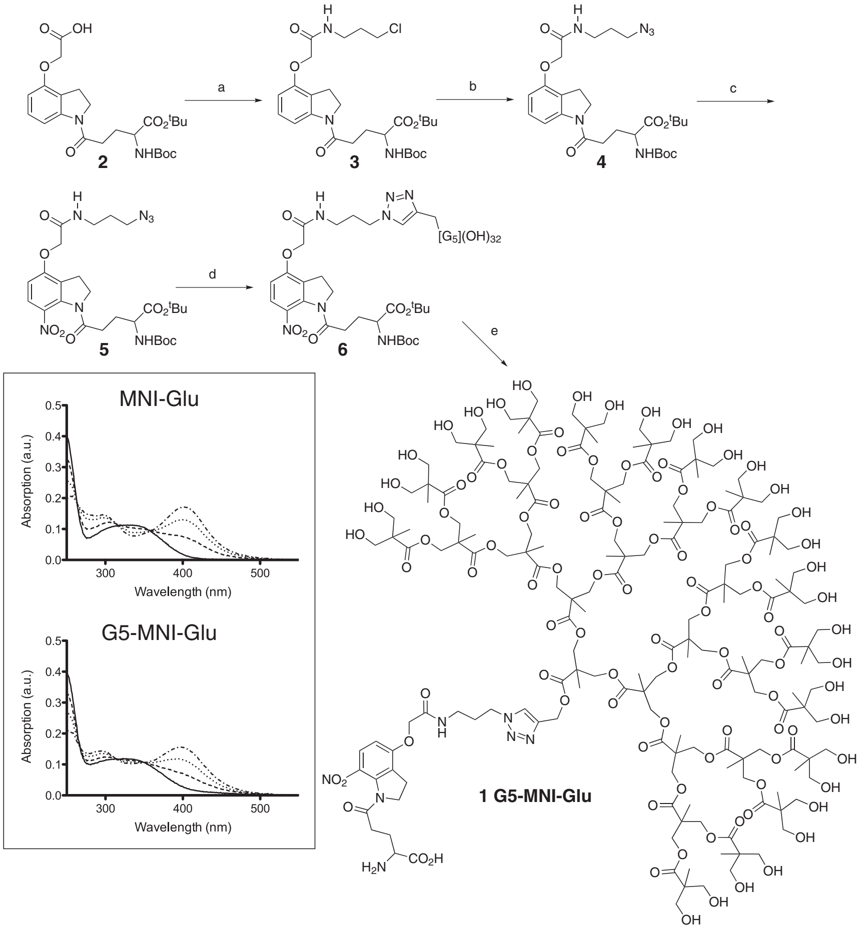

Fig. 1. Synthesis and photochemistry of G5-MNI-Glu. Reagents and conditions: a, 3-chloropropylamine, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; b, NaN_3; c, Claycop, $(AcO)_2O$; d, $Cu(II)SO_4$, Na ascorbate; e, trifluoroacetic acid. (*Inset*) Change in absorption spectra of MNI- and G5-MNI-Glu when irradiated at 365 nm.

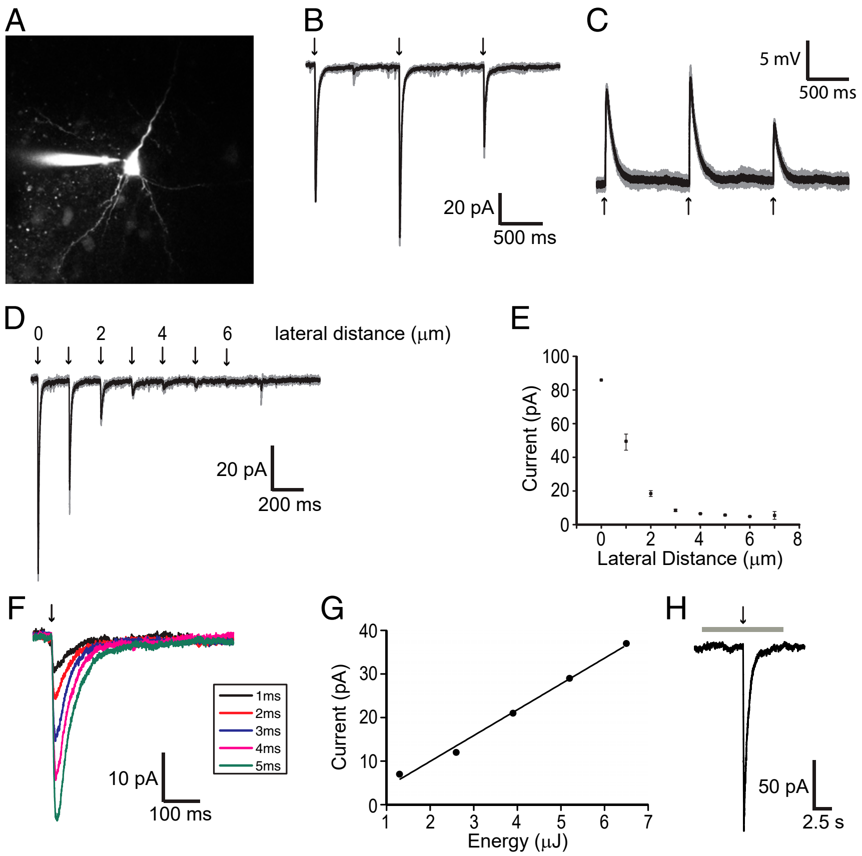

Fig. 2. Uncaging on cells in vitro. G5-MNI-Glu was locally perfused onto neurons in acutely isolated brain slices from glass pipettes positioned just above the slice surface. Arrows indicate the timing of using a 410-nm laser and 1.3-mW (*A*–*G*), or 385-nm LED (*H*). (*A*) Two-photon fluorescent image of a patch-clamped CA1 neuron filled with Alex594. (*B*) Currents evoked from CA1 neurons from laser irradiation (5 ms) at three points around the soma. (*C*) Changes in membrane potential evoked under the same conditions as *B*. (*D* and *E*) Currents evoked by moving the laser focus laterally with 1-μm steps. (*F*) Photo-evoked currents produced by increasing the uncaging period. (*G*) Evoked current increased linearly with energy ($r^2 = 0.99$). (*H*) Current evoked by photolysis (full-field LED illumination) on a VTA dopamine neuron. The gray bar illustrates the perfusion period.

positioned in the middle of the field of view, and light was delivered through the epi-fluorescent port of the microscope. Photolysis again produced an inward current (Fig. 2*H*).

Having shown previously that the unsubstituted G5-OH dendrimer has no detectable antagonism in the mM range (29), we used the effect on miniature inhibitory postsynaptic currents (mIPSCs) as means to test the GABA-A receptor antagonism of G5-MNI-Glu. The probe was bath-applied to brain slices in four successively increasing concentrations (0.25, 0.50, 0.75, and 1.0 mM), followed by washout of the recording chamber. The mIPSCs from CA1 neurons were recorded in the presence of tetrodotoxin (to block action potentials), APV (to block NMDA receptors), and CNQX (to block non-NMDA receptors). Over the entire concentration range, there was no significant reduction in the amplitude of the mIPSCs (Fig. 3*A*; Student t test, $P > 0.05$; $n = 3$ cells), suggesting no antagonism of GABA-A receptors. Overall, there was no significant reduction in the frequency of the mIPSCs either (Fig. 3*B*; Student t test, $P > 0.05$; $n = 3$ cells), except at 0.25 mM, there was a slight increase (Fig. 3*C*; Student t test, $P = 0.0098$; $n = 3$ cells). Fig. 3*C* shows representative recordings of mIPSCs at the beginning and end of one such experiment, with mIPSCs from the highest [G5-MNI-Glu] tested shown. These data suggested to us that G5-MNI-Glu could be useful for glutamate uncaging in experiments in which the balance of excitation and inhibition is important; for example, in freely behaving animals. Thus, we tested the probe during Pavlovian conditioning, using optofluidic uncaging in the ventral tegmental area (VTA), a midbrain region with dopaminergic nuclei critically involved in reward-related behaviors.

Stimulation of dopamine neurons in the VTA is critical for behavioral learning in conditioned place preference tests (38, 39). Classical methods such as electrical stimulation or infusion of drugs of abuse have been used to induce reinforcement. Thus, a simple conditioned place preference test is a very robust means to assay whether uncaging of glutamate can cause increase in firing of dopamine neurons in vivo so as to encode learning or experience. Using the stereotaxic coordinates for the VTA (*SI Appendix*), we positioned unilaterally optofluidic devices on 11 mice. The cannulas were then lowered carefully to be above the VTA so as to allow drug and light delivery without mechanically disturbing the brain region itself (Fig. 4*A*). Two to three weeks after surgical implantation, mice were subjected to a conditioned place-preference protocol in which they were allowed to explore two chambers for a 900-s period on day 1 (pretest). Each environment is novel and neutral in this behavioral test; however, the walls and floors are distinct, and can be distinguished subsequently. On days 2 to 4, mice were placed on each side for 600 s, and **1** was infused continuously at 2.5 mM during these periods (pairing; i.e., the conditioning period). However, only on one side was light applied (390 nm, 10 mW, 200 ms, 2 Hz). Note that we have previously shown that there is a fivefold dilution of caged glutamate during uncaging experiments in vivo (13), implying that there was ~0.5 mM G5-MNI-Glu available for uncaging in the mouse brain. On day 5 (test), both chambers are available for

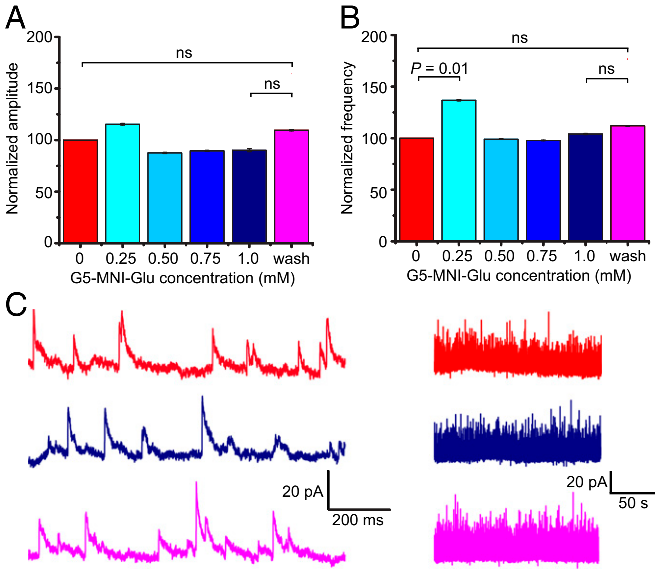

Fig. 3. G5-MNI-Glu is nonantagonistic against GABA-A receptors. Miniature inhibitory postsynaptic currents (mIPSCs) from CA1 neurons were recorded in presence of 0, 0.25, 0.50, 0.75, and 1.0 mM G5-MNI-Glu. The probe was applied in a circulating solution (10 mL) bathing each brain slice. Cells were monitored for 20 min, after an initial period of 4 min. The final wash with normal buffer lasted 20 min. (*A* and *B*) Normalized amplitude and frequency for each concentration (n = 3 cells). (*C*) Representative mIPSCs recordings with [G5-MNI-Glu] = 0 (red) and 1 mM (dark blue), followed by washout (i.e., 0 mM, pink).

exploration, and the residence of the mouse recorded (Fig. 4*C*). For the pairing days, a light guide and tube were connected to the cannula (Fig. 4*A*), but not during the pretest and test days. The residence times for each mouse on pretest day 1 was compared with that on test day 5 (Fig. 4*D*). Overall, we found there was a significant increase in residency time in the chamber associated with uncaging compared with the other chamber for the 11 mice tested, indicating behavioral conditioning (Fig. 4*D*; $P > 0.05$, paired, nonparametric Wilcoxon rank test). Since G5-MNI-Glu is inert up to 1 mM (Fig. 3), and the neuronal response from uncaging is linear (Fig. 2), our data imply one could use a wide variety of energies and concentrations to achieve similar effects.

Discussion

With the recent introduction of single-piece, highly flexible implantable optofluidic probes (16), or even more sophisticated entirely head-mounted devices for wireless photopharmacology (15), the need for photoactivatable drugs will increase for such devices to be used widely. Such probes will enable precise phasic delivery of drugs via optofluidics in a manner that complements the tonic delivery normally realized by slow infusion into the rodent brain. To test the feasibility of the development of such caged compounds for in vivo use, we selected glutamate as the drug test bed. First, the pharmacological concentration demand for glutamate is very high, as the target receptors have very low affinities for the neurotransmitter (ca. 10 to 100 micromolar), and our caged glutamate probe was effective in the 0.25 to 1.0 millimolar range (Fig. 2). Second, caged glutamate probes have well known off-target side effects (21, 40), providing scope for testing our cloaking method for reduction of such. The cloaked caged compound, G5-MNI-Glu, showed no GABA-A receptor antagonism up to 1 mM (Fig. 3). Third, in principle, uncaging glutamate allowed the probe to be used in a well-defined behavioral assay, one that could be validated by comparison with other temporally well-defined methods. In vivo uncaging of glutamate on dopamine neurons in the VTA produced a robust behavioral effect (Fig. 4), one that equaled other well-established approaches (14, 39). Our data suggest that cloaked caged compounds might provide one general method for delivery of caged prodrugs with optofluidics. In particular, we could imagine caged receptor-specific antagonists using such technology. Often such drugs are quite lipophilic, and the addition of aromatic caging chromophores only exacerbates this problem. Another benefit of cloaking is that dendrimers are highly biocompatible and soluble in physiological buffer (41, 42). Photopharmacology is a topic of intense current interest (43–48); our work is an example of using optofluidic delivery of a caged compound in freely moving rodents, and thus describes a useful paradigm for this type of experiment with future caged probes that will allow phasic manipulation of the behavior of higher order animals.

Methods

Chemical Synthesis and Photochemistry. See *SI Appendix* online for full synthetic procedures. The quantum yield for photolysis of **1** was measured by comparison with the rate of photolysis with MNI-Glu. The rate of change of absorption of 0.1-mM solutions of MNI-Glu and **1** in Hepes buffer (40 mM at pH 7.4, 100 mM KCl) were measured in a 1-cm cuvette during photolysis with a 365-nm LED (Thorslabs).

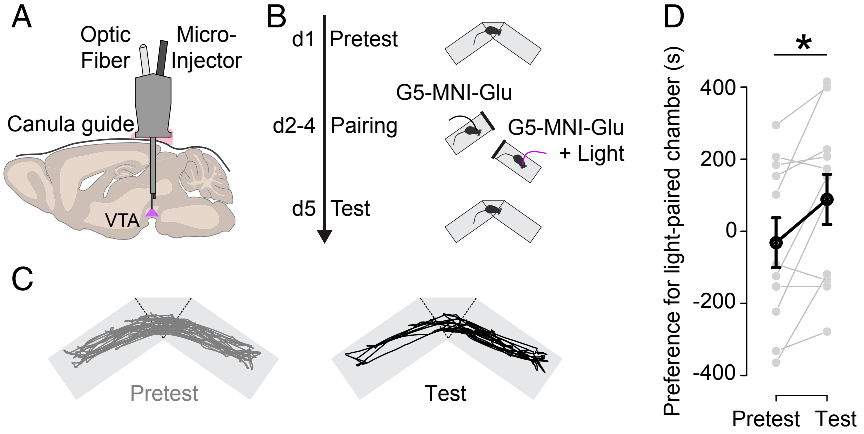

Fig. 4. Optofluidic uncaging in freely moving mice. (*A*) Cartoon of optofluidic device in the mouse brain. (*B*) Outline of the conditioned-place preference protocol: pretest on day 1 (d1), when implanted mice explore both chambers freely for 900 s; pairing (d2 to d4), when G5-MNI-Glu was infused with or without light; and test (d5), when mice were allowed to explore the reconnected chambers freely for 900 s (no infusion, no light). (*C*) Representative trajectories of a mouse before conditioning (pretest) and after conditioning (test). (*D*) Average (black) of the preference for the light-paired chamber (G5-MNI-Glu + light) before (pretest) and after (test) after pairing. Individual mice (11) shown in gray.

Physiology. All animal experiments were approved and performed at Mount Sinai according to guidelines from the NIH (49) and according to the recommendations issued by the European Commission directives 219/1990, 220/1990, and 2010/63, and approved by Sorbonne Université. Brain slices were prepared acutely from C57BL/6J mice, as described in ref. 50. Brain slices were transferred to the recording chamber and perfused with carbogenated artificial cerebral spinal fluid at RT. Whole-cell recordings were made from hippocampal CA1 pyramidal or midbrain dopamine neurons. Patch pipettes with a resistance of 3 to 5 MΩ were filled with different internal solutions. For uncaging experiments, cells were patch-clamped at −60 mV. For mIPSC recordings, cells were clamped at +10 mV and recorded in the presence of 1 μM tetrodotoxin, 10 μM CNQX, and 50 μM D-AP5 applied via the perfusion system, and analyzed in LabVIEW (National Instruments).

Laser uncaging on CA1 neurons was performed on an Olympus BX61 microscope fitted with a Prairie Technologies Ultima dual-galvo scan head and Vision II Ti:Sapphire laser, a continuous-wave 410-nm laser, and an EPC10 amplifier. Dopamine neurons were characterized in current clamp mode, as described (14). Whole-cell recordings were performed using an Axoclamp 200B amplifier. LED uncaging used full-field illumination through the epifluorescence pathway (36) with a 385-nm LED (100 ms, pE-2, CoolLED), with a light output of 6.5 mW, corresponding to 5 mW/mm^2 at the focal plane.

Mice (C57BL/6JRj) were implanted unilaterally with a chronic multiple-fluid/optical injector (Doric Lenses Inc.) at coordinates (from bregma, in millimeters): AP = 3.1 to 3.3; ML = 0.5 to 0.6; DV = 4. The guides (length = 4 mm from skull surface; OD = 450 to 650 μm) combined a fluid injection needle (protruding to 4.5 mm from skull surface) for delivering G5-MNI-Glu and an optic fiber (200 μm core, NA = 0.66, protruding to 4.3 mm from skull surface) for light delivery coupled to a ceramic ferrule (1.25 mm). Between experiments, a plug was used to close the cannula and seal the implant. The implant was attached to the skull with dental cement (SuperBond, Sun Medical).

The conditioned place preference apparatus used a Y-maze (Imetronic, France) with one closed arm and two other arms with manually operated doors. Two rectangular chambers (11 × 25 cm) with different cues (texture and color) were separated by a central neutral triangular compartment (side of 11 cm). One pairing compartment had gray textured floor and walls, and the other smooth black and white striped walls and floor. See *SI Appendix* online for a detailed description of the full physiological procedures.

The data that support the findings of this study are available at http://doi.org/10.5281/zenodo.3689250.

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