Stimulating neurons with light

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Recent technological advances have enabled the use of different optical methods to activate neurons, including 'caged' glutamate, photoactivation of genetically engineered cascades, and direct two-photon excitation. The ability to use light as a stimulation tool provides, in principle, a non-invasive method for the temporally and spatially precise activation of any neuron or any part of a neuron. When combined with two-photon excitation, excellent spatial control can be achieved even in complex and highly scattering preparations, such as living nervous tissue. Different methods that have been developed in the last several decades have been used to probe neuronal sensitivity, mimic synaptic input, and elucidate patterns of neural connectivity.

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Abbreviations

AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
Bhc-glutamate	*N*-(6-bromo-7-hydroxycoumarin-4-ylmethoxycarbonyl)-L-glutamate
CNB-glutamate	L-glutamic acid γ-(α-carboxy-2-nitrobenzyl) ester
MNI-glutamate	4-methoxy-7-nitroindolinyl glutamate
NMDA	*N*-methyl-D-aspartate

Introduction

Experimental investigations into neuronal function require the ability to manipulate neuronal activity. In most cases, this has been accomplished by electrically stimulating neurons, using electrodes inserted into the tissue of interest. This method has a long tradition going back to Galvani's electrical stimulation of frog nerves. Although very useful, using electrodes to stimulate is cumbersome, provides only crude spatial resolution, requires mechanical stability of the electrodes, does not allow sequential stimulation at multiple closely spaced sites, and can result in substantial mechanical damage to the tissue. In addition, electrical stimulation activates not only neurons with somata at the stimulation site, but also axons of passage; stimulation at multiple sites requires implantation of multiple electrodes, which induces extensive tissue damage.

All of these problems can be solved by stimulating neurons with light. Light can be easily focused with fine resolution, it can be delivered quickly, and it is relatively harmless. It is not surprising then, that neuroscientists have devoted considerable effort to the development of optical stimulation methods and, in particular, to the use of optical methods to 'uncage' glutamate, because this neurotransmitter is able to excite most mammalian neurons. The basic approach is to convert inactive 'caged' glutamate to active glutamate with ultraviolet light. This method has been used to mimic synaptic input and to map the glutamate sensitivity of the dendritic arbors of single cells (Figure 1b). Glutamate uncaging can also be used to generate action potentials with fine spatial resolution in small populations of neurons in brain slices; by combining this method with intracellular recording, it is possible to map the locations of neurons connected to a single cell (Figure 1a). Connectivity can also be investigated by direct photoactivation of action potentials in neurons. Finally, neurons can be made sensitive to light by using genetic methods to express photosensitive proteins. Here, we review the use of both single-photon and two-photon optical methods to stimulate neurons in order to unravel neural circuits, mimic synaptic connections, and investigate glutamate sensitivity.

Early development of optical stimulation methods

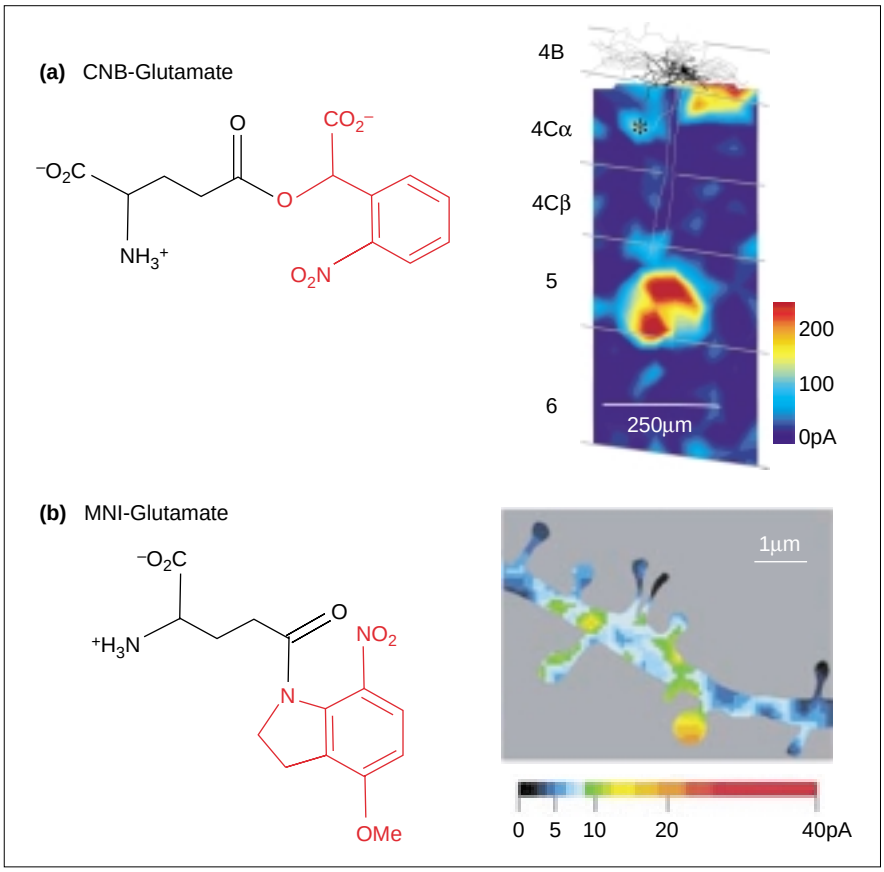
The invention of lasers enabled the development of the first optical stimulation methods. In 1971, Fork [1] focused laser light on *Aplysia* abdominal ganglion neurons and reported that the cells were depolarized to action potential threshold by the illumination. Although the mechanism of the depolarization was not characterized, this effect was reversible and the neuron returned to a resting membrane potential. In this pioneering study, Fork proposed the systematic use of optical methods to unravel neural circuits.

Farber and Grinvald [2] continued these efforts in 1983, by using a fluorescent dye to stain the membrane of leech neurons. They then used a laser to excite the dye, which, presumably through the production of oxygen free radicals at the membrane, produced a reversible depolarization and generated action potentials in the neuron. They applied this method to sequentially activate multiple neurons while recording from a single cell, thus mapping the sources of input to the recorded neuron.

Excitation with caged glutamate – single-photon

During the last decade, investigators have developed light-sensitive bioactive compounds, typically referred to as caged compounds [3] (see also [4]). These compounds are molecules that are rendered inactive by the addition of chemical groups, typically nitrobenzyl groups (Figure 1a), which are broken up by the absorption of light. Because virtually every neuron in the mammalian central nervous system can be stimulated by glutamate, caged glutamate is as an ideal compound for conferring light sensitivity to neurons and has been the focus of most research efforts. Here, we discuss stimulation of glutamate receptors using caged glutamate; we divide the studies into one-photon and two-photon uncaging

Figure 1



Structure and use of caged glutamate compounds. **(a)** Chemical structure and example of the use of CNB-glutamate in mapping connections to single cortical neurons. **(b)** Chemical structure and example of the use of MNI-glutamate in mapping the glutamate sensitivity of dendrites. For both CNB-glutamate and MNI-glutamate, caging groups are indicated in red and glutamate in black. The right panel of (a) shows the locations of neurons that make functional excitatory connections to a single inhibitory neuron located in layer 4B of a monkey primary visual cortex brain slice. Ultraviolet laser light was focused onto discrete sites in the brain slice, causing uncaging of CNB-glutamate and generation of action potential in neurons with somata near the stimulation site. The resulting synaptic currents, indicative of connections from neurons at the stimulation site to the intracellularly recorded layer 4B inhibitory neuron, were measured. The pseudocoloring of the input map is indicative of the magnitude of the measured synaptic currents, observed following photostimulation at each site, as indicated by the colored scale bar. The smoothed map is based on linear interpolation of the values measured following stimulation at discrete sites. This neuron received strong excitatory input from neurons in cortical layers 4Cα and 5, but only weak input from layers 4Cβ and 6. An anatomical reconstruction of the recorded neuron is also overlaid on the input map. Gray lines indicate laminar boundaries and the laminar identities are indicated to the left of the map. Reproduced with permission from [21], © American Association for the Advancement of Science, 2001. The right panel in (b) shows a high-resolution map of the glutamate sensitivity of a dendrite. Two-photon excitation was used to uncage MNI-glutamate while measuring the resulting inward currents at the neuron's cell body. This method resulted in an incredibly high-resolution view of the dendritic sites that were most sensitive to glutamate. Even the responses of single dendritic spines could be resolved. The pseudocoloring over the dendritic outline corresponds to the magnitude of the inward currents measured, as indicated by the colored scale bar below. Reproduced with permission from [40••], © Nature Publishing Group, 2001.

sections. Due to our focus, we do not discuss optical inactivation methods or uncaging of agents other than glutamate.

Mimicking synaptic input and optical mapping of receptors

Several studies have used caged glutamate to stimulate different parts of the dendritic tree. They followed the large body of work carried out with glutamate iontophoresis. These types of experiments are important because they can provide both a method to systematically probe the dendritic tree, and a way of mimicking the function of the presynaptic terminal. They thus serve as a method to explore the contribution of the presynaptic terminal to synaptic function and plasticity. At the same time, however, these experiments suffer from the unwanted uncaging that occurs above and below the focal point (see below) and the potential contamination of the results by the stimulation of non-synaptic receptors.

One of the first experiments uncaging glutamate on dendrites was done by Kandler *et al.* [5] on CA1 pyramidal neurons in slices. They reported the long-term depression of glutamate responses after the pairing of uncaging with depolarization of the neuron. A similar result was obtained with bursts of uncaging by Dodt *et al.* [6]; these authors used an infrared-guided laser stimulation system to uncage glutamate on the apical dendrites of layer 5 neocortical pyramidal neurons. The same group also used glutamate uncaging to perform a mapping study of *N*-methyl-D-aspartate (NMDA) versus α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors on these dendrites; they reported that receptors were differentially distributed [7] and that hot spots of receptor activation existed along the dendritic tree [8].

In these same neuronal types, Schiller *et al.* [9] paired glutamate uncaging with backpropagating action potentials and reported the activation of NMDA receptors and subsequent calcium influx after pairing. Also, they subsequently reported the triggering of local dendritic spikes after uncaging [10]. The dendritic spikes were spatially

restricted to the activated area of the dendrite and were interpreted as arising from an NMDA receptor-mediated regenerative effect. Finally, in the cerebellum, Wang *et al.* used caged glutamate to mimic synaptic inputs and to map the spatial extent of long-term depression [11•]. Single-photon uncaging of glutamate therefore proves to be a useful method for mimicking synaptic input and probing neuronal sensitivity without the complications introduced by activation of the presynaptic terminal.

Mapping circuits with caged glutamate photostimulation

'Photostimulation' of action potentials in candidate presynaptic neurons by uncaging glutamate, combined with intracellular recordings from single neurons, has been an important technological advance in the study of cortical circuits. This method has been used most extensively to investigate interlaminar connectivity in the cerebral cortex [12–17,18••,20–22,23••,24] (for an example, see Figure 1a). In addition, photostimulation has been used to map connections in the superior colliculus [25], the suprachiasmatic nucleus [26], the hippocampus [27,28], and the song system in birds [29]. Along with other complementary methods, such as intracellular dye filling, simultaneous intracellular recording and stimulation of multiple neurons [30], and optical measures of activity [4,31], photostimulation has contributed importantly to an increasingly detailed understanding of functional connectivity.

Photostimulation is typically used to identify the sources of functional input to single identified neurons in living brain slices. To map functional connectivity, caged glutamate is used to elicit action potentials in candidate presynaptic neurons while intracellular recordings are made from a postsynaptic neuron. The successful implementation of this method requires that sufficient glutamate uncaging is achieved to generate action potentials; however, the population of neurons generating action potentials should be restricted to sites close to the focused light pulse. Thus, activation of distant neurons whose dendrites pass through the region of glutamate uncaging must be contained to subthreshold levels. Several papers have reported results from control experiments demonstrating the ability to achieve adequate spatial resolution with caged glutamate-based photostimulation (e.g. [18••,23••]).

Although photostimulation with caged glutamate has been very valuable for mapping neural circuits, it has some limitations. Most notably, action potentials are generated in all neuron types near the stimulation site and their timing is not tightly controlled (c.f. [18••,23••]). Because different cell types at any given location are likely to differ in their connections to candidate postsynaptic cells, methods that allow more selective stimulation would be useful. The potential to overcome this limitation by using two-photon excitation to activate single cells [32•] or by using genetic methods [33••], along with cell-type-specific promoters, is considered below. Two-photon methods may also provide greater temporal resolution, allowing the study of synaptic dynamics with an optical stimulation method. Caged glutamate-based photostimulation is also likely to be limited to *in vitro* studies, because delivery of the caged compound *in vivo* would likely be problematic. However, two-photon based excitation, either directly or based on genetic methods, may also overcome this limitation.

Two-photon excitation

A problem with optical stimulation methods that use traditional (one-photon) light sources is that the light is scattered by living tissue and the distribution of energy, and therefore the uncaging region, is not very precise. For example, the light from a tightly focused, diffraction limited (0.2 μm) spot at the surface of a brain slice diffuses in the slice such that the lateral spread will become comparable to the depth beneath the surface (D Kleinfeld, personal communication). Even though the light is focused at the focal point, a similar energy stimulates (and uncages) molecules below and above the focal point.

The invention of two-photon microscopy [34] provided a solution to these inherent limitations of traditional fluorescence excitation. In a two-photon excitation, two infrared photons are simultaneously absorbed by the fluorophore or caged compound and produce an excited state similar to that from the absorption of a single photon of twice their energy [35]. Due to the nonlinear nature of the excitation, the space where the reaction occurs is essentially limited to the focal volume [34]. As an added bonus, because the original photons are of low-energy (infrared), they pass through the tissue without any ability to uncage fluorophores or produce appreciable photodamage. Thus, two-photon excitation is a veritable 'magic wand' that only stimulates the focal volume; it appears to be the ideal tool for spatial control of photochemistry and uncaging experiments [34], because the only region of space where the concentration of photons is high enough to sustain photoexcitation or uncaging is the focal point. Therefore, the spatial resolution of the uncaging is, in principle, as good as the focal point (<1 μm^3 in most two-photon experiments).

Two-photon microscopy relies on the simultaneous absorption of two photons to achieve the nonlinear excitation. An alternative 'chemical two-photon' method was developed to uncage compounds with high spatial precision [36]. The idea was to attach two caging groups to glutamate, as opposed to one. Therefore, for the glutamate to be active, the molecule needs to sequentially absorb two photons. The authors reasoned that, because the probability of absorbing one photon increases at the focal point, the requirement for subsequent absorption of the second photon prevents the uncaging of glutamate above and below the focal point. In practice, this method has been used to obtain good spatial resolution in brain slice experiments [11•].

Mapping sensitivity with two-photon glutamate uncaging

One of the earliest uses of two-photon excitation to uncage compounds was in a study done by Denk [37] on cultured

neurons in the presence of caged carbamoylcholine. He used whole-cell recording of the currents elicited by systematically focusing the laser, and thus uncaging neurotransmitter, over the cultured neuron to obtain a functional map of the distribution of cholinergic receptors along its membrane. This experiment used a random selection of stimulated pixels to prevent artifactual activation or desensitization of receptors during sequential stimulation of neighboring sites. Since this study, the use of two-photon uncaging of glutamate has been attempted by many groups, including our own, with mixed success. The major problem is that the traditional uncaging groups, such as nitrobenzyls, are designed for one-photon uncaging. This, together with the very small focal volume of the two-photon excitation, makes sufficient stimulation of glutamate receptors difficult for clear detection of the currents over noise.

This situation has changed with the design of newer caged glutamate compounds. Furuta *et al.* [38•] designed a bromo-hydroxycoumarin-caged compound that they refer to as Bhc-glutamate (*N*-(6-bromo-7-hydroxycoumarin-4-ylmethoxycarbonyl)-L-glutamate). Bhc-glutamate is more than an order of magnitude more sensitive than carboxy-nitrobenzyl caged glutamate (CNB-glutamate; L-glutamic acid γ-(α-carboxy-2-nitrobenzyl) ester) [38•]. The synthesis of Bhc-glutamate allowed the creation of three-dimensional maps of the glutamate sensitivity of pyramidal neurons in brain slices.

More recently, a methoxy derivative of nitroindolino-glutamate (MNI-glutamate; 4-methoxy-7-nitroindolinyl glutamate) has been developed [39,40••] (see also G Ellis-Davies in [4]). Matsuzaki *et al.* [40••] used two-photon uncaging of MNI-glutamate to map glutamate receptors along dendrites of CA1 pyramidal neurons. The spatial resolution of the activation using two-photon excitation in these experiments was superb, even in brain slices, enabling the authors to not only map receptors in different dendritic spines, but also estimate the number of AMPA receptors per spine using optical fluctuation analysis. These methods are likely to lead the way in the use of caged neurotransmitters to functionally probe the dendritic tree.

Direct two-photon stimulation

Using a different approach, a recent study has reported the direct activation of neurons by a two-photon laser source [32•]. As a two-photon version of the Fork experiment, focusing a femtosecond laser on the membrane of neocortical neurons in slices depolarizes them and causes them to fire. This occurs in the absence of any dye and has a nonlinear relation with the average power of the illumination, a hallmark of a two-photon or three-photon reaction. There appear to be at least two different mechanisms involved in this effect. Whereas lower intensity, longer illumination produces a slow depolarization mediated by oxygen free radicals (and presumably subsequent inactivation of potassium channels), high intensity, shorter illumination produces very fast depolarization through a mechanism that is unknown but may be related to the creation of membrane microholes. By combining this method with intracellular recording, it may be possible to rapidly probe functional connectivity between numerous cell pairs and identify single neurons that connect to the recorded cell. Connected cell pairs could then be targeted for simultaneous intracellular recording and characterization of synaptic properties, and subsequent dye-filling and staining would also allow detailed anatomical characterization. These methods might also work *in vivo*.

Genetic methods for photostimulation

Finally, another novel approach for increasing the sensitivity of neurons to light is to express a genetically engineered photoactivatable sensor in them [33••]. Zemelman *et al.* [33••] coexpressed arrestin, rhodopsin and a subunit of a G-protein in cultured hippocampal neurons, to artificially recreate the *Drosophila* phototransduction cascade and sensitize neurons to light. The depolarization of the cells occurs, presumably, through the opening of cation channels by a poorly understood mechanism.

Although several improvements are likely to be necessary to obtain photostimulation with good temporal resolution, there are major advantages to this type of genetic method. Most notably, it should be possible to limit photostimulation to selected subsets of neurons by restricting gene expression with cell-type-specific promoters. Also, because this method does not require the delivery of caged compounds, it is likely to be useful for photostimulation *in vivo*, particularly when combined with two-photon excitation to reduce light scattering and improve spatial resolution.

Conclusions and future directions

Overall, we are very optimistic about the future use of optical stimulation methods. In the last few years, the relatively few studies performed with caged glutamate have already demonstrated that these can be powerful methods to probe dendritic function and to map circuits. In addition, we feel that the 'optical revolution' is only starting and novel optical methods using either novel caging groups, genetically based probes, or two-photon excitation, as well as the combination of optical stimulation with imaging methods to monitor neuronal activity, are likely to have a major impact in neuroscience.

Acknowledgements

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