Caged compounds for multichromic optical interrogation of neural systems

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Abstract

Caged compounds have widely used by neurophysiologists to study many aspects of cellular signaling in glia and neurons. Biologically inert before irradiation, they can be loaded into cells via patch pipette or topically applied in situ to a defined concentration, photolysis releases the caged compound in a very rapid and spatially defined way. Since caged compounds are exogenous optical probes, they include not only natural products such neurotransmitters, calcium and IP$_3$, but non-natural products such as fluorophores, drugs and antibodies. In this Technical Spotlight we provide a short introduction to the uncaging technique by discussing the nitroaromatic caging chromophores most widely used in such experiments (e.g. CNB[1], DMNB, MNI and CDNI). We show that recently developed caging chromophores (RuBi and DEAC450) that are photolyzed with blue light (ca. 430–480 nm range) can be combined with traditional nitroaromatic caged compounds to enable two-color optical probing of neuronal function. For example, one-photon uncaging of either RuBi-GABA or DEAC450-GABA with a 473-nm laser is facile, and can block non-linear currents (dendritic spikes or action potentials) evoked by two-photon uncaging of CDNI-Glu at 720 nm. We also show that two-photon uncaging of DEAC450-Glu and CDNI-GABA at 900 and 720 nm, respectively, can be used to fire and block action potentials. Our experiments illustrate that recently developed chromophores have taken uncaging out of the "monochrome era", in which it has existed since 1978, so as to enable multichromic interrogation of neuronal function with single synapse precision.

Keywords

uncaging; two-photon; multichromic; dendritic spikes; spines

Caged compounds are organic molecules that are biologically or functionally inert until they are activated by light(Ellis-Davies, 2007). These optical probes are similar to pro-peptides found in cells or pro-drugs made by medicinal chemists in that their activity is latent because of covalent modification of some functionality that is essential for activity. Thermal chemical reactions are used to liberate peptides and drugs from their precursors; the distinctive feature of caged compounds is that light compatible with normal microscopes is used the break the covalent bond (Fig. 1A). The fact that light easily penetrates cell membranes and brain tissue is advantageous as uncaging can evoke rapid responses from single cells in a complex biological environment (Fig. 1B). Since almost all of the biochemistry of cells is mediated by organic molecules, caged compounds have been used to manipulate the concentration of a wide variety of such signaling molecules. Importantly, uncaging can be used for molecules that are not natural products, thus drugs and fluorophores have been caged.

Most published studies use caged compounds with nitroaromatic chromophores that are activated by light in the 340–410 nm range (Adams & Tsien, 1993; Ellis-Davies, 2008; Klan *et al.*, 2013). Despite the huge success of these probes many other chromophores have been used to cage biological signaling molecules(Klan *et al.*, 2013). An interesting feature of some of these molecules is the extension of the absorption spectrum of uncaging into the blue range (440–500 nm), one that is chromatically complementary to traditional nitroaromatic chromophores. This is proving to be an important addition to the optical arsenal available to neurophysiology, as some recent studies have featured wavelength-selective uncaging of two biological signaling molecules(Lovett-Barron *et al.*, 2012; Chiu *et al.*, 2013; Hayama *et al.*, 2013). In this technical spotlight we discuss the chemical probes that enable such studies. We provide an introduction to uncaging chromophore design and the basics of uncaging for neurophysiological studies. We illustrate these with examples of experiments using whole-cell, single-spine and multi-spine uncaging of neurotransmitters such as glutamate and GABA. Using multi-spine uncaging of glutamate in one optical channel we then show some of the capabilities of two-color uncaging combining either two-photon and one-photon excitation or dual two-photon excitation. Our goals are to provide a clear picture of the advantages and disadvantages of caged compounds for activating neurons and glia, and introduce readers to the new method of two-color uncaging for neuroscience.

Introduction to caged compounds and chromophore design

Chromophores for caged compounds were initially developed by organic chemists for use as photochemical protecting groups in the synthesis of small organic molecules. Thus, the *ortho*-nitrobenzyl(Barltrop *et al.*, 1966) and the dimethoxy- *ortho*-nitrobenzyl(Patchornik *et al.*, 1970) photochemical protecting groups were widely used(Binkley & Flechtner; Amit *et al.*, 1974). With the invention of caged compounds (Kaplan *et al.*, 1978) these chromphores became widely used by physiologists for most important signaling molecules such as IP$_3$(Walker *et al.*, 1987), cGMP(Nargeot *et al.*, 1983), calcium(Tsien & Zucker, 1986), neurotransmitters(Milburn *et al.*, 1989), peptides(Walker *et al.*, 1998), lipids(Xia *et al.*, 1997), proteins(Marriott, 1994), RNA(Chaulk & MacMillan, 1998), and DNA(Monroe *et al.*, 1999). Subsequently, novel photochemical protecting groups (or caging chromophores)

tended to be developed by biologists(Furuta *et al.*, 1995; Hagen *et al.*, 2001) seeking alternatives to the original nitrobenzyl caging chromophores. The increasing usefulness of caged probes for biology then stimulated chemists to re-enter the area(Zayat *et al.*, 2003; Smirnova *et al.*, 2005; Momotake *et al.*, 2006; Specht *et al.*, 2006; Gug *et al.*, 2008). However, the nitrobenzyl protecting groups are still the most important for organic synthesis(McGall & Christians, 2002) and remain widely used in physiology(Ellis-Davies, 2007; 2008).

The *ortho*-nitrobenzyl cages(Barltrop *et al.*, 1966; Patchornik *et al.*, 1970) and related nitroaromatic chromophores, MNI (Papageorgiou & Corrie, 2000) and CDNI(Ellis-Davies *et al.*, 2007), absorb light quite well in the 300–380 nm range. The absorption tail of the nitroindolinyl chromophore extends beyond 400 nm, so violet lasers, that are a common part of confocal microscopes, can be used to uncage these probes(Trigo *et al.*, 2009a). Until recently, it has proved difficult to extend the absorption spectrum of such nitroaromatic cages beyond this range(Aujard *et al.*, 2006). Importantly, Specht and co-workers have found that conjugation of an aminophenyl group to the 4-*ortho*-nitrobenyl chromophore (ANBP) shifts the absorption maximum to ca. 400 nm with the tail extending up to 500 nm(Donato *et al.*, 2012). A similar absorption maximum (385 nm) has been reported for the coumarin-based caging chromophore, DEAC(Hagen *et al.*, 2001). An even larger red shift in the absorption maximum has been reported by Etchenique for the "RuBi" chromophore(Zayat *et al.*, 2003; Zayat *et al.*, 2006). Recently, we have developed a new red-shifted coumarin cage, DEAC450, which also absorbs in a similar region(Olson *et al.*, 2013a). Thus, there is now a variety of caging chromophores and caged compounds with different photochemical properties available for neurophysiological studies. However, only some of these chromophores allow two-color uncaging and we discuss the design considerations for these in the next section.

Chromophore requirements for two-color uncaging

The requirements for wavelength-independent uncaging of two biological signaling molecules are probably obvious to anyone who has used multi-color fluorescence imaging. Not only must each chromophore have a distinct absorption maximum, but there must also be distinct absorption *minima* for each chromophore so as to prevent optical cross talk when imaging at different wavelengths of light. The same basic constraint applies to caging chromophores. The ideal situation is that each chromophore absorbs light entirely independently of the other (Fig. 2A). It is almost always possible to have a region of unique absorption in the redder part of the absorption spectrum for the longer wavelength caging chromophore (open arrow, Fig. 2B). However in the shorter wavelength range, the ideal is that the longer wavelength chromophore absorption approaches zero as in Fig. 2A. This stipulation is much harder to satisfy completely (we are aware of only one published example (Scott *et al.*, 2009)), as there is normally significant absorption of both chromophores in this region (filled arrow, Fig. 2B). Many recent studies by chemists and biologists have illustrated this overlap problem(Kotzur *et al.*, 2009; Goguen *et al.*, 2011; Menge & Heckel, 2011; Priestman *et al.*, 2011; Schaäfer *et al.*, 2011; Priestman *et al.*, 2012; Rodrigues-Correia *et al.*, 2013). All these reports show that to use two caged molecules *in approximately equal concentrations*, one must first photolyze the longer wavelength cage

completely before photolyzing the short wavelength cage (Box 1). The reason for this is that because there is significant optical cross talk between the two channels, attempts to photolyze the shorter wavelength cage would give co-liberation of both compounds, leading to confounding co-stimulation of two processes. This is analogous to what can be observed in fluorescence imaging with yellow channel emission from blue channel excitation. Interestingly, the absorption spectra of ChR2(Nagel *et al.*, 2003) and Halo(Duschl *et al.*, 1990) have considerable overlap (Fig. 2D), and initial report of bidirectional control of membrane potentials(Han & Boyden, 2007; Zhang *et al.*, 2007) have not been followed by more studies using the probes as partners probably because of the overlap.

Box 1

Two versions of wavelength-selective two-color uncaging

(A) Absorption spectra of nitroaromatic (purple), DEAC (blue) and DEAC450 (red) caging chromophores. Note that microscope glass is normally opaque below 330 nm. (B) Irradiation of a mixture of nitroaromatic- and DEAC-caged compounds with λ_1 causes uncaging of both cages, whereas irradiation with λ_2 enables wavelength-selective photolysis of the DEAC caged compound. Typically, experiments with such chromophore mixtures feature *essentially complete* photolysis of the DEAC-cage (i.e. the "long wavelength") with λ_2 before applying λ_1 to the cells. (C) Irradiation of a mixture of nitroaromatic- and DEAC450-caged compounds with λ_1 or λ_3 produces wavelength-selective uncaging of either caged compound, no matter which wavelength of light is used first. Thus λ_1 and λ_3 can be applied in an *arbitrary order* such that photolysis with λ_1 can bracket photolysis with λ_3, see (Olson et al., 2013a) and experiments herein.

Recently we have developed a new caging chromophore, called DEAC450, that is the first caging chromophore made to address this problem(Olson *et al.*, 2013a; Olson *et al.*, 2013b; Amatrudo *et al.*, 2014). DEAC450, as the name implies, has a large absorption maximum in the 450–460 nm range, importantly it also has a distinct absorption minimum in the 340–360 nm range (black trace, Fig 2C). Independently of our own work, Jullien's group recently reported similar findings with another DEAC-based chromophore, 4-thio-DEAC(Fournier *et al.*, 2013). Using DEAC450-caged Glu(Olson *et al.*, 2013b), cAMP(Olson *et al.*, 2013a) and GABA(Amatrudo *et al.*, 2014)we have established that the DEAC450 chromophore may be used for optically independent uncaging at longer wavelengths of light when partnered with the appropriate nitroaromatic caged compound, using one- and two-photon excitation, so as to modulate membrane potential in a bi-directional manner (see below).

One and two-photon uncaging of neurotransmitters on cells and single spines

Most studies of LTP use electrical stimulation of afferent axons onto target neurons to induce the measured change in synaptic efficacy. Uncaging bypasses the presynaptic release machinery used by such methods, providing a well defined stimulation at a known location and as such provides some useful features when compared to traditional stimulation methods(Ellis-Davies, 2007).

Uncaging of neurotransmitters using one or two-photon excitation (Fig. 3A) has been a core method in many neurophysiological studies (reviews: (Eder *et al.*, 2004; Alvarez & Sabatini, 2007; Ellis-Davies, 2013)). An important feature of both means of photolysis is that they can produce stereotypical responses. Thus, broad scaled functional mapping using one-photon uncaging of neurotransmitter has been used by several groups(Eder *et al.*, 2004). Two-photon uncaging is more useful for high-resolution functional mapping of receptor densities across the surface of individual dendritic segments(Matsuzaki *et al.*, 2001; Asrican *et al.*, 2007; Katona *et al.*, 2011). Within both types of experiments a stereotypical amount of neurotransmitter is uncaged, albeit on different scales, so that the measured responses can be correlated with strength of synaptic connectivity or receptor density. The basic difference between the effects of one-photon and two-photon excitation is the of spatial resolution of the evoked signals, as large quantities of glutamate from one-photon uncaging fire action potentials whereas small quantities from two-photon uncaging mimic quanta (Fig. 3B). For example, when a continuous-wave 473nm-laser was focused onto a microscope objective so as to overfill the back aperture we found that the spatial resolution of GABA uncaging was modest (Fig. 3C) in comparison to the signal evoked by two-photon uncaging (Fig. 3D). Other studies have reported similar resolution for one-photon uncaging of GABA(Chalifoux & Carter, 2011) and two-photon uncaging of GABA(Gross *et al.*, 2013). While other reports feature much more refined resolution for one-photon uncaging of GABA (2 and 7 microns (Trigo *et al.*, 2009b), or 3 and 25 microns(Rial Verde *et al.*, 2008b) in x and z, respectively). Such differences in resolution for these one-photon studies may arise from different laser powers, shutter times and uncaging depths. Careful control of uncaging conditions enable diffraction-limited one-photon uncaging of glutamate in brain slices(DiGregorio *et al.*, 2007). Crucial factors in this were uncaging with low power and that only cells near the surface of the slice (20 microns) were targeted so as to reduce light scatter. However with deeper penetration into tissue such scatter becomes important. Thus, when confocal imaging was compared quantitatively with 2P microscopy, it was found that the former gave comparable signals to latter only close to the tissue surface; at 50–70 microns into tissue only the latter gave bright, clear images(Centonze & White, 1998). Similarly uncaging with near-IR (e.g. 720 nm) or IR light (e.g. 900 nm) enables much more effective depth penetration of the uncaging beam into brain tissue than the much shorter wavelengths of light (e.g. 400–470 nm) used for linear uncaging. For example, Higley and co-workers have reported 2P uncaging at single spines at depths of 200–300 microns(Chiu *et al.*, 2013).

The first example of diffraction-limited two-photon uncaging was published by Kasai and co-workers(Matsuzaki *et al.*, 2001). Using brief pulses (50 microseconds) of 720nm-light, MNI-Glu was photolyzed on cultured hippocampal neurons using low average power (7 mW) so as to mimic quantal release at single synapses for the first time(Matsuzaki *et al.*, 2001). Interestingly, even though some studies have suggested that uncaging flashes longer than 0.3 ms would reduce the spatial resolution of 2P uncaging due to excited molecule leaving the focal volume before release, or simply by uncaged glutamate diffusing beyond the excited volume(Brown *et al.*, 1999a; Brown *et al.*, 1999b; Kiskin & Ogden, 2002), many studies have reported that diffraction-limited uncaging of glutamate at single spines is possible with longer pulse durations (e.g. 1 ms)(Matsuzaki *et al.*, 2001; Smith *et al.*, 2003;

Carter & Sabatini, 2004; Sobczyk *et al.*, 2005; Araya *et al.*, 2006; Beique *et al.*, 2006; Asrican *et al.*, 2007; Katona *et al.*, 2011).

In 2004 Carter and Sabatini used two-photon uncaging of MNI-Glu and two-photon imaging of calcium in medium spiny neurons (MSN) to establish that that Ca^{2+}-permeable AMPA receptors are a major source of spine Ca^{2+}, and that this Ca^{2+} signal was independent of spine NMDA receptors and voltage-gated Ca^{2+} channels(Carter & Sabatini, 2004). The amount of Ca^{2+} conducted through AMPA receptors was found to be "state dependent". In other words, it was controlled by subthreshold depolarizations of MSN (the up and down states). From a technical point of view, it should be noted this was the first study combining two-photon uncaging and Ca^{2+} imaging at single spines. Two-photon uncaging of glutamate has been used in conjunction with 2pFRET-FLIM to understand more fully signal cascades involved in LTP and LTD (Harvey & Svoboda, 2007; Harvey *et al.*, 2008; Patterson *et al.*, 2010). For a review of this field see(Patterson & Yasuda, 2011).

After two-photon uncaging at single spines became an established technique, several studies took advantage of the ability of galvanometer-based beam steering to direct uncaging to *several optically designated spine heads in rapid succession* (Figs. 4A,B). When the time between uncaging pulses at a clustered group of individual spines is large (e.g. 100 ms) the effect of each pulse is isolated temporally (Fig. 4C, upper panel). However when the inter-pulse interval is reduced to 1 ms, the evoked signal becomes supralinear (Fig. 4C, the integrated signal in the lower panel is 2-fold larger than the upper panel). An alternative means of producing such dendritic non-linearities is gradually increasing the total uncaging energy at a group of clustered spines, an approach that was first reported using 1P uncaging in 2000 by Schiller and co-workers(Schiller *et al.*, 2000), an approach that can be effected using 2P uncaging (see below). However the contribution of single spines, and thus the importance of small voltage changes for dendritic spikes, is seen even more clearly with the experiment shown in (Fig. 4D). Here, the gradually increasing somatic response is plotted when 2–10 spines (Fig. 4B) were irradiated (50 mW), and it showed a marked non-linearity when more than 7 spines were stimulated. This experiment is facile because of the fine three-dimensional resolution that is inherent to two-photon uncaging. As well as one-photon uncaging, local glutamate perfusion or local synaptic stimulation have all been used to produce dendritic spikes. However, such methods have not been used to mimic the temporal summation shown in Figs. 4C or D. It is this feature that several groups have used to study many of the fundamental properties of dendritic integration that had remained closed to synaptic physiology due to the technical inability to stimulate visually defined single spines in an arbitrary manner (Gasparini & Magee, 2006; Losonczy & Magee, 2006; Branco *et al.*, 2010) (Losonczy *et al.*, 2008; Govindarajan *et al.*, 2011; Plotkin *et al.*, 2011; Zhai *et al.*, 2013). For a detailed review see(Major *et al.*, 2013).

Comparison with other methods of actuation

Solution exchange, rapid mixing or "puffing" all offer alternative means to apply agonists to biological tissue so as to evoke a temporally or spatially defined response. It must be noted that these methods do not allow rapid access to the intracellular compartment, so uncaging is almost always the preferred means to evoke responses with intracellular second messengers

such as cAMP or Ca^{2+}(Ellis-Davies, 2007), however for application of extracellular messengers "non-photochemical" means of agonist application are extremely useful. Importantly *any* molecule can be easily applied topically. However, solution exchange and rapid mixing are relatively slow and offer poor spatial control when compared to uncaging. In contrast, puffing can be very fast and highly localized. For example, iontophoresis allows glutamate to be applied to cultured neurons with single synapse spatial resolution so as to evoke quantal-sized EPSCs(Murnick *et al.*, 2002). Importantly it delivers only the natural biomolecule at the correct time and place, without prior bath application of a compound. Using this method one can circumvent the well-documented problem that most caged neurotransmitters have some antagonism towards GABA-A receptors(Rial Verde *et al.*, 2008b; Matsuzaki *et al.*, 2010). (Note, however we have not detected any epileptiform activity in our experiments, even though the caged compounds are bath-applied at concentrations above the IC_{50}.) Another important advantage of puffing in brain slices is that agonists may (easily) be applied to cell processes at different depths or in very different parts of cells(Muller *et al.*, 2012; Oikonomou *et al.*, 2012).

One-photon uncaging of glutamate has also been used to generate dendritic spikes in several studies(Schiller *et al.*, 2000; Jadi *et al.*, 2012) that are very similar to those shown in Fig. 4C,D. Given the very large cost of two-photon lasers, what advantages does the technique have compared to these other methods of local stimulation? First, two-photon uncaging has a slightly superior spatial resolution, signals drop to zero when photostimulation is moved 2–3 microns from the target (Fig. 3D), whereas with other methods this distance is larger, typically 7–10 microns (see (Muller *et al.*, 2012) supplemental figure 2). For studies involving co-activation of small clusters of spines this latter value is quite useful, however the ability to activate a group of single spines in a temporally dispersed or clustered way (Fig. 4C) is inherent to two-photon uncaging such that the study of spatial integration of individual spines in short or long dendritic segments is facile(Gasparini & Magee, 2006; Losonczy & Magee, 2006).

Two-color uncaging

From the very first glutamate uncaging experiment in brain slices(Callaway & Katz, 1993), the potential of two-color uncaging of two signaling molecules was obvious[2]. From the point of view of neurophysiological studies, wavelength-selective, two-color uncaging of glutamate and GABA would allow sophisticated analysis of the synaptic integration of inputs from these neurotransmitters, in a way that local perfusion does not(Liu, 2004; Hao *et al.*, 2009). Recently a number of studies have shown this is now possible, by combining two-photon uncaging of glutamate with one-photon uncaging of GABA(Lovett-Barron *et al.*, 2012; Chiu *et al.*, 2013; Hayama *et al.*, 2013).

Combing two-photon uncaging of glutamate with one-photon uncaging of GABA

RuBi-GABA is efficiently uncaged with blue light(Rial Verde *et al.*, 2008a). Application of this probe in the micromolar concentration range to brain slices allows one-photon uncaging

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[2]GCRE-D discussed the idea with the late Larry Katz at Cold Spring Harbor in 2003.

to produce large currents from activation of GABA-A (Fig. 3C) or GABA-B receptors(Chalifoux & Carter, 2011). These outward currents can be used to block action potentials evoked by current injection from a patch pipette(Rial Verde *et al.*, 2008b) (Fig. 5A). Two-photon irradiation of RuBi-GABA at such low concentrations does not evoke any currents (RuBi compounds have a two-photon absorption maximum at 800 nm, this falls by only 65% at 720 nm, see (Fino *et al.*, 2009a) supplemental information). Since nitroaromatic-caged probes do not absorb blue light (Fig. 2C), two-photon uncaging of MNI- or CDNI-Glu can be easily combined with RuBi-GABA. Thus two-photon uncaging of glutamate at 720 nm at multiple spines on a dendrite can evoke action potentials with excellent spatial resolution (Fig. 5B). In separate experiments we found that the presence of RuBi-GABA has no effect on these spikes unless a brief pulse of blue light preceded the two-photon uncaging (Fig. 5C). Note in the latter experiment the two laser beams were directed at the same dendritic segment, so does not rely on differential distribution of excitatory and inhibitory receptors for the success of such two-color uncaging. In 2012 Losonczy and co-workers combined two-photon uncaging of glutamate with one-photon uncaging of GABA for the first time(Lovett-Barron *et al.*, 2012). This study found that colocalized two-color uncaging on a CA1 radial oblique dendrite (cf. Fig. 5D) was more effective in reducing dendritic non-linearities compared to the effects of GABA uncaging on the cell body. Interestingly, in a study also published in 2012, Schiller and coworkers have arrived at different conclusions by combining one-photon uncaging of glutamate on layer 5 neuron basal dendrites with iontophoresis of GABA on the same two compartments(Jadi *et al.*, 2012). They found that dendritic colocalization reduced the spike threshold, but not height, whereas cell-body inhibition reduced spike height but not threshold. The subtly different conclusions reached in these two reports probably did not arise from the means of glutamate delivery, but may reflect that different neurons were examined.

The localization of GABA-A receptors to dendritic spine heads has been known for sometime(Kubota *et al.*, 2007), but the function of such receptors is poorly understood. Higley and co-workers showed recently that selective stimulation of such synapses using two-photon photolysis of CDNI-GABA or ChR2 infected interneurons reduced Ca^{2+} influx induced by backpropagating action potentials (bAPs) at about 50% of spines on layer 2/3 pyramidal neurons in the PFC. Two-color uncaging of CDNI-Glu and RuBi-GABA in a manner similar to Figs. 5C,D revealed that inhibition was confined to individual spines within a small cluster(Chiu *et al.*, 2013). Kasai and coworkers used two-color uncaging of CDNI-Glu and RuBi-GABA to show that inhibition could help induce LTD within a small cluster of spine heads, after the stimulation of a single spine(Hayama *et al.*, 2013). STDP can be induced at single spines photochemically when two-photon uncaging of glutamate precedes a bAP at 1 Hz for about 1 min ((Harvey & Svoboda, 2007) and follows the "pre before post" timing rule(Feldman, 2012)). However Kasai's laboratory found that a "post before pre" protocol did not induce LTD unless it was preceded by local uncaging of GABA within 50 ms of the AP. LTD appeared as spine shrinkage, with shrunken spines showing reduced currents. Such effects had single spines as their initiation point, but the LTD effect was seen in spines covering an adjacent 15-micron segment. Phosphorylated cofilin was found to be the molecule that spread from the activated spine to its near neighbors. Interestingly, prior sLTP at single spines within a cluster protected this spine from a

subsequent LTD protocol, suggesting that such spines were "write protected" from GABA-mediated spine erasure.

The blue lasers used to uncage RuBi-GABA can also be used to activate ChR2(Chiu *et al.*, 2013). Since ChR2 does not generate experimentally useful currents by non-linear excitation at 720 nm, it has been combined with two-photon uncaging of glutamate at this wavelength in single neurons so as to induce sLTP(Zhang *et al.*, 2008). This is an "all photochemical" version of the pre before post STDP used by the Kasai and Svoboda laboratories. Oertner's laboratory are the only group to publish this type of two-color experiment, but it is a strong proof of principle that the "old" and "new" methods of neuronal actuation can be combined for neurophysiological studies (e.g.(Yagishita *et al.*, 2014)).

Other compounds for 2P/1P, two-color uncaging

Etchenique and co-workers have synthesized several other RuBi caged compounds, these include dopamine(Araya *et al.*, 2013), tryptamine, serotonin(Zayat *et al.*, 2006), nicotine(Filevich *et al.*, 2010), and glutamate(Fino *et al.*, 2009b). In principle, any amino containing molecule could be caged with this technology, e.g. important amino acids such as glycine and D-aspartate. All RuBi-caged compounds are highly active towards one-photon uncaging with blue lasers, so these caged compounds could all be partnered with two-photon uncaging of MNI- or CDNI-caged neurotransmitters at 720 nm, to enable the optical interrogation of the bimodal interaction of such bio-molecules. Recently we have developed DEAC450-cAMP(Olson *et al.*, 2013a) and used blue laser light to uncage this inside neurons (discussed next section). Since DEAC450 is not effectively photolyzed at 720 nm(Olson *et al.*, 2013b; Amatrudo *et al.*, 2014), this caged second messenger could also be used with two-photon photolysis of caged compounds at 720 nm. DEAC450 could be used to cage other molecules such cGMP, ATP, and IP$_3$. Thus, there is quite an array of compounds (potentially) available for "2P/1P" two-color uncaging experiments.

Two-color, one-photon uncaging

The wide availability of continuous-wave lasers on confocal microscopes makes the development of compounds that can be photolyzed in a wavelength selective manner using only linear excitation a very attractive proposition. As noted above, many such experiments have been attempted, but the "normal" spectral overlap of most caged compounds (filled arrow, Fig. 2B) has prevented such selectivity. Recently we have shown that DEAC450-cAMP can be photolyzed with excellent wavelength selectivity in the presence of CDNI-GABA using two wavelengths of visible light(Olson *et al.*, 2013a). Importantly, irradiation of neurons with both caged compounds present with 355-nm light only manifested effects from CDNI-GABA. Thus violet and blue light could be applied to neurons in an arbitrary order (Box 1) so as to modulate the firing rate of striatal cholinergic interneurons in a bidirectional manner. The two-color, one-photon uncaging method is analogous to partnering one-photon uncaging with iontophoresis(Jadi *et al.*, 2012) and dual iontophoresis(Muller *et al.*, 2012; Oikonomou *et al.*, 2012) in terms of resolution and rate of neuronal response.

Two-color, two-photon uncaging

The development of DEAC450-Glu, which allowed optically selective two-photon uncaging at long wavelengths of light for the first time(Olson *et al.*, 2013b), suggested that this probe could be partnered with nitroaromatic caged compounds like CDNI-GABA(Matsuzaki *et al.*, 2010) so as to enable a clean two-color uncaging experiment using non-linear excitation with two wavelengths of light. In Fig 6 we now show that this is feasible.

First, we established that DEAC450-Glu could be uncaged at 900 nm with lateral resolution (Fig. 6C) that was comparable to that reported in many studies for MNI-Glu(Matsuzaki *et al.*, 2001; Smith *et al.*, 2003; Carter & Sabatini, 2004; Sobczyk *et al.*, 2005; Araya *et al.*, 2006; Beique *et al.*, 2006; Asrican *et al.*, 2007; Katona *et al.*, 2011). We have recently shown that when DEAC450-GABA was uncaged around the trigger zone of a CA1 neuron with 900 nm irradiation the resulting hyperpolarization can block action potentials(Amatrudo *et al.*, 2014). Similarly, when CDNI-GABA was bath applied to a brain slice, irradiation around the trigger zone at 720 nm could be used to block action potentials generated by current injection from the patch pipette (Fig. 6B). The experiments shown in Figs. 3B,D were performed with only one caged compound present, when DEAC450-Glu (0.6 mM) and CDNI-GABA (1 mM) were co-applied to a brain slice, bidirectional modulation of membrane potential with chromatically selective two-color, two-photon uncaging was possible (Fig. 6D). Irradiation at 15 points along a basal dendrite at 900 nm fired an action potential, this was reversibly blocked by irradiation at 720 nm (Fig. 6D, in separate experiments uncaging CDNI-Glu at 720 nm and DEAC450-GABA at 900 nm produced similar effects, data not shown). Importantly, when the two lasers were directed to the same neuronal segment, only individual, wavelength-specific effects were apparent (Fig. 6E). These experiments show that two-color, two-photon uncaging of Glu and GABA is practical but do not reveal the quantitative color separation that the DEAC450 chromophore enables. Therefore we measured the optical selectivity of such currents by applying the same power at 720 nm or 900 nm at single spine heads, the difference in currents was 30-fold (Fig. 6F), and the power dosage difference for uncaging CDNI-Glu and DEAC450-Glu at 720 nm was found to be highly significant ($P < 0.0007$, Fig. 6G). In practice, the amount of energy required to see any current from uncaging DEAC450-Glu at 720 nm (50 mW, 5 ms) was much more than was needed for CDNI-Glu uncaging at 720 nm. For the latter, 50–60 mW for 0.5 or 25 mW for 1.0 ms at 720 nm could evoke currents reliably, such powers produced no measurable current from DEAC450-Glu. This series of experiments are similar to those we published in 2010(Kantevari *et al.*, 2010), except with the new DEAC450-caged glutamate almost no photolysis is induced at short wavelengths of light. DCAC-GABA(Kantevari *et al.*, 2010), a DEAC analog, was *more* photoactive at short wavelengths than long wavelengths of light(Kantevari *et al.*, 2010). With CDNI- and DEAC450-caged compounds we find that it is straightforward to establish conditions in which there is effectively *no optical cross talk* between uncaging at each wavelength (Box 1). These experiments, we believe, demonstrate the real potential of two-color, two-photon uncaging for the first time. Since it has been established that only two-photon uncaging allows rapid, high-resolution stimulation of single synapses, we suggest that dual-color two-photon uncaging offers unprecedented capabilities in controlling neuronal signaling.

Summary of possible two-color actuation modalities

Wavelength-selective, two-color actuation is based upon, most obviously, the ability of chromophores to absorb in spectrally isolated parts of the electromagnetic spectrum (Fig. 2). The biological responses of the target receptors is also important as "bi-directional weight" of two such receptors acting in concert is probably never equal. Until the development of DEAC450(Olson *et al.*, 2013a), all attempts at multi-color actuation using linear excitation required sequential and complete uncaging of the longer wavelength cage(Kotzur *et al.*, 2009; Goguen *et al.*, 2011; Menge & Heckel, 2011; Priestman *et al.*, 2011; Schaäfer *et al.*, 2011; Priestman *et al.*, 2012; Rodrigues-Correia *et al.*, 2013), or for the caged compounds to be present in very different concentrations(San Miguel *et al.*, 2011; Klan *et al.*, 2013). Initial reports for the genetically encoded probes ChR2 and Halo suggested that retinal-based actuators could circumvent the constraints of the absorption spectra (Fig 2D), and no doubt that for particular circumstances this can be observed. However, a more recent quantitative comparison of the currents generated by spectral variants of several excitatory opsin-based probes revealed that clean actuation at two wavelengths is very difficult to achieve(Prigge *et al.*, 2012). This may go some way to explaining why bi-directional actuation with ChR2 and Halo (or their many equivalents) has been difficult to deliver for in vivo studies of mouse behavior. But note that Gottschalk and co-workers have used ChR2 variants with blue- and red-light sensitivities to fire different classes of neurons in *C. elegans* so as to control behavior bimodally(Erbguth *et al.*, 2012). Recently Boyden and co-workers have found and mutated new opsin proteins that allow decent power windows for blue and red light activation of spikes in pyramidal cells in brain slices(Klapoetke *et al.*, 2014). However, they do emphasize that *all* opsins can be activated by blue light.

Another important issue has been raised recently for the partnering of ChR2 and Halo (or equivalents) is that expression of two proteins under different promoters will always produce widely different protein levels in cells, introducing an almost insurmountable problem for *consistent* bi-directional control. Such problems are not seen with caged compounds, as concentrations are defined. Recently, the discoverers of ChR2(Nagel *et al.*, 2003), Bamberg and co-workers, developed a solution to this problem by linking two opsin probes in one integral membrane protein(Kleinlogel *et al.*, 2011). Many research groups continue to improve the properties of light-gated ion channels and pumps to address these issues(Knopfel *et al.*, 2010).

With all these constraints, it is perhaps no surprise that there have been relatively few two-color neurophysiological actuation experiments(Zhang *et al.*, 2008; Lovett-Barron *et al.*, 2012; Chiu *et al.*, 2013; Hayama *et al.*, 2013). However, these studies show clearly that two-photon uncaging of glutamate at short wavelengths (720–740 nm range) can be partnered with one-photon actuation with blue light (450–473 nm range). This is because nitroaromatic caged glutamate probes are not photolyzed with blue light and RuBi-GABA *at low concentrations* does not produce any current with non-linear excitation at 720 nm. ChR2 has a much larger two-photon cross-section at 720 nm than MNI-Glu(Andrasfalvy *et al.*, 2010), but the small nature of the excitation volume (Fig. 3A) coupled with the low single channel current of ChR2 mean that point excitation produces no effective current(Rickgauer

& Tank, 2009; Prakash *et al.*, 2013). In contrast, RuBi-GABA and ChR2 produce large currents with blue light(Zhang *et al.*, 2008; Chiu *et al.*, 2013).

What other 2P/1P modalities are easily available with commercially available probes? MNI-Glu at 720 nm can be partnered with linear activation of RuBi-dopamine and probably RuBi-Glu, as well C1V1, optoXR, eArch3.0(Yizhar *et al.*, 2011). Unfortunately DEAC450-based probes are not commercially available, but DEAC450-cAMP offers another potential 1P partner for 2P uncaging of MNI-Glu. In terms of mixed 1P/1P modalities, uncaging MNI-Glu at 355 nm could be partnered with Halo and eArch3.0 (450–600 nm light(Yizhar *et al.*, 2011)). Recently, Deisseroth and colleagues have shown that some newer opsin-based probes are usefully two-photon active(Prakash *et al.*, 2013), but only at very long wavelengths (>900 nm), thus these probes could also be partnered for 2P/2P experiments with MNI-Glu at 720 nm or RuBi-Glu at 800 nm. An advantage of two-photon excitation is that its inherent non-linearity can enhance differences in linear absorption. For example, DEAC450-Glu is about 11-fold more active at 450 nm compared to 350 nm(Olson *et al.*, 2013b), and it is about 18-fold more fluorescent at 900 nm compared to 720 nm when two-photon excitation is used. These data suggest that dual two-photon excitation has the capability of always providing better wavelength selectivity for two-color experiments. Certainly dual two-photon uncaging is uniquely powerful for subcellular interrogation of dendritic processes (Fig. 6). Our hope is that this Technical Spotlight will help further the application of two-color actuation experiments, especially using dual two-photon methods of uncaging.

Acknowledgments

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Appendix

Materials and methods

Caged compounds and fluorescent dyes

MNI-Glu (Papageorgiou & Corrie, 2000) (Matsuzaki *et al.*, 2001), CDNI-Glu(Ellis-Davies *et al.*, 2007), CDNI-GABA(Matsuzaki *et al.*, 2010; Ellis-Davies, 2011), PEG-DEAC450-GABA(Amatrudo *et al.*, 2014)] and DEAC450-Glu(Olson *et al.*, 2013b) were prepared as previously described. RuBi-GABA was purchased from Tocris. Alexa-594 and Oregon Green BAPTA-1 were purchased from Molecular Probes.

Animals and brain slice preparation

All animal handling was performed in accordance with NIH guidelines approved by institutional IACUC review. Under isoflurane anesthesia, mice were decapitated and transverse brain slices (300–350 microns thick) were cut in ice-cold external solution, bubbled with 95% O_2 and 5% CO_2, containing (in mM): 124 NaCl, 25 $NaHCO_3$, 1 KCl, 2 KH_2PO_4, 10 glucose, 2.5 $CaCl_2$, 1.3 $MgCl_2$. Slices were then transferred to artificial cerebrospinal fluid (ACSF) containing (in mM): 127 NaCl, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 2.5 KCl, 1 $MgCl_2$, 2 $CaCl_2$ and 20 glucose, bubbled with 95% O_2 and 5% CO_2. The slices were maintained at 20–22°C for at least 60 minutes until use.

Two-photon microscopy and physiology

Imaging and uncaging experiments were performed as described previously(Crowe *et al.*, 2010; Kantevari *et al.*, 2011; Amatrudo *et al.*, 2014). An Olympus BX61 microscope (Penn Valley, PA, USA) fitted with a Prairie Technologies (Middleton, WI, USA) Ultima dual-galvo scan head (Haydon *et al.*, 2008) controlled by Prairie View 5 and three Chameleon Ti:sapphire lasers (Coherent, Palo Alto, CA, USA) modulated by 350–80 Pockels cells (Conoptics, Danby, CT, USA), a continuous-wave 473-nm laser (Laserglow, Toronto, ON, Canada), an EPC9 or EPC10 amplifier (Heka Instruments, Bellmore, NY, USA) and an Olympus 60x lens (LUMFLN60XW, 1.1 NA) was used. Whole-cell patch-clamp recordings were obtained from cortical pyramidal cells identified with IR differential interference contrast microscopy. Patch-clamp recordings were made with glass electrodes (3–5 MΩ) filled with (in mM): potassium gluconate (135), HEPES (10), $MgCl_2$ (4), Na_2ATP (4), NaGTP (0.4), and sodium creatine phosphate (10), adjusted to pH 7.3 with KOH. Unless other wise noted in the figure legend, the internal solution contained Alexa-594 (0.1 mM) for visualization at 1070 nm. Caged compounds were dissolved in a small volume of ACSF

and bath-applied to brain slices by recirculation. Off-line data analysis was performed using IgorPro, imageJ, Excel, or Prism.
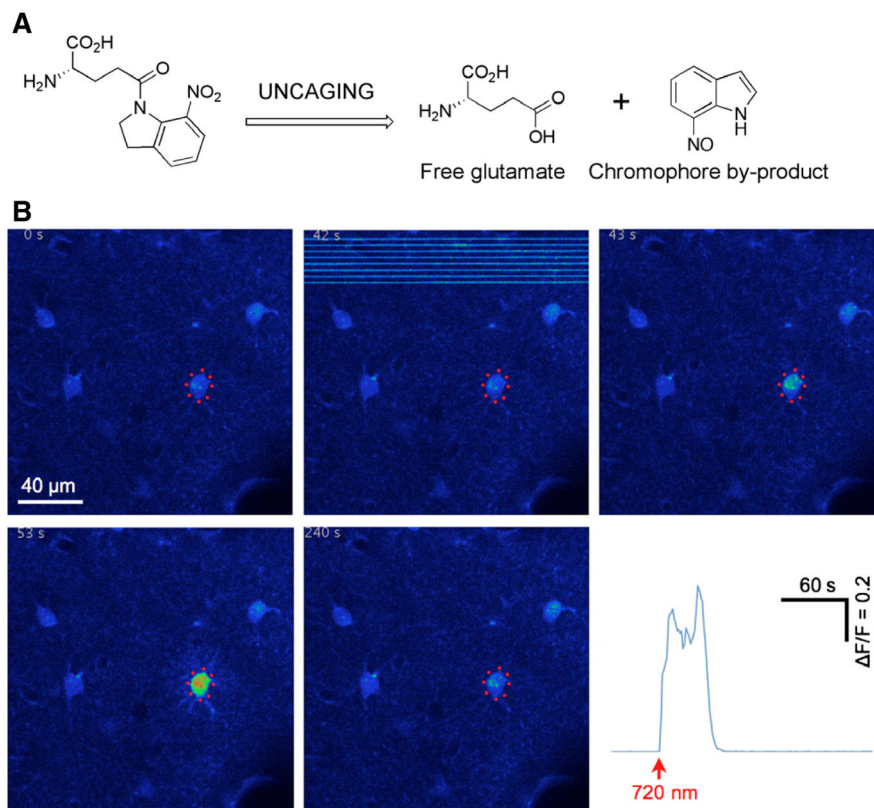
Fig. 1.
Example of uncaging on living cells. (A) Photorelease of glutamate from MNI-Glu. (B) Example of the rapid biological effects of uncaging. In this experiment MNI-Glu was topically applied to the brain of an anesthetized mouse and uncaging was directed to eight points near an astrocyte. Before applying the caged compound, the dura was removed and a Ca^{2+}-sensitive dye was loaded into the astrocytes (Crowe *et al.*, 2010). Two-photon imaging at 0.8 Hz was at 950 nm and uncaging was at 720 nm (15 mW for 10 ms per point). Warmer colors in the individual image frames represent higher Ca^{2+} concentration in the targeted cell. The graph shows a plot of the time course of the change in fluorescence in proportion to resting fluorescence.
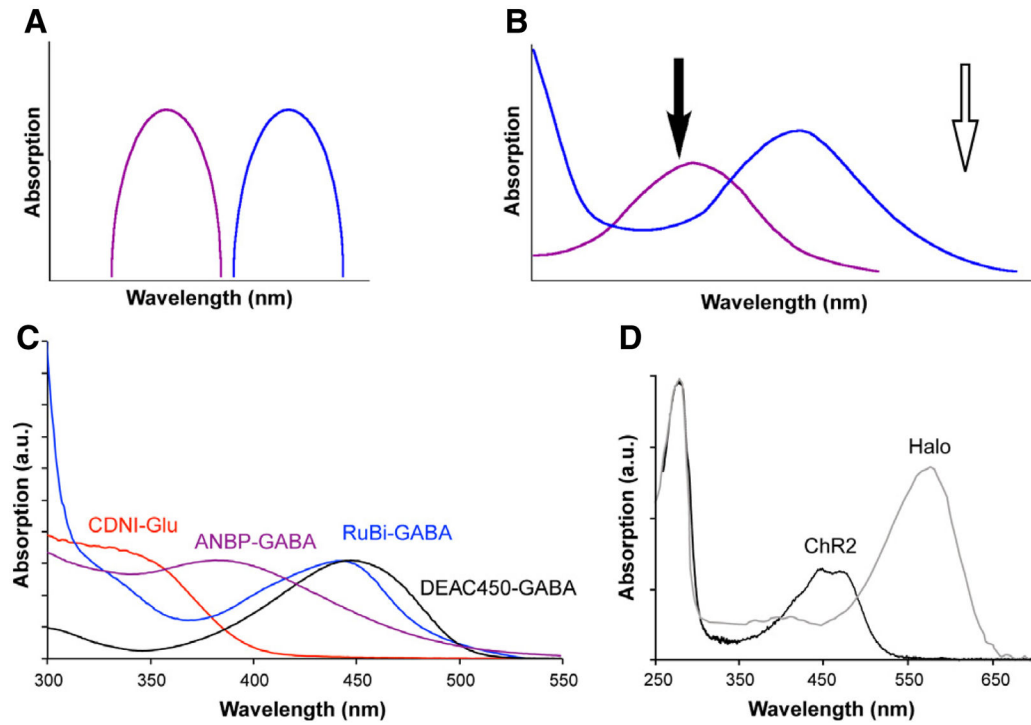
Fig. 2.
Design of chromophore absorption for two-color photochemical actuation. (A) Cartoon of ideal chromatically independent absorption spectra for wavelength-selective two-color actuation. (B) Normally two chromophores with distinct absorption maxima have a non-overlapping region of the electromagnetic spectrum where only the longer wavelength caging chromophore (blue trace) absorbs (open arrow). However, the absorption maximum of the short-wavelength chromophore (purple) typically overlaps significantly with the longer wavelength chromophore (black arrow). (C) Absorption spectra of CDNI-Glu (red), ANBP-GABA (purple), RuBi-GABA (blue) and DEAC450-GABA (black). Note the minimum of DEAC450 is close to the maximum of CDNI. In the remaining figures we 'color-code' 1P uncaging with blue, 2P uncaging at 720 nm with red and 2P uncaging at 900 nm with black or white. (D) Absorption spectra of ChR2 and Halo when normalized to their absorption maximum have sizable overlap at 450 nm.
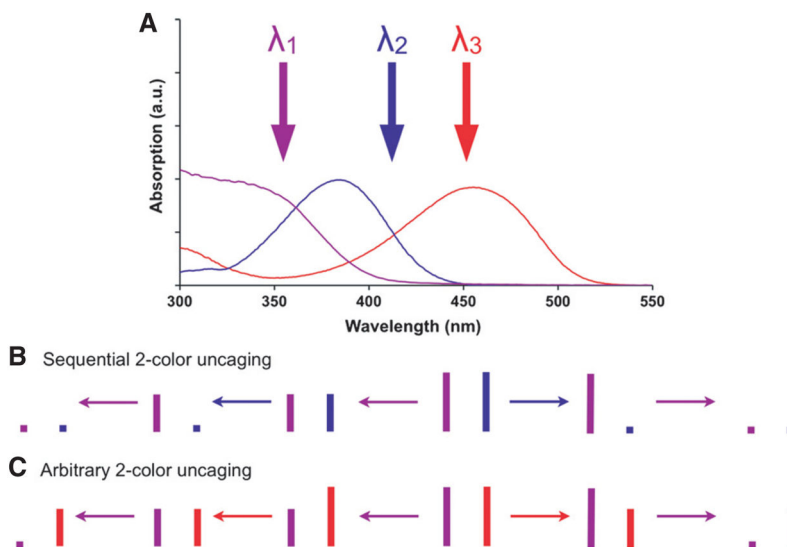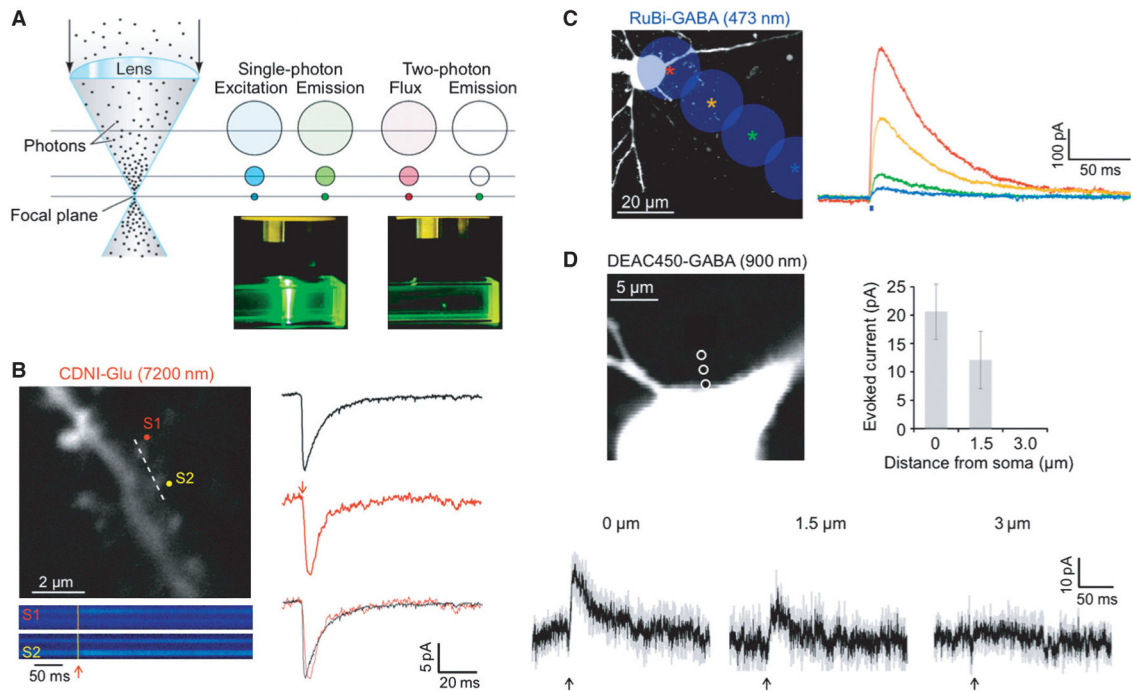
Fig. 3.
Two versions of wavelength-selective two-color uncaging. (A) Absorption spectra of nitroaromatic (purple), DEAC (blue) and DEAC450 (red) caging chromophores. Note that microscope glass is normally opaque below 330 nm. (B) Irradiation of a mixture of nitroaromatic- and DEAC-caged compounds with λ_1 causes uncaging of both cages, whereas irradiation with λ_2 enables wavelength-selective photolysis of the DEAC caged compound. Typically, experiments with such chromophore mixtures feature essentially complete photolysis of the DEAC cage (i.e. the 'long wavelength') with λ_2 before applying λ_1 to the cells. (C) Irradiation of a mixture of nitroaromatic- and DEAC450-caged compounds with λ_1 or λ_3 produces wavelength-selective uncaging of either caged compound, no matter which wavelength of light is used first. Thus λ_1 and λ_3 can be applied in an arbitrary order such that photolysis with λ_1 can bracket photolysis with λ_3; see Olson *et al.* (2013a) and experiments herein.

Fig. 4.
Introduction to 1P and 2P uncaging. (A) Left, cartoon of a lens focusing light. Middle, 1P excitation occurs throughout the light absorption path and total excitation is equal in each *z*-section. Right, 2P emission is confined to the focal plane due to the nonlinear nature of the creation of the excited singlet state. (B) Left, image of a dendritic segment of a CA1 pyramidal cell filled with Oregon Green BAPTA-1 (0.2 mM) using 2PE at 820 nm showing spines that were targeted for 2P uncaging of CDNI-Glu (1 mM) with 720 nm light (50 mW). Spine-selective 2P uncaging of CDNI-Glu was revealed by line-scan imaging (dotted line) with a period of 0.7 ms (146 pixels, 4 μs/pixel). Increases in $[Ca^{2+}]$ are shown in pseudocolor traces and reveal that S1 and S2 were stimulated separately. Right, currents (red traces, *n* = 24 from five cells) from uncaging of CDNI-Glu (1 mM, 720 nm, 1 ms) at single spines closely mimicked miniature EPSCs (black traces, *n* = 108). (C) A 473-nm laser was targeted on a cell soma and, at 20-μm increments (colored stars), the corresponding current traces from photolysis of RuBi-GABA (10 μM, 2 ms, 20 mW) are shown. (D) Two-photon photolysis of DEAC450-GABA (0.2 mM, 900 nm, 5 ms, 100 mW) evoked outward currents that decreased as the laser was moved away from the cell body. Each trace is an average of three events recorded from three cells, with grey indicating the SEM. For C and D, CA1 pyramidal cells were held at −40 mV. Compared to 1P uncaging, 2P uncaging generates a much smaller spatially restricted input. Brian slices were isolated as described in the Appendix.
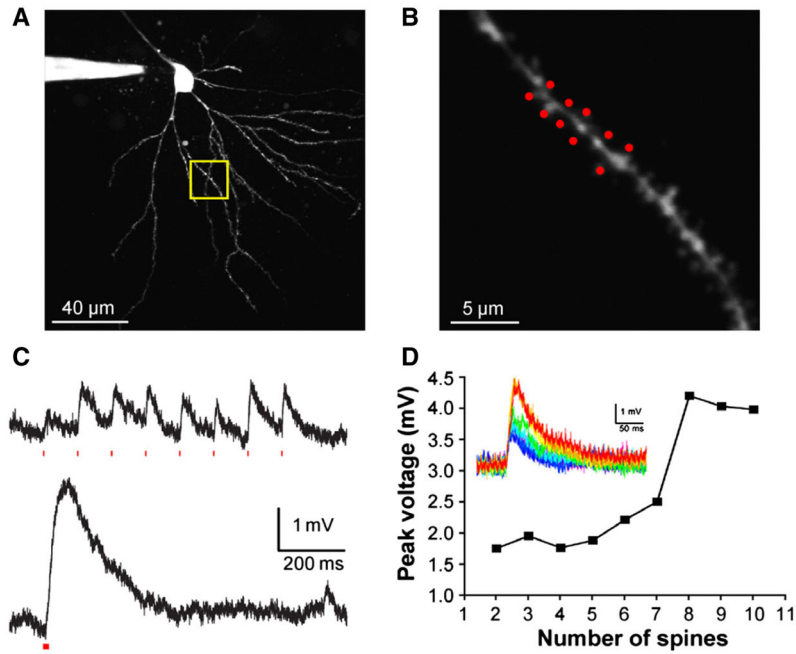
Fig. 5.
Temporal integration of excitation at multiple spine heads. (A) Two-photon fluorescence image of a CA1 pyramidal cell. (B) Detail of dendrite shown in the yellow box in (A), with a cluster of 10 spines targeted for the experiment in (D). (C) CDNI-Glu (1 mM) was uncaged at 720 nm at eight spines along a dendrite, compressing the inter-pulse interval between uncaging events from 100 ms (upper) to 1 ms (lower), producing a nonlinear response. Individual responses from each spine varied between 0.25 and 1.0 mV, and temporal clustering doubled the integrated voltage response. A similar nonlinear dendritic response (dendritic spike) can be clearly seen in (D), where the inter-pulse interval was 0.12 ms. (D) Plot of the somatic voltage responses from uncaging at two to ten spines, with the associated voltage traces inset. The evoked response was approximately linear for two to seven spines, but showed a marked nonlinearity when uncaging was directed to eight or more spines.
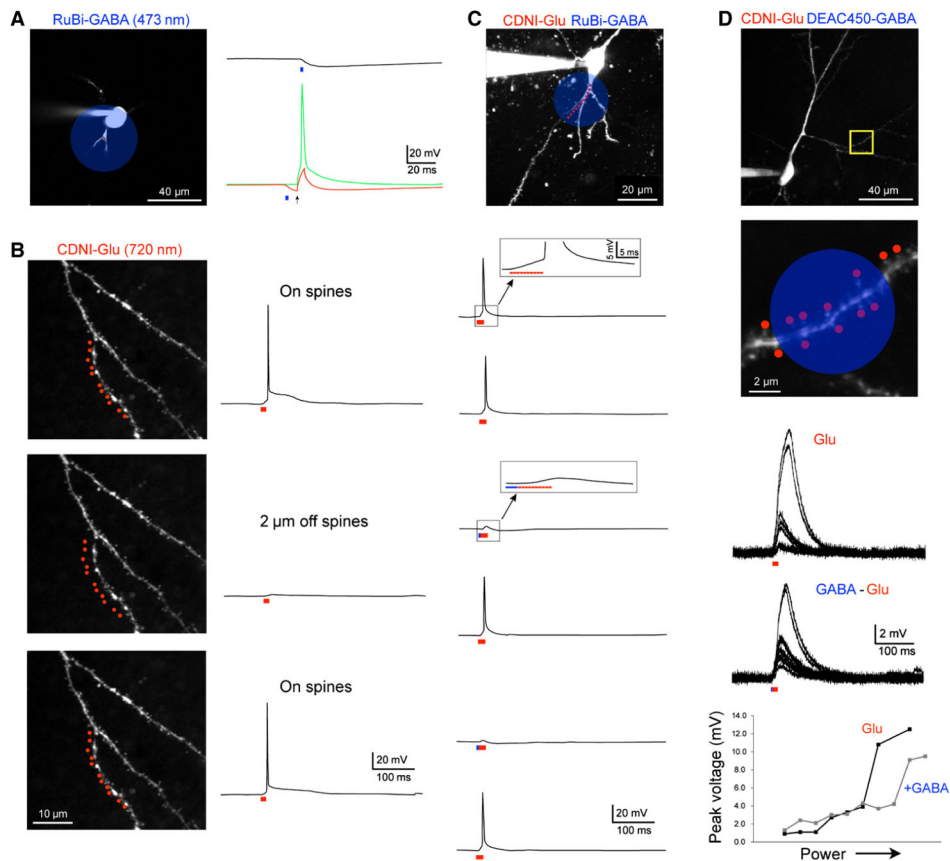
Fig. 6.

Two-color uncaging combining 2P and 1P excitation. (A) A 473-nm laser was targeted on a layer 2/3 pyramidal cell soma (blue circle); the hyperpolarization from photolysis of RuBi-GABA (10 μM, 2 ms, 20 mW) is shown (right). This stimulus generated an inhibitory input (top black trace) of sufficient amplitude to block an electrically evoked (1 nA, 1 ms) action potential (bottom green and red traces). Uncaging was 10 ms prior to the electrical stimulus. (B) Two-photon uncaging of CDNI-Glu (1 mM, 720 nm, 1 ms, 70 mW) at 11 spines along the dendrite of a CA1 pyramidal cell generated sufficient depolarization to evoke an action potential (top). When the uncaging flashes were targeted 2 μm away from the dendrite, no action potential was generated (middle); the spike was restored when uncaging was returned to the original position, demonstrating the precise nature of 2P inputs. (C) When CDNI-Glu (1 mM) and RuBi-GABA (10 μM) were co-applied to a slice, rapid bi-directional control of the membrane potential was effected when the 720-nm light was or was not preceded by a flash of 473-nm light (2 ms, 20 mW). Two-photon photolysis was with 720-nm light at eleven points (0.5 ms, 60 mW) along the basal dendrite of a CA1 pyramidal cell (held at −55 mV). Note that both of these inputs are occurring along the same portion of the dendritic tree, demonstrating the optical selectivity these two caged compounds under these conditions. (D) Two-photon fluorescence image of a CA1 pyramidal neuron used for the following physiological experiment, with a more detailed image in the area in the yellow box. Red dots indicate the location of 2P photolysis and the blue circle represents the area for 1P uncaging. CDNI-Glu (1 mM) and DEAC450-GABA (20 μM) were co-applied to the

brain slice. Irradiation with 720-nm light of increasing power (55–90 mW) at 14 spines along an oblique dendrite elicited a dendritic spike. The dendritic spike was modulated when the 720-nm light stimulus was preceded by 473-nm light (2 ms, 15 mW). The plots of peak spike amplitude vs. laser power shows how the dendritic spike is altered by inhibition, as the maximal amplitude is reduced and the threshold (i.e. the point at which the signal becomes nonlinear) is increased.
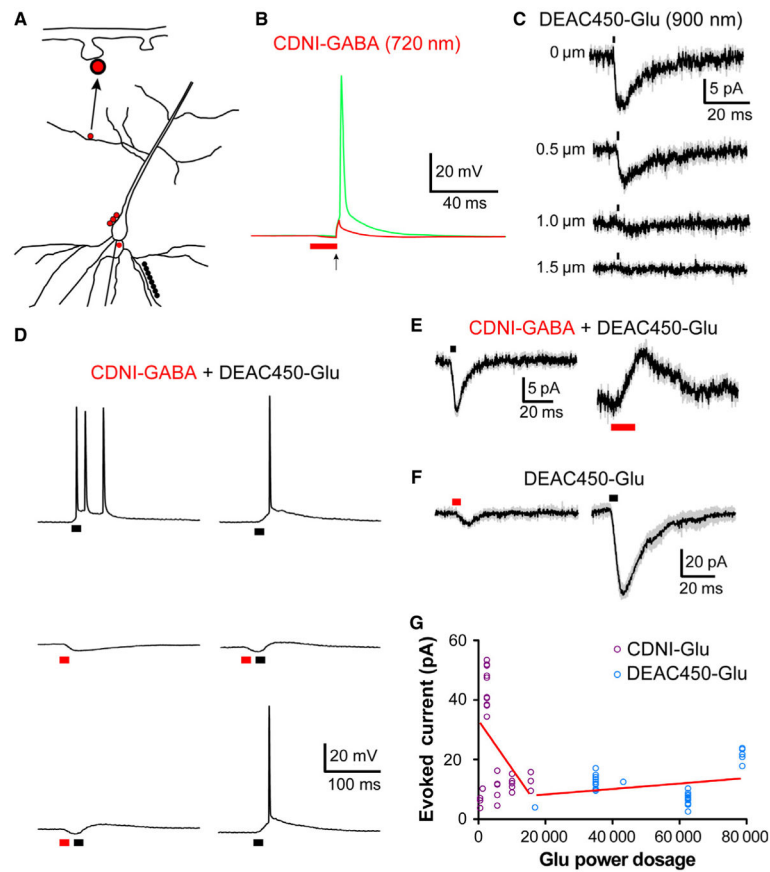
Fig. 7.
Two-color 2P uncaging. (A) A schematic representation of the various photolysis positions on CA1 pyramidal neurons of the two mode-locked Ti: sapphire lasers used in (B)–(F). The two uncaging wavelengths are color-coded in each part with red representing the 720-nm laser and black the 900-nm laser. Neurons were imaged with a third laser at 1070 nm. For (C) and (F) the same points are targeted with both uncaging lasers. (B) CDNI-GABA (0.2 mM) was uncaged with 720 nm light (2 ms, 60 mW) at 12 points around the axon hillock, which generated an inhibitory input capable of blocking (red trace) electrically evoked action potentials. The green trace represents a control action potential in which only the electrical stimulus (2 ms, 500 pA) was applied. The pyramidal cell was held at −55 mV. (C) DEAC450-Glu (1 mM) was uncaged with 900-nm light (1 ms, 35 mW) at a single spine head. Each trace represents an average of 5 or 10 responses from one cell (grey represents SEM), which is representative of four cells on which this experiment was performed. (D) CDNI-GABA (1 mM) and DEAC450-Glu (0.6 mM) were co-applied to a slice and a CA1 pyramidal cell (held at −60 mV) was recorded from. Irradiation at 900 nm at 10 spines along a basal dendrite (1 ms, 120 mW) produced sufficient depolarization to evoke a burst of action potentials (top left trace). Irradiation at 720 nm at three points adjacent to the soma (5 ms, 125 mW) generated an inhibitory input of several millivolts (middle left). When these two inputs were paired in rapid succession, the action potentials were blocked (bottom left trace). Action potentials were blocked reversibly by interleafing dual-color irradiation at 720 and 900 nm (right middle trace) with 900 nm photolysis (right top and bottom traces). (F)

CDNI-GABA (1 mM) and DEAC450-Glu (0.6 mM) were co-applied to a slice, and selectively activated at three points along a CA1 pyramidal cell soma (held at −50 mV). Stimuli were 720 nm, 5 ms for 90 mW and 900 nm, 1 ms for 50 mW. Traces are an average of five responses from a cell with a holding potential of −50 mV, and are representative of responses observed in four cells (grey indicates SEM). (F) DEAC450-Glu (0.5 mM) was uncaged at single spine heads; the same stimulus (5 ms, 50 mW) was used at both wavelengths. Uncaging at 900 nm evoked 30-fold larger response than that at 720 nm. Each trace is an average of five events from one cell, representative of six cells on which this experiment was performed (grey indicates SEM). Note that when a stimulus duration of 1 ms was used, DEAC450-Glu did not generate a measurable response at 720 nm (data not shown). Furthermore, the typical power dosage at 720 nm used to uncage CDNI-Glu (0.5–1 ms, 25–60 mW) did not produce any detectable 2P EPSCs from DEAC450-Glu. (G) Summary of all uncaging experiments for CDNI-Glu and DEAC450-Glu when uncaged at 720 nm at single spine heads. The evoked currents from many cells for CDNI-Glu and DEAC450-Glu uncaging at 720 nm (hence red lines for both data groups) are plotted vs. the 'Glu power dosage' (the caged compounds are color-coded according to their absorption spectra). This latter quantity is the product of the $(power)^2 \times (time) \times [cage]$. As DEAC450 is ~ 10× more photoactive for 2P excitation (Olson *et al.*, 2013b) this adjustment factor is also used. The graph shows an *n* of 35 for each cage compound. The linear regression line for each compound was computed and an analysis of covariance showed the two slopes were significantly different ($F = 12.76$, $P = 0.00075$).