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Two-photon uncaging: New prospects in neuroscience and cellular biology

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ABSTRACT

An uncaging process refers to a fast and efficient release of a biomolecule after photochemical excitation from a photoactivatable precursor. Two-photon excitation produces excited states identical to standard UV excitation while overcoming major limitations when dealing with biological materials, like spatial resolution, tissue penetration and toxicity and has therefore been applied to the uncaging of different biological effectors. A literature survey of two-photon uncaging of biomolecules is described in this article, including applications in cellular- and neurobiology.

1. Introduction

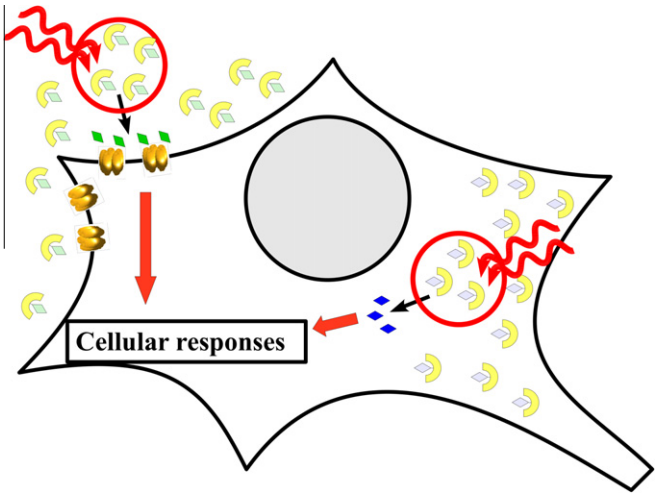
Biology is entering a new phase due to the development of sophisticated methods to scrupulously investigate the extraordinary complexity of cellular processes. These methods necessitate to comply with a spatial and temporal organization of the biological system of interest. Control of cellular processes can only be attained using triggering methods which are orthogonal to their cellular environment. Photochemical activation of an inert biological precursor offers a unique, orthogonal way to attain this spatiotemporal control. Photoactivatable precursors of biological effectors, called 'caged compounds', encompass different biological domains involving light-activatable neurotransmitters, second messengers, enzyme or receptor binders as well as small molecule gene regulators. A series of well-established criteria are closely associated to these caged biomolecules. First, the photolytic reaction requires to be efficient and fast to generate a localized concentration jump of the biomolecule in a time frame in agreement with the elicited cellular process. The photoresponsive precursor should be functionally inert as well as non toxic to the cellular system and so should be the photolytic fragment.

A series of recent reviews have extensively described the different caging groups[1] as well as the biological applications thereof.[2] Among the more recent outcomes related to this methodology is the two-photon (2P) uncaging process which induces a photolytic reaction with a remarkable spatial resolution, allowing to analyze cellular processes with a finer spatiotemporal control. Scheme 1 illustrates the two-photon uncaging concept.

2. Results and discussion

2.1. Two-photon uncaging: principle

After two-photon excitation, a molecule reaches an excited state and can either return to its ground state by light emission,



Scheme 1. Two-photon uncaging for focused biological effects.

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leading to two-photon induced fluorescence and application thereof in imaging such as two-photon excitation microscopy, or can be subject to chemical reactions leading to bond cleavage or rearrangements. This later process is of particular interest in the photoliberation of biologically active substances. Indeed, the fine spatiotemporal localization of the liberation at the focal point of the laser system, due to a quadratic dependence of the excitation probability versus the electric field of the exciting light, allows subcellular control of the released substance. In addition, the use of IR light to induce this two-photon process is less damaging to cells than more energetic UV light used in classical illumination.[2c] Furthermore, the penetration of IR light in living tissues is much more efficient than UV. The efficiency of a two-photon induced photochemical reaction is usually described as $\delta_a\Phi_u$, in the same manner as for one photon ($\varepsilon\Phi_u$).

The practical use of two-photon excitation to induce uncaging of biomolecules has been described for the first time a decade ago.[3] The strategy developed in this direction consisted first in testing the two-photon uncaging efficiency of the different caging groups described so far in the literature for one-photon excitation. Table 1 summarizes these results showing the structures of these caging groups and the corresponding two-photon action cross sections ($\delta_a\Phi_u$) expressed in Göppert–Mayer (GM) units (1 GM = 10^{-50} cm^4 s photon^{-1}). The efficacy of the photoliberation is not only linked to the two-photon absorption cross section δ_a, but also to the efficiency of the photochemical reaction Φ_u. Thus both δ_a and Φ_u have to be optimized.

Classically described cages are based on dipolar donor–acceptor systems (typically methoxy/nitro for nitrobenzyl, nitrophenylalkyls and nitroindolinyl platforms, Fig. 1a).[1c] This architecture is well known in non-linear optics for leading to efficient materials. Numerous works on molecular engineering of such probes proved that an increase in the donor or acceptor efficiency, or an elongation of the conjugated system (Fig. 1b) led to an increased δ_a.[4] We have to note that the introduction of aromatic heterocycles (such as thiophene, furan...) can also improve the 2P absorption cross section. Recently, we described new architectures derived from symmetrical acceptor–acceptor systems based on a fluorenyl core (Fig. 1c) showing impressive δ_a.[5] For these systems, the elongation of the delocalization pathway should also lead to an increase of δ_a. For the improvement of Φ_u the relation between structure and properties are less explored, and result mainly from experimental observations. The selected molecules referred mostly to the uncaging of second messengers (Ca^{2+}, cAMP) or neurotransmitters such as glutamate, GABA or glycine.

2.2. Two-photon uncaging: a survey

For all these systems, the chromophoric part can be described as electron donor and/or acceptor groups linked together by conjugated systems. Nevertheless, few classes of cages can be highlighted: the 2-nitrobenzyl series (*o*-NB), 2-nitrophenylalkyl series, 6-bromo-7-hydroxycoumarin-4ylmethyl series (Bhc-), nitroindolinyl series (NI-), 8-bromo-7-hydroxyquinoline series (BHQ-) and *trans*-cinnamate ester series. Generally, the two-photon uncaging action cross sections of described cages are measured, at best, at two wavelengths, around 740 nm (higher efficiency for most cages, but corresponding to low laser efficiency and stability) and around 800 nm (most available lasers). However, only a few cages are described with two-photon uncaging action cross section spectra from 740 to 900 nm.[3b,5,12,20]

Initially, the wildly used *o*-NB and Bhc series were tested for two-photon uncaging, with low efficiencies for the *o*-NB series (<0.3 GM) and better efficiency for Bhc series, both at 740 and 800 nm (0.4 GM and 2 GM, respectively for a broad range of caged substances). Since then, many groups of chemists began to perform molecular engineering on these cages to increase their two-photon sensitivity (either δ_a or Φ_u). For example, the efficiency of the photochemical reaction of the *o*-NB group has been increased by introduction of an electronegative atom in benzylic position[8] compared to the parent simple *o*-NB.[3] Surprisingly, the elongation of the conjugated system by an ethynylbenzene or a vinylbenzene (which should increase the δ_a) did not lead to an increase in the 2P uncaging action cross section,[8] probably due to competitive side photochemical reactions and a poor uncaging quantum yield. In addition, the substitution of the methoxy group by a better electron donating group (mainly OH, NH_2 or NR_2), which should also increase the δ_a, did not increase significantly the two-photon uncaging action cross section, probably because of undesirable photochemical reactions due to these groups.[8] A great improvement in the *ortho*-nitro series was achieved by replacing the benzylic group of the *o*-NB derivatives by a phenethyl group in such systems, leading to more efficient photolysis. The two-photon efficiency of such systems was improved compared to the parent *o*-NB analogs.[17,18] A further improvement was obtained by elongating the conjugated system of these compounds with a stable benzene ring. Such systems can be seen as methoxynitrobiphenyl platforms, which are known to be efficient in two-photon absorption. This modification points out the crucial role of the link between the leaving group and the cage. Indeed, this methoxynitrobiphenyl system linked by a benzylic atom to the released group (*o*-NB series) does not display significant two-photon uncaging action cross section, but with an ethyl junction (phenethyl series) the 2P uncaging action cross sections were significantly increased. Various substances were caged (glutamate, and fluorophores: coumarin or acridinone (DDAO) derivatives) with efficient 2P induced photolysis (about 4 GM at 740 nm and 0.4 GM at 800 nm[19,20]). Another system has been described in 2006, based on a nitrodibenzofurane moiety, leading to $\delta_a\Phi_u$ of 0.6 GM at 720 nm. Structurally, this molecule is similar to a constrained analog of the previously described methoxynitrobiphenyl cage.

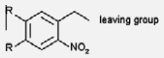
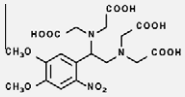
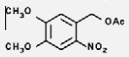
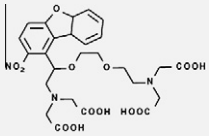
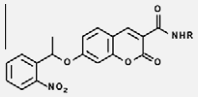
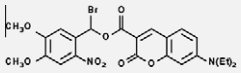
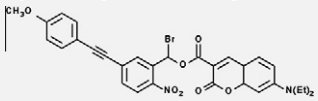
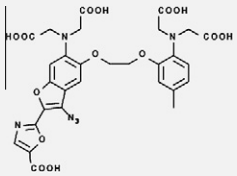
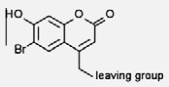
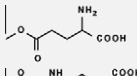
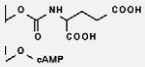
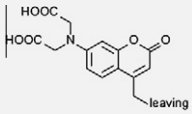
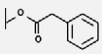
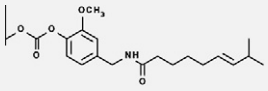
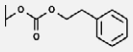
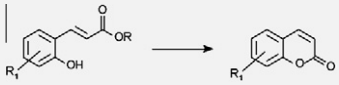
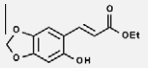
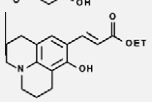
In 2008, another type of systems was introduced, based on symmetrical chromophores contrarily to all previously described donor–acceptor systems. A system based on a symmetrical bis-nitrostilbene was prepared and exhibited unprecedented two-photon uncaging action cross sections (over 3 GM in the 740–800 nm range with a maximum of 5 GM at 800 nm).[5] It takes advantage both of the efficient link between the cage and the biological molecule (nitrophenethyl) leading to very good uncaging quantum yields, and the very high δ_a of the symmetrical bis-acceptor chromophore.

The heteroaromatic quinoline core has also been used to build interesting caging groups for carboxylic acids or phosphates.[16] The 8-bromo-7-hydroxyquinoline (BHQ-series) present biologically relevant 2P uncaging action cross sections in the 0.4–0.6 GM range at 740 nm, while its efficiency drops at 800 nm ($\delta_a\Phi_u$ = 0.087 GM).

Another very elegant and efficient way to release a caged alcohol concomitant to a fluorescent reporter is to use the photoisomerization of *trans*-cinnamate esters.[12] The two-photon sensitivity of such photochemical reactions is excellent (up to 4.7 GM at 750 nm) but, due to the low velocity of the uncaging process following photoisomerization, such cages have to be used to study slow biological processes.

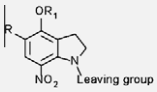
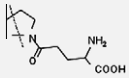
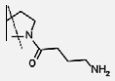
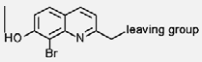
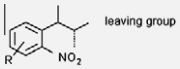
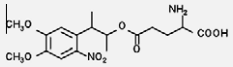
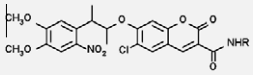
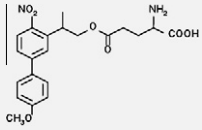
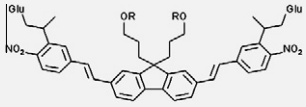
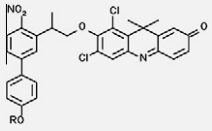
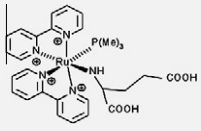
Methoxynitroindolinyl caging groups are commercially available with moderate efficiencies upon two-photon excitation (0.06 GM at 730 nm for glutamate uncaging). Various chemical modifications were also performed on this system, leading first to MDNI, by the introduction of a second nitro group on the benzene ring.[13,15] Unfortunately, the two-photon efficiency $\delta_a\Phi_u$ is not significantly affected by this introduction of an electron

Table 1a
Two-photon sensitive caging groups (**1**/**2**)

Caging group		Two-photon cross section (wavelengths)
2-Nitrobenzyl (*o*NB-) series		
	DM-nitrophen (Ref. 3a)	0.013 GM (730 nm) caged Ca^{2+}
	(Ref. 3b)	0.03 GM (740 nm); 0.01 GM (800 nm) caged acetate
	NDBF-EGTA (Ref. 6)	0.6 GM (720 nm) caged Ca^{2+}
	NPE-hydroxycoumarin (Ref. 7)	0.68 GM (740 nm) caged fluorescence
	(Ref. 8)	0.065 GM (750 nm) caged carboxylate
	(Ref. 8)	0.05 GM (750 nm) caged carboxylate
Azid-**1**		
	AZID-1 (Ref. 3a)	1.4 GM (700 nm) caged Ca^{2+}
6-Bromo-7-hydroxycoumarin-4ylmethyl (Bhc-)series		
	(Ref. 3b)	1.99 GM (740 nm); 0.42 GM (800) caged acetate
	(Ref. 3b)	0.89 GM (740 nm); 0.42 GM (800 nm) caged glutamate
	(Ref. 3b)	0.95 GM (740 nm); 0.37 GM (800 nm) caged glutamate
	(Ref. 9)	2.28 GM (740 nm); ~ 0.30 GM (800 nm) caged cAMP (equiv isomer)
	(Ref. 9)	1.95 GM (740 nm) caged cGMP (equiv isomer)
7-Bis(carboxymethyl)amino]coumarin-4-ylmethyl (BCMACM)series		
	(Ref. 10)	0.38 GM (740 nm)
	(Ref. 10)	2.47 GM (740 nm)
	(Ref. 10)	1.43 GM (740 nm)
	(Ref. 11)	1.24 GM (740 nm)
Coumarins from *trans*-cinnamate esters isomerization		
	(Ref. 12)	3.8 GM (750 nm) caged ROH
	(Ref. 12)	4.7 GM (750 nm) caged ROH

withdrawing group. Nevertheless, substitution of the methyl group on the phenolic part by a carboxylic acid chain increased by a factor 4 the two-photon uncaging action cross section (around 0.24 GM at 720 nm).

Table 1b
Two-photon sensitive caging groups (2/2)

Caging group		Two-photon cross section (wavelengths)
Nitroindolinyl (NI-) series		 R = H; R_1 = CH_3 (MNI) R = NO_2; R_1 = CH_3 (MDNI) R = H; R_1 = CH_2COOH (CNI) R = NO_2; R_1 = CH_2COOH (CDNI)
	MNI-Glu (Ref. 13) MDNI-Glu (Ref. 13) CNI-Glu (Ref. 14) CDNI-Glu (Ref. 15)	0.06 GM (730 nm) caged glutamate 0.06 GM (730 nm) caged glutamate CNI ~1 × MNI (720 nm) caged glutamate CDNI ~4 × MNI (720 nm) caged glutamate
	CNI-GABA (Ref. 14) CDNI-GABA (Ref. 14)	CNI ~1 × MNI (720 nm) caged GABA CDNI ~4 × MNI (720 nm) caged GABA
8-Bromo-7-Hydroxyquinoline (BHQ-) series		
	BHQ-OAc (Ref. 16)	0.59 GM (740 nm); 0.087 GM (780 nm) caged acetate
	BHQ-$OPO(OCH_3)_2$ (Ref. 16)	0.43 GM (740 nm) caged phosphate
o-Nitrophenylalkyl series		
	DMNPB-Glu (Ref. 17)	0.17 GM (720 nm) caged Glutamate
	DMNPB-coumarin (Ref. 18)	0.21 GM (740 nm) caged fluorescence
	PMNB-Glu (Ref. 19)	0.45 GM (800 nm) caged glutamate
	BNSF-Glu (Ref. 5)	5.0 GM (800 nm) caged glutamate
	PENB-DDAO (Ref. 20)	3.7 GM (740 nm) caged fluorescence
Rubidium complex		
	RuBI-Glu (Ref. 21)	

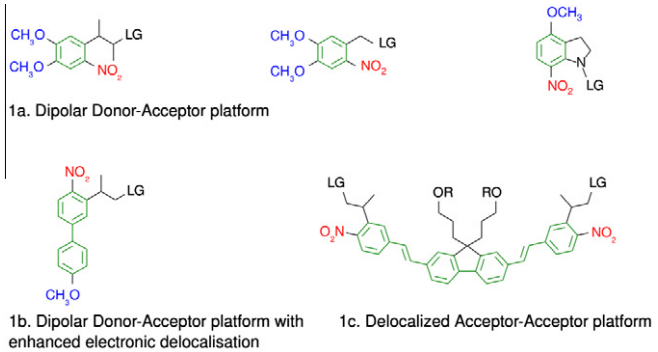


Figure 1. Platforms structures of commonly used cages showing donor groups (blue), acceptor groups (red) and conjugated system (green) (LG = leaving group).

Recently, some coordination complexes have been used for glutamate uncaging, but without determination of the $\delta_a\Phi_u$.[21]

2.3. Photoactivatable fluorescence

Fluorescent imaging techniques have considerably increased our ability to unravel finer details of cellular events. One significant reason for these advances has been the development of fluorescent proteins, because proteins can be genetically encoded and targeted to defined cell types. A further refinement came from 'photoactivatable' fluorescent proteins (PAFPs), a small category of fluorescent proteins[22] that allow for example to lower the diffraction barrier to nanometer resolution and has been used in photoactivated localization microscopy (PALM).[23] The fluorescence characteristics of photoactivatable proteins can be controlled by irradiation at a specific wavelength, intensity and duration, leading to tracking studies in living cells and organelles in a well define, spatiotemporal manner.[24] However, the large size of PAFPs combined with their 'limited' photophysical properties can restrict trafficking studies applications. Those limitations can be overcome by labeling fusion proteins with synthetic, small 'photoactivated' fluorophores (also called caged fluorophores). Besides their advantage in terms of

size, new caged fluorophores specifically engineered for two-photon excitation may limit photoinduced cellular damage and should become important research tools for tracking proteins in cells. This technology may open the way to developments in emerging microscopies with nanometer resolution, such as PALM, which takes advantage of the intrinsic 3D resolution of the two-photon excitation (2P-PALM).[25]

The general strategy for masking fluorescence is to perturb the electronic structure of the fluorophore by attaching a photoremovable group, thereby rendering the molecule either non-fluorescent or very weakly so. The phenolic group of fluorescein was first targeted in this respect;[26] subsequent extensions include rhodamine,[27] Q-rhodamine,[28] resorufin,[28] fluorescein[29] and hydroxycoumarin[7] using *o*-nitrobenzyl (*o*-NB) derivatives as caging groups. Hydroxycoumarin was also modified using the *o*-nitrophenethyl (DMNPB)[18] caging group, resulting in an enhanced two-photon uncaging sensitivity. A different photochemical reaction was recently described on a masked push–pull fluorophore by photochemical conversion of a non-fluorescent aromatic azido-DCDHF label into the corresponding fluorescent anilino derivative.[30] To date, 7-hydroxycoumarin-3-carboxamide derivatives are the only caged fluorophores for which two-photon uncaging excitation has been measured,[7,18] leading to two-photon cross sections $\delta_a\Phi_u \leqslant 0.6$ GM at 740 nm for the 1-(2-nitrophenyl)ethyl cage.

Unfortunately, most of these 'caged' fluorophores will probably have limited use in cell biology either because of the poor 2P uncaging efficiencies of the used photoremovable groups or the unadapted biophysical properties that is, fluorophores which are prone to bleaching or which require too low excitation wavelengths. We have recently developed a new red emitting 'caged' fluorophore,[20] which is excited at wavelengths where tissue turbidity and cellular autofluorescence are low.[31] This molecule uses the chemical and photophysical properties of a 1,3-dichloro-9,9-dimethyl-9*H*-acridin-2(7)-one (DDAO) derivative,[32] a far-red emitting fluorophore (λ_{em} = 658 nm) which was caged using a donor-biphenyl-acceptor platform described previously for caging glutamate[19] and displaying unprecedented two-photon uncaging cross sections at 740 nm ($\delta_a \cdot \Phi_u$ = 3.68 GM).[5]

There is a clear need in caging more suitable fluorophores (red emitting) with better cages. But even with the best 'photoactivatable' fluorophore in hand, one major limitation for visualizing molecular events in living cells remains the specificity of the signal, that is the attachment of the fluorophore specifically to the cellular protein of interest. This limitation can be overcome by using small genetically encoded peptide tags and complementary small organic affinity probes[33] with sufficient specificity and affinity to be used in living cells. To be of practical use, such an approach has to provide a highly specific labeling reaction both in vitro and in vivo.[34]

2.4. Two-photon uncaging in neurobiology

The nervous system is an extremely complex network of highly-specialized cells (around 100 billions) that sense external stimuli and carry information back and forth throughout the body. Each of these neurons is connected to thousands other neurons, making the task of mapping functional connectivity between cells extremely challenging. A prerequisite to understanding such a circuitry is the ability to manipulate precisely the activity of individual neurons. Mapping connectivity between neurons can be done by stimulating a pre-synaptic neuron and recording simultaneously excitatory or inhibitory responses from the post-synaptic cell. Because electrode-based recordings are limited to a few neurons at a time, it quickly became clear that optical methods would enable stimulating multiple areas of the brain, thereby generating precise input maps for neurons.[35] Nearly all neurons are excited by glutamate, making photorelease of glutamate an ideal strategy to stimulate pre-synaptic cells. Yet, one-photon uncaging of glutamate suffers from lack of spatial specificity, because light is scattered by living tissues, resulting in a stimulated area that contains not only the cell of interest but also distant neurons whose dendrites pass by. To circumvent this issue, two-photon uncaging of glutamate has been developed. The first advance came from 'chemical two-photon uncaging', where a single molecule of glutamate is caged by two α-carboxy-2-nitrobenzyl (CNB) caging groups. Photorelease of glutamate in this case requires the sequential absorption of two photons, increasing the probability of double-uncaging at the focal point, and resulting in a significant improvement in the resolution of the stimulated area.[36,37] Later two caged-glutamates with two-photon cross section were developed: Bhc-Glu[3b] and MNI-glutamate.[38,39] MNI-glutamate is commercially available and has contributed importantly to the detailed understanding of functional connectivity. Importantly, two-photon sensitive cages allow a finer spatial resolution but also a deeper tissue penetration, allowing mapping in thick brain slices.[40]

Another great challenge in neurobiology is to study synaptic function and plasticity, that is the modulation of the synaptic strength with time. Two-photon photorelease of glutamate is sufficiently precise that it can be restricted to individual dendritic spines, and sufficiently time-controlled that it can mimic the kinetics and amplitude of unitary excitatory post-synaptic currents.[39,41] Two-photon uncaging of MNI-glutamate has been used to measure the number, properties and location of receptors in single post-synaptic densities, and to induce synaptic plasticity, a remodeling of the synapse widely believed to be the mechanism by which memory is encoded and stored in the central nervous system (CNS).[42–44]

Inhibition in the adult CNS is mainly controlled by the neurotransmitter GABA. Surprisingly, the development of adequate caged-GABA lags behind the caged-glutamates. A number of caged-GABAs have been engineered but they all have unfavorable photochemical (no two-photon cross section) and pharmacological properties (antagonist before photolysis), limiting their application in neurobiology.[38,45–48] Antagonist properties are not desired for at least two reasons: (1) since photorelease is only partial, very high concentrations of caged-compounds are needed, and the excess of uncaged antagonist may mask the effect of photoreleased agonist; (2) incubation with an antagonist may change the physiology of the tissue over time, and induce epileptic events in case of caged-GABA. Recently a visible-light sensitive caged-GABA based on an inorganic ruthenium complex (RuBi-GABA) has been developed.[49] RuBi-GABA shows no antagonistic properties at concentrations used for photolysis. Furthermore because visible light is used instead of UV-light, this allows for a deeper uncaging in tissues than with classical cages. It will be very interesting to see if, like RuBi-glutamate,[21] RuBi-GABA is sensitive to two-photon irradiation. More recently, the first two-photon sensitive caged GABAs have been engineered using the CNI, CDNI and N-DCAC photoremovable groups.[50] Unfortunately, those compounds also interfere with the GABA receptor function before photolysis, questioning the applicability of those probes outside of receptor mapping.

Besides extracellular photorelease of neurotransmitters, photomanipulation of intracellular chemistry (second messengers, transcription factors, RNA...) offers great promise in neurobiology. Second messengers like Ca^{2+}, inositol-1,4,5-triphosphate (IP3), cyclic nucleotides or ATP have been caged and used to control signal transduction cascades with one-photon excitation (for a recent review see.[2c]) Two-photon sensitive caged Ca^{2+}, IP3 and cyclic nucleotides are still under development and haven't been used yet in neurobiology.[3a,6,11,51,52] Two-photon intracellular uncaging can also be used to photoregulate transgene expression with high spatial resolution. This approach has very recently been applied to control the expression of genes in hippocampal cultures using a two-photon sensitive caged-doxycycline, a member of the tetracy-

cline antibiotics that can be used to control transcriptional activation.[53]

Detailed investigations into the complex CNS require the development of better probes. The first need is in two-photon sensitive cages with fast photorelease and good photochemical efficiency, to allow the reduction of both the laser power needed for uncaging (and therefore photodamage to tissues) and the concentration of caged-compound used (and therefore adverse pharmacology). It has been shown for example that the millimolar range of concentrations classically used for MNI-glutamate dramatically antagonizes the GABAergic transmission.[19] Following the development of MNI-Glu, new caged-glutamates with improved photochemical properties have been developed: MDNI-glu,[13] DMNPB-Glu,[17] CDNI-Glu,[15] PMNB-Glu,[19] BNSMB-Glu and BNSF-Glu.[5] The last two compounds largely surpass the others in terms of two-photon cross section, and their use in neurobiology is greatly awaited.

3. Conclusion

Photochemistry provides a non-invasive means to investigate dynamic processes in biology.[2] Photocontrolling the activity of biomolecules can be achieved in different ways, including illumination of naturally photoreactive proteins (i.e., channelrhodopsins), the use of optical switches for reversible control of proteins and finally the uncaging process to release biomolecules. A major progress in this latter field was achieved using two-photon excitation which allowed a tremendous improvement in the spatial resolution while using less harmful and deeper penetrating irradiation wavelengths in the IR range. While there is plenty of room for new chemical developments in the two-photon uncaging field, applications in neurobiology and cellular biology are just beginning and are open to sophisticated developments.

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