Laser photolysis of caged compounds at 405 nm: Photochemical advantages, localisation, phototoxicity and methods for calibration

Federico F. Trigo, John E.T. Corrie, David Ogden

Laboratoire de Physiologie Cérébrale CNRS UMR 8118, Université Paris Descartes, Paris 75006, France
MRC National Institute for Medical Research, London NW7 1AA, United Kingdom

ABSTRACT

Rapid, localised photolytic release of neurotransmitters from caged precursors at synaptic regions in the extracellular space is greatly hampered at irradiation wavelengths in the near-UV, close to the wavelength of maximum absorption of the caged precursor, because of inner-filtering by strong absorption of light in the cage solution between the objective and cell. For this reason two-photon excitation is commonly used for photolysis, particularly at multiple points distributed over large fields; or, with near-UV, if combined with local perfusion of the cage. These methods each have problems: the small cross-sections of common cages with two-photon excitation require high cage concentrations and light intensities near the phototoxic limit, while local perfusion gives non-uniform cage concentrations over the field of view. Single-photon photolysis at 405 nm, although less efficient than at 330–350 nm, with present cages is more efficient than two-photon photolysis. The reduced light absorption in the bulk cage solution permits efficient wide-field uncaging at non-toxic intensities with uniform cage concentration. Full photolysis of MNI-glutamate with 100 µs pulses required intensities of 2 mW µm⁻² at the preparation, shown to be non-toxic with repeated exposures. Light scattering at 405 nm was estimated as 50% at 18 µm depth in 21-day rat cerebellum. Methods are described for: (1) varying the laser spot size; (2) photolysis calibration in the microscope with the caged fluorophore NPE-HPTS over the wavelength range 347–405 nm; and (3) determining the point-spread function of excitation. Furthermore, DM-Nitrophen photolysis at 405 nm was efficient for intracellular investigations of Ca²⁺-dependent processes.

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1. Introduction

Photolysis to release neurotransmitters, hormones or neuro-modulators in the extracellular solution from inert ‘caged’ precursors is widely applied in neuroscience to mimic the timescale and localisation of physiological responses. Recent improvements in the chemistry of caged neurotransmitters, particularly the development of the hydrolytically stable, fast (sub-µs) nitroindoline-caged amino acids (Papageorgiou et al., 1999; Morrison et al., 2002), permits the use of photolysis with near-UV excitation (reviewed in Thompson et al., 2005) or pulsed IR with the two-photon effect (Matsuzaki et al., 2001; Smith et al., 2003) to mimic synaptic activation. The precision and speed of laser scanning microscopes (Gasparini and Magee, 2006; Losonczy and Magee, 2006) or of holographic techniques (Lutz et al., 2008) can be used to localise release. Photolysis in the extracellular solution in brain slice or in vivo preparations utilises water-dipping objectives with working distances of 2–3 mm to permit micropipette recording and stimulation while retaining the optical resolution needed to optimise imaging and photolysis. However, study of neurotransmitter receptor properties in the physiological range requires high (mM) neurotransmitter concentrations at the receptors and therefore mM cage concentrations in the external solution. At the peak absorption wavelength of 320–360 nm for commonly used near-UV cages, the light absorption in the cage solution itself between the objective and the preparation is so strong at concentrations greater than about 1 mM that little light reaches the preparation.

Two approaches have been taken to minimise this problem, first the use of two-photon excitation where there is no light absorption outside the focus, and second, in near-UV photolysis, with application of the cage by local perfusion to minimise the depth of the absorbing medium. Both approaches have problems. The first method, two-photon photolysis, is problematic because of poor two-photon light absorption by the cages. At non-toxic light intensities, of average power around 5 mW, the small two-photon cross-sections of currently available cages (less than 0.1 GM, see Brown et al., 1999; Matsuzaki et al., 2001; Kiskin et al., 2002) give much less than 10% conversion of the cage concentration in the two-photon excitation volume with the short exposures needed for localisation of ligand release. For this reason in studies with...
two-photon uncaging the light intensities appear to be near the limits imposed by phototoxicity (for photolysis see Kiskin et al., 2002; for imaging see Koester et al., 1999; Hopt and Neher, 2001; Ji et al., 2008) and exposures are usually several fold longer than the optimum of <100 µs required to confine release to the excitation volume. The second approach, local perfusion with cage solution combined with efficient near-UV photolysis, has problems of variable penetration of the cage into the slice and non-uniform concentrations in the tissue away from the pipette tip (Dittman and Regehr, 1997; DiGregorio et al., 2007; see Fig. 12 below). The concentrations are reduced and unknown over most of the microscope's field of view, precluding the rational use of local perfusion in quantitative studies with wide-field methods such as multi-spot uncaging or with large area holographically shaped uncaging regions.

The method described here uses excitation at 405 nm, where light absorption by the cage is reduced sufficiently to permit penetration to the preparation. The reduced photolysis efficiency at the longer wavelength can be compensated by using higher light intensity, while remaining at non-toxic levels. This paper provides an empirical assessment of the use of 405 nm laser light for photolysis, showing its advantages for wide-field applications particularly with the nitroindoline cages. It describes the overall efficiency of photolysis at 405 nm, a method for calibration of photolysis in the microscope at 405 nm and near-UV wavelengths, and the efficiency relative to phototoxicity in synaptic experiments. A custom optical arrangement producing a small diffraction-limited spot and/or a larger area laser spot with independent sub-millisecond timing in a slice microscope is described, as well as a convenient adaptation allowing commercial microscopes to use 405 nm laser input via the epifluorescence condenser.

2. Materials and methods

2.1. Electrophysiological recording

Sprague Dawley rats aged 15–23 days old were used to prepare sagittal cerebellar slices following institutional guidelines. Briefly, the animal was placed under deep anesthesia using isoflurane and was decapitated. The cerebellar vermis was quickly removed. Parasagittal slices (200 µm thick) were cut with a vibrissclicer (Leica VT1200S; Leica Microsystems, Wetzlar, Germany) in an ice-cold artificial cerebrospinal fluid (ACSF) and then placed in an incubating chamber for 60 min at 34 °C. Thereafter slices were kept at room temperature. The same bicarbonate-buffered saline was used as the cutting and storing solution; composition (in mM): 122.5 NaCl, 2.5 KCl, 26 NaHCO3, 1.3 NaH2PO4, 25 glucose, 2 CaCl2 and 1 MgCl2, osmolality adjusted to 300 mOsm kg−1 H2O and pH set at 7.4 by the continuous bubbling of a mixture of 5% CO2 and 95% O2. When the animal was placed under deep anesthesia using isoflurane (Cambridge Electronic Design, Cambridge, UK) and analysis was in Image J or Igor Pro.

Two optical arrangements are described, one using optical components arranged as a breadboard microscope, the other an adaptation of commercial microscopes for stationary spot photolysis with CCD imaging.

2.2. Custom two-spot microscope

The microscope was based around a Leica infinity corrected 63 × 0.9 NA water-dipping objective (HCX Apo U-V1) mounted in a PIFOC (Physike Instrumente, Germany) piezo focus vertically on a linear translation stage. Light transmission in the objective at 405 nm was estimated as 50%. Transmitted light was from a green LED (Luxeon K2) via a 0.9 NA darkfield condenser. Epifluorescence illumination was achieved by a blue LED (470 nm peak, Luxeon K2 with excitation filter 470/40 nm) gated ON only during camera exposure to minimise bleaching. The preparation was viewed with an EMCCD camera (Andor Ixon, 512 × 512, effective pixel size 0.25 µm, controlled by Andor software) via an ET series FITC dichroic reflector and emission filter set (Chroma Technology, Rockingham, USA). The preparation stage and micromanipulators were moved together on a micrometer controlled x–y stage, with 30 µm travel piezo elements inserted between the micrometers and the stage to permit 100 nm precision fine movements. The CCD camera was mounted on a rotating x–y translation stage to permit precise orientation of the camera chip for subsequent alignment with filled neurons.

2.2.1. Laser inputs

Two 405 nm laser inputs with independent timing and position could be used in the same experiment or independently. One was arranged to produce a large area irradiation (<80 µm2), the other a small, sub-µm spot. The first was a Compass 405-50 (Coherent, Germany; 50 mW CW) with free space emission and a shutter (Uniblitz, Vincent Associates, Brattleboro, USA) to prevent excitation by the diode leakage light, the other an Illex 2000 405 nm (33 mW CW; Point Source, Southampton, UK) coupled with a polarisation-maintaining single mode fibre. Laser timings were controlled by a Digimter D4040 (Digitemter, Welwyn, UK) via TTL buffer amplifiers, and a computer interface (CED Power 1401 Cambridge Electronic Design, UK), running either Spike 2 (CED) or WinWC (Dr John Dempster, University of Strathclyde).

A diagram of the light paths is given in Fig. 1A. The laser inputs were arranged with orthogonal polarisations and combined in a polarising beam combiner (Halio Optics PBC20, Chelmsford, UK). The Compass 405-50 output was via a rotatable half-wave plate to permit variable attenuation of this path in the polarising beam combiner. Beam expanders were placed in the output of both lasers; the output of the Point-Source laser filled the back of the objective by 90% (e−2) to generate a submicron spot in the objective focus. To generate a large area spot the output of the Compass 405-50 was either focused into the back focal point of the objective (as standard Koehler epifluorescence illumination) producing a cylindrical beam with Gaussian profile and diameter varied by an iris diaphragm, or the spot size was varied by the degree of defocus of an expanded beam. The optical components were arranged on a vertical breadboard and light output was delivered to the microscope via a Z405RDC dichroic reflector (Chroma Technology, Rockingham, VT). Before entering the microscope 10% of light was reflected by a cover slip onto a large area photodiode (1 cm2, T2010 Thorlabs, Ely, UK), reverse biased and with a current/voltage amplifier, to record the total input laser power to the microscope with 1 µs time resolution. Laser power exiting the objective was calibrated against photodiode current with a Fieldmation power meter (PD-VIS detector, Coherent, France).
2.3. Implementation in commercial microscopes

The epifluorescence condenser in an imaging microscope is designed to produce an output focused at the back focal point of the microscope objective when given a collimated input from the lamphouse. To produce a sub-micron spot in the focal plane of the objective requires an expanded collimated beam with diameter similar to the objective back aperture and was achieved with a commercial microscope by introducing a strong negative lens to diverge a collimated laser input. The collimated output of a fibre-coupled 405 nm laser (Point Source Iflex2000, single mode fibre, collimated output 1.3 mm diameter) was diverged with a 25 mm focal length negative lens in a dual LED lamphouse (OptoLED, Cairn Research, Faversham, UK) reflecting the light with a 45° dichroic reflector (425 DCXR, Chroma) into the epifluorescence condenser of a Zeiss Axioskop FS1 microscope. The dichroic mirror could be tilted in two dimensions to align with the optical axis of the microscope, the laser input and negative lens were adjusted in three axes to position and focus the spot. When implemented in an Axioskop FS1 the optical length from the fibre output to epifluorescence input was increased to 230 mm by a focusing extension tube (Comar, Cambridge) between the LED lamphouse and microscope. A minimal spot of approximately 1 μm diameter could be formed at the focus seen in 100 μM pyranine (HPTS) solution viewed with the CCD camera. The second, straight-through path of the lamphouse contained a blue LED with collimating lens and excitation filter for simultaneous conventional 470 nm epifluorescence excitation. This optical arrangement, shown in Fig. 1B, should be generally applicable in commercial epifluorescence microscopes by simple adjustments of the optical lengths and by ensuring the epifluorescence dichroic reflector of the microscope reflects 405 nm to the objective.

2.4. Comparison of photolysis efficiencies of MNI-glutamate and NPE-HPTS at 347 nm and 405 nm

Details of the structure and characteristics of photolysis at near-UV wavelengths of NPE-HPTS (the 1-(2-nitrophenyl)ethyl ether of pyranine) are given by Jasuja et al. (1999; see also Canepari et al., 2001; Kiskin et al., 2002).

2.4.1. Calibrations at 347 nm

The efficiencies of photolysis of MNI-glutamate and NPE-HPTS were compared at 347 nm as follows. Single 20 ns pulses of 90 ± 5 mJ energy were delivered to a 1 mm path length cell from a frequency doubled ruby laser (Lumonics QSR2). Twenty-five aliquots, each of 15 μl of NPE-HPTS (0.465 mM) or 0.47 mM NPE-HPTS were irradiated. Solutions were prepared in 25 mM Na phosphate buffer pH 7 containing 5 mM dithiothreitol. The aliquots of MNI-glutamate were combined and analysed by reverse-phase HPLC (Merck Lichrospher 250 mm × 4 mm RP8 column) with mobile phase 25 mM Na phosphate pH 6 – MeCN (5:1 v/v) at flow rate 1.5 ml min⁻¹, retention time 6.3 min. The extent of photolysis was estimated as 30.1% from comparison of the peak height with that of a non-irradiated control. The extent of photolysis of the NPE-HPTS was estimated by fluorescence methods. An aliquot of 200 μl of the irradiated NPE-HPTS solution was diluted to 2 ml with CAPS buffer 100 mM pH 10.5 and measured on a Cary Eclipse fluorimeter (excitation 454 nm 2.5 nm slit, emission 510 nm, 5 nm slit). Standard solutions of HPTS in the CAPS buffer were prepared to make a linear calibration curve in the range 0.5–8 μM. The unphotolysed solution gave a reading corresponding to 1 μM free HPTS and the photolyzed solution 23.4 μM, a net production of 22.4 μM HPTS corresponding to 4.8% photolysis. Thus, the relative efficiency of photolysis of MNI-glutamate to NPE-HPTS at 347 nm is 6.3:1.

2.4.2. Calibrations at 405 nm

Experiments to determine the relative efficiencies were made with long exposures at low laser power. The beam of a Compass 405-50 (Coherent; 1/4” beam diameter 3 mm) irradiated a 4 mm path-length cuvette, volume 185 μl. The 405 nm light in CW (continuous wave) regulated modulator was shuttered to give periods of 50 s exposure at 2 mW, monitored continuously with a photodiode. Solution was mixed between exposures and the total exposure time was 600 s. For MNI-glutamate the initial concentration was 267 μM, estimated from the absorption coefficient of MNI-glutamate at 405 nm (see Fig. 2) to absorb approximately 10% of the laser light passing through the cuvette. Solution contained NaCl 140 mM, 10 mM HEPES pH 7.3 and glutathione 5 mM to reduce the reactive nitrosoindole by-product for long-term storage. The solution was collected at the end of exposure and analysed for free glutamate (Pharmacia AlphaPlus Analyser with ninhydrin detection). Parallel non-irradiated control samples were treated and analysed in the same way. The free glutamate concentrations in the irradiated and non-irradiated aliquots of MNI-glutamate were 12.1 μM and 0.9 μM, respectively from an initial cage concentration of 267 μM, giving 4.2% conversion. The output of the photodiode was integrated and the results were expressed as the fraction photoyster per J of photon energy applied, giving 31.1 × 10⁻³ J⁻¹. Data were also obtained for GABA release from the nitroindoline GABA cage DPNI-
GABA (Papageorgiou and Corrie, 2007) and gave 4.3% conversion over a similar period of exposure, yielding $3.65 \times 10^{-3}$ J$^{-1}$.

Photolysis at 405 nm of NPE-HPTS (Tocris, Bristol, UK) in the 4 mm cuvette was measured as the fluorescence of free HPTS generated during photolysis. Fluorescence excitation was by the 405 nm laser light used for photolysis and emission monitored by a photodiode and 520 nm bandpass filter (FWHM 40 nm). The laser power was 2 mW CW and the NPE-HPTS concentration was 75 µM (in 100 mM borate buffer pH 9), calculated to give 10% absorption. Initially fluorescence was recorded with 75 µM HPTS solution in the cuvette to check for stable fluorescence records, and the solution was then changed to 75 µM NPE-HPTS in borate buffer to monitor fluorescence generated by photolysis. As above, exposures were for 50 s periods with solution mixing between each exposure. At the end of the photolysis period the solution was replaced by 75 µM HPTS to obtain a second fluorescence reading corresponding to complete photolysis. The fluorescence due to photoreleased HPTS at the end of 50 s periods was normalised to that of 75 µM free HPTS and plotted against the cumulative light exposure in J. The data showed a linear increase of normalised fluorescence with integrated light energy, with slope 0.099 J$^{-1}$. Conversion of MNI-glutamate relative to NPE-HPTS is therefore 0.314:1 at 405 nm and of DPNI-GABA 0.369:1.

2.4.3. Procedure for preparation of NPE-HPTS aqueous vesicles in Sylgard

The following components are required: Sylgard 184 (Dow-Corning, components mixed 10:1); 1 ml Epipendorf tubes; micro-electrode glass with flame-polished ends for mixing; a hot plate regulated at 50–60 °C; 10–22 mm coverslips; NPE-HPTS at 50 µM in Na borate buffer 100 mM pH 9.0. Room lighting should be free of near-UV, e.g. by tungsten light. The procedure is to mix 10 µl of the 50 µM NPE-HPTS solution into 100 µl Sylgard by stirring; vigorous mixing generates small vesicles <5 µm, while gentle mixing gives larger vesicles up to 30 µm diameter. 1 drop of vesicle suspension is placed onto each coverslip and left for >30 min (preferably overnight) for air bubbles to disperse. The Sylgard is cured by warming at 60 °C, covered with a lid, for 7–10 min. The vesicle size range is checked in a microscope. Vesicles and NPE-HPTS are stable for many months in the dark at 4 °C.

3. Results

3.1. Comparison of light loss at 405 nm vs. 351 nm at high cage concentration

The basis for use of 405 nm excitation can be seen by reference to Fig. 2, which shows quantitative near-UV–vis absorption spectra for MNI-caged glutamate (solid curve) and for the nitrosoindole by-product following complete photolysis (dashed curve). At 405 nm absorption by MNI-glutamate is 10% of the peak absorption at 335 nm, resulting in better penetration of light through the cage solution above the preparation. However, photolysis at 405 nm is useful only if the greater intensities needed for full photolysis are demonstrably non-toxic, and, secondly, if the nitrosoindole by-product does not interfere with excitation. The nitrosoindole absorbs strongly at 405 nm, approx 18 times more than MNI-glutamate, and has been noted previously to interfere with fluorescence excitation in imaging measurements (Canepari et al., 2004).

To measure directly the attenuation of light a second photodiode was mounted beneath the bath in addition to that measuring light input, and the cage concentration in HEPES-buffered saline (pH 7.3) was progressively decreased by dilution. Fractional light transmission at different MNI-glutamate concentrations was measured relative to zero concentration controls obtained before and after. The arrangement of components is shown in Fig. 3A. The laser focus was set just above the chamber base and the light at different intensities and pulse durations transmitted to the photodiode PD2 under the experimental chamber were recorded. Data were obtained at pulse durations of 0.02–10 ms and intensities up to the maximum power available from each laser, in the presence or absence of MNI-glutamate at concentrations up to 5.5 mM. Fig. 3B shows the output, $I_s$ of the bath photodiode PD2 recorded in the absence or presence of 2.7 mM and 5.5 mM of MNI-glutamate with a submicron spot in the focal plane at input power $I_0 = 1$ mW after the objective. The fractional transmission recorded in a series of measurements is plotted in Fig. 3C against the MNI-glutamate concentration, c, present in the bath. The data were fitted with the relation $log (I_s/I_0) = \epsilon_{405} c \times x$ with $x = 0.23$ cm, the working distance of the water-dipping objective. The estimated absorption coefficient was $\epsilon_{405} = 510 \text{ M}^{-1} \text{ cm}^{-1}$, in good agreement with the value of 488 M$^{-1}$ cm$^{-1}$ obtained at 405 nm with the quantitative near-UV–vis absorption of MNI-glutamate shown in Fig. 2.

The fractional transmission of photolysis light to the specimen calculated for the same conditions of path length 0.23 cm and the same range of MNI-glutamate concentration but at shorter wavelength of 351 nm, near the peak near-UV absorption, is plotted for comparison in Fig. 3C (blue curve). Data are calculated from the measured absorption coefficient $\epsilon_{351} = 4420 \text{ M}^{-1} \text{ cm}^{-1}$. The transmission declines steeply with cage concentration, at 1 mM MNI-glutamate it is 0.11 of control, at 2 mM 0.011 and at 5 mM 0.025 of control. At 405 nm corresponding transmissions are 74% at 1 mM, 55% at 2 mM and 23% at 5 mM. The relative photolysis efficiency at the two wavelengths can be calculated by assuming that the quantum yield of MNI-glutamate is independent of wavelength (for evidence see description of Fig. 6), so the fraction photolysed at a given intensity is proportional to the ratio of absorption coefficients, in this case 11% on going from 351 nm to 405 nm.
Comparison of the overall photolysis between 405 nm and 351 nm excitation, taking account of light loss in the bath and of the reduced photolysis efficiency, generates the relation shown in Fig. 3C as the dashed line, plotted here on a log ordinate (right-hand axis). Equal conversion at 405 nm and 351 nm for 2.3 mm solution depth was at MNI-glutamate concentration of 1.12 mM, and overall conversions for the same intensities after the objective at 405 nm compared with 351 nm are five-fold greater at 2 mM and 1642-fold greater at 5 mM cage concentration.

3.2. Loss of light by absorption in the nitrosoindole by-product

The nitrosoindole by-product shown in Fig. 2 has an absorption coefficient 18 times higher than MNI-glutamate at 405 nm. The interference with photolysis excitation at 405 nm was investigated here in three optical configurations by recording light transmitted to the bath photodiode PD2 (configuration shown in Fig. 3A). The configurations tested were (i) the submicron spot produced by the objective nor a large spot created by a defocused expanded beam (Fig. 4A) showed time-dependent changes in transmission. Thus, accumulation of the nitrosoindole by-product in the optical path is expected to reduce photolytic efficiency over ten millisecond time-periods only with collimated beams of 405 nm light. The lack of interference in the case of the focused beam (Fig. 4A) is due to the $1/z^2$ dependence of intensity on displacement, generating nitrosoindole at a high rate close to the focus and correspondingly slower rates further away as the intensity drops steeply. In contrast the approximately constant intensity with distance of a collimated beam generates the nitrosoindole over a longer path. Consequently, the optical convenience of a collimated uncaging beam (Fig. 4B), which is easily changed in diameter by an iris and in intensity with the laser modulation, is offset by a requirement to operate with short exposures of <4 ms to avoid nitrosoindole accumulation.

In contrast to the nitrosoindoles, the nitrosocarbonyl by-products produced by nitrobenzyl or (nitrophenyl)ethyl cage photolysis have been found not to interfere with fluorescence measurements, for example comparing MNI-glutamate with a (nitrophenyl)ethyl caged glutamate (1-[(2-nitrophenyl)ethoxycarbonyl]-caged glutamate; Canepari et al., 2004). Furthermore, when necessary they can be inactivated by including glutathione or dithiothreitol in the solution (Walker et al., 1988; Barth et al., 1997). With the nitrosoindole by-product of nitroindoline photolysis the thiol reaction has been shown to be too slow to be useful in this respect (half-time approximately 40 s with 1 mM DTT; Papageorgiou et al., 2008).

3.3. Localisation of photolysis—the point-spread function of the minimised laser spot

The distribution of light intensity in the minimised focal spot, the point-spread function of the laser spot, was recorded at different z displacements of the objective by the light reflected from a plane mirror back through the objective and the 405 nm dichroic, sufficient light reaching the CCD camera to map the distribution of light at the objective focal plane with 0.25 μm pixel resolution. The laser spot size seen as fluorescence at the focus of the objective in a HPTS solution (100 μM, pH 7.5) was first minimised by adjusting the beam expander. The emission filter was removed and the focus stepped in 0.1 μm increments over ±4 μm from the central position, with a 128 × 128 frame recorded in each position. The stack of images generated correspond to confocal planes separated by 0.2 μm in z, with 0.25 μm pixel size in the x–y plane. This convenient optical arrangement is equivalent to a second objective used with paired light pulses and with beams of different diameter showed that the slow change was due to diffusional equilibration of the by-product with the bath solution rather than a slow step in the generation of the by-product. In contrast, neither the minimal focal spot (Fig. 5A) produced by a collimated expanded beam at the back of the objective nor a large spot created by a defocused expanded beam (Fig. 4A) showed time-dependent changes in transmission.

Fig. 5. Distribution of light intensity near the focus. Light from a focused beam in the microscope (shown in Fig. 1A) was reflected back to the camera by a plane mirror and the focus stepped through ±4 μm in 0.1 μm steps, thus using the microscope objective as a telescope and the camera to record the intensity distribution in each plane separated by 0.2 μm. (A) Intensity on a black-low to white-high 8 bit pseudocolour representation at the best focus. (B) Line profile in x across the brightest pixel in (A) fitted with a Gaussian function \( (\frac{1}{\sqrt{2\pi}}) \) diameter 1.1 μm). (C) Pseudocolour representation of the z-section projected from the line intensity profile in (B). (D) Shows the line profile in z of intensity plotted through the pixel of highest intensity in (C) and (A). The distribution of intensity is asymmetric, positive values away from the objective; best fit Gaussian has \( (\frac{1}{\sqrt{2\pi}}) \) diameter 4.8 μm. Scale bars 2 μm in (A) and (C).

to view the planes above and below the focus (D. Wokosin, personal communication; Botcherby et al., 2007). The images were analysed with stack processing routines available in Image J (NIH). The profiles obtained are shown by the images and plots of Fig. 5. Data were fitted by Gaussian functions to obtain the \( (\frac{1}{\sqrt{2\pi}}) \) width. At the focus the x–y profile shown in panel 5A was symmetrical with \( (\frac{1}{\sqrt{2\pi}}) \) diameter 1.1 μm determined from the graph plotted in panel 5B. The intensity distribution in the z-axis at the peak x–y intensity is shown by the 8-bit pseudocolour z–x representation in panel Fig. 5C. When plotted the data were asymmetric, showing a steeper slope above than below the principal focus, with approximate \( (\frac{1}{\sqrt{2\pi}}) \) diameter of 4.8 μm shown in Fig. 5D.

3.4. Comparison of responses to photoreleased glutamate with spontaneous glutamatergic synaptic currents

The ability to mimic the fast time course of synaptic activation by photorelease of l-glutamate from MNI-glutamate with 405 nm laser light was tested here by comparing the time course of photolysis-evoked currents with spontaneous post-synaptic currents in cerebellar molecular layer interneurons. Fig. 6 shows currents evoked in 2 mM MNI-glutamate by 100 μs pulses at 0.8 mW in a molecular layer interneuron (MLI) of a 17 day rat cerebellum in TTX-containing solution. Panel Aa shows spontaneous currents superimposed and aligned (black) and the averaged trace (white). Panel Ab shows spontaneous currents superimposed and aligned (black) and the averaged trace (white). Panel B shows spontaneous post-synaptic currents on the left and photolysis-evoked currents of similar amplitude on the right, and panel C averaged records normalised to the same peak. It can be seen that the rise of the photolysis currents is slow compared to the synaptic events, 10–90% rise in 0.85 ms vs. 0.32 ms for the miniature synaptic currents. The peak of the photolysis current is broader and the decline slower than the synaptic currents, indicating that the localisation is not as good for the photolytic release relative to the synaptic events.

3.5. Calibration of photolysis with the caged fluorophore NPE-HPTS

The efficiency of photolysis depends firstly on the photochemical properties of the cage, defined by the absorption coefficient (or cross-section) at the excitation wavelength, the quantum yield and the release kinetics, and second, on the efficiency of the microscope in transmitting excitation light to the preparation. The efficiency of photolysis in a particular experimental microscope requires empirical determination and may change progressively due to changes in excitation source efficiency or light transmission to the preparation. Well-characterised caged fluorophores of known photochemical efficiency that are quenched in the caged form and release fluorophore quantitatively on uncaging provide a convenient means of calibration by epifluorescence imaging or photometry of the released fluorophore. This allows the yields of other cages in the same microscope to be calculated by comparison with the known photolytic efficiency of the caged fluorophore. NPE-HPTS has proved suitable in this context, the photolysis effi-
Fig. 6. Comparison of currents evoked by photolysis of MNI-glutamate with spontaneous excitatory post-synaptic currents in a cerebellar molecular layer interneuron. Rat 17 days. Holding potential –67 mV; K-glucurate based internal solution, HEPES-buffered external solution containing 0.5 μM TTX and 2 mM MNI-glutamate. Panel Aa: Spontaneous events (black traces) and the average (white trace). Panel Ab: Currents evoked by photolysis with 100 μs pulses of 0.8 mW (black) and the average (white). Panel B: Single spontaneous events (left traces) and photolysis-evoked currents (right). Panel C: Averaged spontaneous current and photolysis-evoked currents normalised and superimposed.

Fig. 7. Near-UV–vis spectra were determined for MNI-glutamate and for NPE-HPTS in 25 mM Na phosphate pH 7.0. Dotted curve: MNI-glutamate. Solid curve: NPE-HPTS. Absorption coefficients for MNI-glutamate and NPE-HPTS: at 347 nm: 4606 M⁻¹ cm⁻¹ and 7405 M⁻¹ cm⁻¹, respectively; at 405 nm: 488 M⁻¹ cm⁻¹ and 17805 M⁻¹ cm⁻¹.

Fig. 3.5.1. Photolysis efficiencies of MNI-glutamate and NPE-HPTS at 347 nm and 405 nm

Quantitative near-UV–vis spectra of MNI-glutamate and NPE-HPTS are shown in Fig. 7. The absorption coefficients vary considerably in the range of interest for photolysis between 300 nm and 405 nm, thus with monochromatic light of different wavelengths the photolysis efficiencies might be expected to vary correspondingly. This was tested here. Comparative data were obtained with laser sources at 405 nm and 347 nm, the latter close to the peak efficiency for near-UV photolysis and to the widely used wavelengths of 351 nm and 364 nm (Argon ion) and 355 nm (DPSS or tripled YAG) lasers.

3.5.1.1. Photolysis at 347 nm. The procedures used for photolysis at 347 nm are described above in Section 2. Briefly, single 20 ns pulses of 90 ± 5 mJ energy were delivered to a 1 mm path-length cell from a frequency doubled ruby laser (Lumonics QSR2). Photolysis of MNI-glutamate (0.47 mM) was assayed by reverse phase HPLC and photolysis of NPE-HPTS (0.465 mM) by fluorimetry of released HPTS. Photolysis of MNI-glutamate was estimated as 30.1%, of NPE-HPTS as 4.8%. Thus, the relative efficiency of photolysis of MNI-glutamate to NPE-HPTS at 347 nm is 6.3:1.

3.5.1.2. Photolysis at 405 nm. Experiments to determine the relative efficiencies at 405 nm were made with long exposures of 600 s at low CW laser power with periodic mixing of the solution (see Section 2). Conversion of MNI-glutamate (0.267 mM) was assayed by amino acid analysis, that of NPE-HPTS by the rate of fluorescence generation in a 75 mM solution relative to that of 75 mM HPTS in the same conditions (pH 9, 100 mM Na borate). Conversion of MNI-glutamate per Joule of input energy was 31.1 × 10⁻³ J⁻¹, that of NPE-HPTS at 405 nm 0.099 J⁻¹, a ratio of 0.314:1 MNI-glutamate relative to NPE-HPTS.

The results show that, for monochromatic light, at 347 nm the relative efficiency for photolysis of MNI-glutamate is 6.3-fold larger than NPE-HPTS and, at 405 nm MNI-glutamate is 0.314-fold that of NPE-HPTS. The 20-fold difference in relative efficiencies at the two wavelengths can be accounted for by comparing the absorption spectra of MNI-glutamate and NPE-HPTS, shown in Fig. 7. The ratios
of absorption coefficients MNI-glutamate to NPE-HPTS is 0.622 at 347 nm and 0.027 at 405 nm, a factor of 23-fold. Since the efficiency at each wavelength is proportional to absorption coefficient times quantum yield \( Q \), the similarity indicates that the changes are accounted for by the large changes in the absorption coefficients with wavelength and that \( Q \) does not change. If \( Q \) is the same at 347 nm and 405 nm, it indicates that it is independent of wavelength and the relative photolysis efficiency of the two cages scales with the ratio of absorption coefficients in the range 347–405 nm. This in turn indicates that the calibration procedure with NPE-HPTS can be applied for sources at other wavelengths, e.g. for argon ion at 351 nm and 362 nm, for DPSS sources at 355 nm and diode lasers at 370–380 nm, by taking the ratio of absorption coefficients and assuming a constant quantum yield.

It is interesting that the absorption spectrum and photolysis of NPE-HPTS differ so markedly from that of the NPE chromophore alone, and suggests a positive interaction between the two chromophores in photolysis, increasing the efficiency at longer wavelengths above that expected for NPE cages such as NPE-ATP (see the action spectrum in McCray, 1998). It has been reported in this context that a nitrobenzyl-caged 7-hydroxycoumarin compound shows evidence of an interaction between its two chromophores that enhances the efficiency of photolysis (Zhao et al., 2001). Our data indicate that a similar interaction occurs between the two chromophores of NPE-HPTS to increase efficiency at 405 nm relative to that expected for the NPE chromophore alone.

### 3.5.2. Calibration in the microscope

The release of HPTS by photolysis of NPE-HPTS can be used to calibrate the efficiency in the experimental microscope by monitoring the fluorescence released by brief flashes of known energy from NPE-HPTS contained in aqueous vesicles suspended in Sylgard. The release of HPTS is detected by epifluorescence with 450–490 nm excitation, 520–550 nm emission. In the example given below detection is with a CCD camera but in a previous description was done with a PMT detector and adjustable diaphragm to isolate fluorescence from individual vesicles (Canepari et al., 2001). The fraction converted per flash, \( \alpha \), is determined by plotting the fluorescence increase \( F \) with flash number \( N \). Taking the exponential approximation for small \( \alpha \) for the series \( F = (1 - \alpha)^N \), data fitting with an exponential curve gives \( F = \exp(-\alpha N) \). The approximation tested numerically shows that values of \( \alpha \) obtained from a least squares exponential fit are estimated well at \( \alpha < 0.1 \), overestimated by 5% at \( \alpha = 0.1 \) and by 10% at \( \alpha = 0.2 \). Thus, the exponential function is a good approximation at values of \( \alpha < 0.1 \), however for larger conversions an empirical correction, \( \alpha(\text{corr}) = \alpha(\text{fit}) - 0.35\alpha(\text{fit})^{1.7} \), can be applied as described by Canepari et al. (2001).

The procedure is illustrated by the results in Fig. 8. Vesicles of a range of diameters were irradiated successively with 1 ms pulses of 405 nm at constant intensities. The progressive change in fluorescence was plotted as shown in Fig. 8C and fitted with an exponential curve to determine the photolysis per 1 ms flash. The total light intensity after the objective was determined separately at each intensity with a power meter, the fraction of the light irradiating the vesicle was determined by image analysis of the fraction of the light falling on an area of corresponding size and the result expressed as energy density over the vesicle area. Finally, the conversions obtained with several vesicles of different sizes and at different intensities were plotted against the energy density. In the example shown in Fig. 8D a slope of 1.58% conversion per nJ \( \mu \text{m}^{-2} \) was obtained in six vesicles. This indicates that 1.00158 ± 64 nJ \( \mu \text{m}^{-2} \) would be required for complete conversion of NPE-HPTS. The relative photolysis efficiency of MNI-glutamate to NPE-HPTS of 0.314 at 405 nm, determined above, gives the result that complete photolysis of MNI-glutamate requires 204 nJ \( \mu \text{m}^{-2} \). The reduced photolysis efficiency at 405 nm relative to the peak at 330–350 nm requires higher light intensities at the preparation than would be the case at shorter wavelengths and raises the possibility that phototoxicity may be a limiting factor. The phototoxicity of 405 nm photolysis in synaptic experiments was determined by monitoring the stability of the amplitude, rise time and decay time of currents repetitively evoked by \( \gamma \)-glutamate release from MNI-glutamate at low and high intensities. Recordings were from cerebellar molecular layer interneurons or from Purkinje neurons. Cells were labelled with fluorophore Alexa 488 to identify dendrites and axons in the CCD camera image and hot-spots of activity were identified on dendritic processes in recordings of the evoked currents. Results of experiments with photorelease of \( \gamma \)-glutamate from MNI-glutamate, activating AMPA-subtype glutamate channels in a MLI are shown in Fig. 9. Pulses of 100 \( \mu \text{s} \) duration and power 0.8, 1.3 or 1.8 mW at the preparation after correction for loss in the microscope from MNI-glutamate (and other nitroindolines) relative to NPE-HPTS assuming constant quantum yield is given by the ratio of the integrals of the cage and NPE-HPTS absorption spectra in that range. For the range of wavelengths 280–370 nm transmitted by the commonly used Schott UG11 filter the absorption of MNI-glutamate to NPE-HPTS at the same concentration is 0.55, and the predicted photolysis is 5.3:1. This is based on quantum yields of 0.085 and 0.0088 for MNI-glutamate and NPE-HPTS, respectively calculated from the ratio of efficiencies of 6.3:1 at 347 nm measured above. For the wavelength range 250–400 nm of Schott UGS5, the absorption is 0.48 and predicted photolysis efficiency 4.6:1. In circumstances where the spectrum of the source or the spectral transmission of the microscope are unknown, a direct calibration detecting proton release with HPTS should be used (Canepari et al., 2001).

### 3.6. Phototoxicity of 405 nm irradiation

The reduced photolysis efficiency at 405 nm relative to the peak at 330–350 nm requires higher light intensities at the preparation than would be the case at shorter wavelengths and raises the possibility that phototoxicity may be a limiting factor. The phototoxicity of 405 nm photolysis in synaptic experiments was determined by monitoring the stability of the amplitude, rise time and decay time of currents repetitively evoked by \( \gamma \)-glutamate release from MNI-glutamate at low and high intensities. Recordings were from cerebellar molecular layer interneurons or from Purkinje neurons. Cells were labelled with fluorophore Alexa 488 to identify dendrites and axons in the CCD camera image and hot-spots of activity were identified on dendritic processes in recordings of the evoked currents. Results of experiments with photorelease of \( \gamma \)-glutamate from MNI-glutamate, activating AMPA-subtype glutamate channels in a MLI are shown in Fig. 9. Pulses of 100 \( \mu \text{s} \) duration and power 0.8, 1.3 or 1.8 mW at the preparation after correction for loss in the microscope from MNI-glutamate (and other nitroindolines) relative to NPE-HPTS assuming constant quantum yield is given by the ratio of the integrals of the cage and NPE-HPTS absorption spectra in that range. For the range of wavelengths 280–370 nm transmitted by the commonly used Schott UG11 filter the absorption of MNI-glutamate to NPE-HPTS at the same concentration is 0.55, and the predicted photolysis is 5.3:1. This is based on quantum yields of 0.085 and 0.0088 for MNI-glutamate and NPE-HPTS, respectively calculated from the ratio of efficiencies of 6.3:1 at 347 nm measured above. For the wavelength range 250–400 nm of Schott UGS5, the absorption is 0.48 and predicted photolysis efficiency 4.6:1. In circumstances where the spectrum of the source or the spectral transmission of the microscope are unknown, a direct calibration detecting proton release with HPTS should be used (Canepari et al., 2001).

### 3.7. Loss of light by tissue scattering determined with confocal detection

An estimate of the scattering of 405 nm focused laser light in slice preparations was obtained by confocal recording of the fluorescence intensity of the fluorophore HPTS (pyranine, 50 or 100 \( \mu \text{M} \)) in HEPE5 buffered saline pH 7.3. HPTS is a highly polar fluorophore that has been used as a probe in studies of diffusion in the
extracellular space (Xia et al., 1998). The slice was equilibrated in HPTS-containing solution for 1 h before the experiment. Emission of fluorescence in a confocal spot due to excitation by the 405 nm laser was estimated in single pixels, in 2 × 2-binned or 4 × 4 binned pixels corresponding to 'pinhole' dimensions of 0.25, 0.5 or 1 μm. The relative intensity of emission as the laser was scanned through the tissue is expected to depend on the proportion of the extracellular fluorophore-containing volume in the spot and on the scattering of excitation (405 nm) and emitted light (520–560 nm). The focus was stepped in 80 increments of 0.85 μm and the images collected at each location were analysed as a z-stack. Points to be scanned were chosen in the molecular layer, the granule cell layer and white matter of 21-day cerebellar slices. The Z-scans from regions in the molecular layer are collected in Fig. 10A (5 scans), from the granule cell layer in Fig. 10B (3 scans) and from the white matter in Fig. 10C (3 scans). Generally, the fluorescence drops sharply by a factor of 3–5 on entering the slice (2-pixel 1) and subsequent pixels show large fluctuations in fluorescence due to variations of fractional extracellular volume (due to intracellular vs. extracellular location). The variability was greatest in the granule cell layer where the number of soma encountered is high, it was less in the molecular layer and least in the white matter where cellular structures are least. To separate the factors of fractional extracellular volume from scattering, the molecular layer data were averaged across five locations to generate the mean as a function of depth, shown as the solid black line in Fig. 10A. These data were fitted by an exponential curve to obtain an empirical length constant. The average intensity dropped to 25% of that in the surface of the slice (corresponding to 50% loss in

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3. Photolysis of DM-Nitrophen

As well as the nitroindoline cages discussed above, nitrobenzyl cages such as DM-Nitrophen, NPE-caged InsP3 and NPE-caged ATP were found here to be photolysed efficiently enough at 405 nm to be useful in experiments. Although the principal advantage of improved light penetration is not required for intracellular photolysis, the convenience of 405 nm diode lasers and compatibility with optical corrections in microscopes are significant factors. The use of 405 nm with a nitrobenzyl cage is illustrated for photolysis of DM-Nitrophen (Kaplan and Ellis-Davies, 1988) in a vesicle calibration experiment shown in Fig. 11. The vesicles contained DM-Nitrophen 5 mM, total added Ca²⁺ 3.4 mM in an internal solution containing 4 mM MgATP, 0.5 mM Na₃GTP, 135 mM K-gluconate and 20 mM HEPES pH 7.3. The Ca²⁺ released was detected with 1 mM Oregon Green Bapta 5N, which has K (Ca) of 37 μM. The vesicles were irradiated with 10 μm diameter laser spots in 0.5 ms pulses at different intensities and the Ca²⁺ released monitored as fluorescence change ΔF. Fig. 11A shows fluorescence of two vesicles in separate experiments with five flashes at powers, 0.8 mW and 2.4 mW. The mean ΔF values following each flash are plotted in Fig. 11B for three vesicles at 0.8 mW, 1.6 mW and 2.4 mW. The fitted exponential curves give 85% conversion/flash at the lowest power (solid symbols) and close to maximal conversion in a single flash at high powers. The results show that Ca²⁺ release from DM-Nitrophen is efficient enough at 405 nm to be useful in experiments to test the Ca²⁺ sensitivity of cellular processes such as secretion or motility.

3.9. Assessment of local perfusion of the cage solution in a slice preparation

To assess problems associated with local perfusion and the importance of bath application of the cage to ensure a spatially uniform and known concentration, we tested the amplitude of responses as the distance from a local perfusion pipette was progressively increased, keeping the laser spot at the same location on the preparation. The perfusion pipette was a large tipped patch pipette of 2–3 MΩ resistance when filled, and cage solution was ejected by a pulse of 1 bar pressure applied by a picospritzer starting 10 s before the laser flash. Responses recorded at different distances from the pipette tip are illustrated in Fig. 12A and summarised for 4 cells in Fig. 12B. The response amplitude declined continuously from the perfusion pipette tip with a half-amplitude at 50 μm.

4. Discussion

The use of 405 nm laser photolysis was developed mainly to address the deficiencies in near-IR two-photon excitation of currently available extracellular cages, such as MNI-glutamate, that...
are too inefficient to safely avoid problems with phototoxicity of pulsed IR. The known two-photon cross-section of $0.02$–$0.06 GM$ ($10^{-50} \text{cm}^4 \text{s/photons}$) for MNI-glutamate (Matsuzaki et al., 2001; D.O. unpublished measurements) predicts a small fractional concentration change in the excitation spot at non-toxic intensities. At an average power of 5 mW and typical TiS pulse parameters (200 fs, 76 MHz) at 720 nm with 0.75 NA water-dipping objective a conversion in the laser spot of <5% of concentration at equilibrium (>0.5 ms) can be calculated (procedures detailed in Kiskin et al., 2002). In contrast, at 405 nm data described here show that 100 μs pulses of 2 mW at the preparation produce complete photolysis and have been demonstrated to be non-toxic in synaptic experiments.

The alternative approach of local cage perfusion to minimise the light path with near-UV excitation at 355 nm was found here to have a spatially non-uniform and variable concentration distribution from trial to trial when tested with 405 nm excitation, in agreement with data reported by Dittman and Regehr (1997). Local cage perfusion is incompatible with uniform and reproducible caged ligand release at known concentration over the entire field of view, as required for multi-point uncaging with confocal or holographic methods.

A second aim was to generalise the use of the caged fluorophore NPE-HPTS for laser photolysis calibration, extending the earlier descriptions by Jasuja et al. (1999) and Canepari et al. (2001). We
The time course of photolysis events was compared with spontaneous synaptic events in molecular layer interneurons, which have the advantage in this context of showing little cable attenuation of distal events recorded at the soma. The rise times and decay times were slower in the photolysis-evoked currents, rise times approximately 0.85 ms compared with 0.31 ms, and decay times slower by a similar factor. The volume over which l-glutamate is released by the laser spot shown in Fig. 5 is larger than the release volume at synaptic contacts, and may be the reason for the prolonged rise and fall of activation. It may be noted that spontaneous excitatory synaptic currents in interneurons are fast compared with other neurons, due in part to fast electrotonic propagation to the soma. Real differences in time-course of synaptic and photoactivated events may not be so well distinguished in less compact neurons if the time-course is determined by cable attenuation.

To test the generality of photolysis at 405 nm, experiments were done to release Ca$^{2+}$ from the dimethoxy nitrobenzoyl cage DM-Nitrophen in the aqueous vesicle preparation described here. With 0.5 ms pulses at high power, 2.4 mW, sufficient Ca$^{2+}$ could be released in a single pulse to saturate the fluorescence change in Oregon Green Bapta 5N, a low affinity Ca$^{2+}$ indicator (K = 37 μM). The results show that laser photolysis of DM-Nitrophen at 405 nm is efficient enough to be used in kinetic investigations of Ca$^{2+}$-dependent processes, and will provide a useful tool for studies that utilise photolysis to investigate second messenger responses.

Diode lasers at 405 nm have been developed to have high power (>100 mW CW), fast modulation (>100 MHz) and high contrast, 10,000:1, sufficient for the concentration range of glutamate or other transmitters that needs to be released in physiological studies. They are readily connected to commercial microscopes with a single mode fibre to produce a high quality input beam. Commercial confocal microscopes are in some cases equipped for scanning with 405 nm for DAPI detection and may be easily adapted for point photolysis. A simple adaptation that utilises the epifluorescence condenser of commercial microscopes and a commercially available dual lamphouse with LED and laser inputs is outlined here. The advantages of light penetration in the cage solution with 405 nm lasers also apply to flashlamp based systems where a broad band of excitation centred around 400 nm will have similar advantages in studies requiring extracellular uncaging at high cage concentrations by light delivered from the microscope objective.

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