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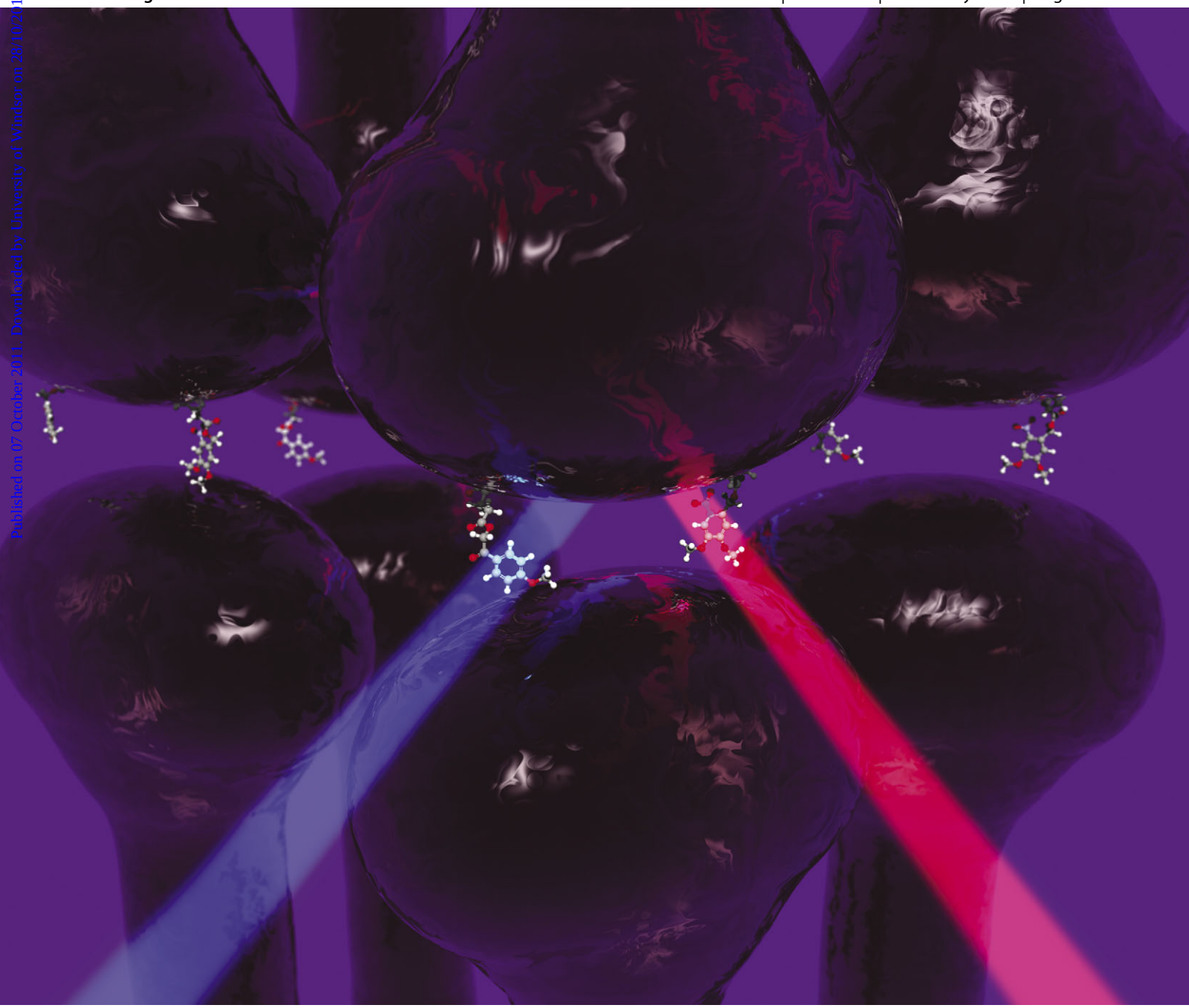

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COMMUNICATION

Wavelength-orthogonal photolysis of neurotransmitters *in vitro*†

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Irradiation of a mixture of 4-methoxyphenacyl-caged (*S*)-glutamate and 4,5-dimethoxy-2-nitrobenzyl-caged γ-amino butyric acid (GABA) on neurons, at ~260 nm, evokes selective photorelease of (*S*)-glutamate (Glu) whereas photolysis at 405 nm causes selective photorelease of GABA.

The lack of photophysically distinct (wavelength-orthogonal) caged biomolecules has been a significant shortcoming in the toolkit of chemical probes for biological investigations. Yet a plethora of applications exist for wavelength-orthogonally protected tools:[1] Wavelength-orthogonal protection of receptor agonists and antagonists could enable temporal and spatial control over receptor activation and inhibition; wavelength-orthogonal protection of two neurotransmitters would allow unprecedented study of synaptic integration of excitatory and inhibitory inputs (Fig. 1); and wavelength-orthogonal caging of residues within proteins would allow temporal and spatial control over post-translation protein modification.

Our aim was to demonstrate proof-of-concept in the application of wavelength-orthogonal protecting groups to the study of biological problems. We focused on (*S*)-glutamic acid (Glu) and γ-amino butyric acid (GABA) as they are the two most significant central nervous system (CNS) neurotransmitters and evoke opposing biological responses. Glu is an excitatory amino acid that mediates its action *via* activation of a large family of Glu receptors, which comprise both ligand-gated cation channels and G-protein-coupled receptors (GPCRs). GABA is an inhibitory transmitter that exerts its action *via* activation of both ligand-gated chloride channels and GPCRs. Consequently, these transmitters have fast biological responses that are clearly distinguishable in neurons. Placing Glu and GABA under wavelength-orthogonal photocontrol is of interest when attempting to mimic the complex signals received by neurons.

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Pairs of caging groups have been defined as wavelength-selective if it is possible to selectively and sequentially photolyse them using two different irradiating wavelengths in one order, but that in the other order appreciable quantities of both groups are cleaved. The term wavelength-orthogonal photolysis has been applied to systems in which the two groups can be cleaved with a high degree of selectivity in either order. Wavelength-orthogonal photolysis was first achieved by Bochet, who demonstrated that a 3,5-dimethoxybenzoin group could be selectively cleaved in the presence of a 4,5-dimethoxy-2-nitrobenzyl (DMNB) group, when irradiated at 254 nm. Irradiation at 420 nm evoked cleavage of the DMNB group and the 3,5-dimethoxybenzoin group remained intact.[2–5]

Wavelength-selective protecting groups have been employed in solid phase synthesis[6,7] and wavelength-orthogonal protecting groups have been applied to surface lithography.[8–10] The first biological application of wavelength-selective caging groups was exemplified by Kotzur *et al.* who demonstrated the selective photorelease of thiol groups in a peptide.[11] Lawrence *et al.* then employed wavelength-selective caging groups to control the photoactivation of cGMP- and cAMP-mediated

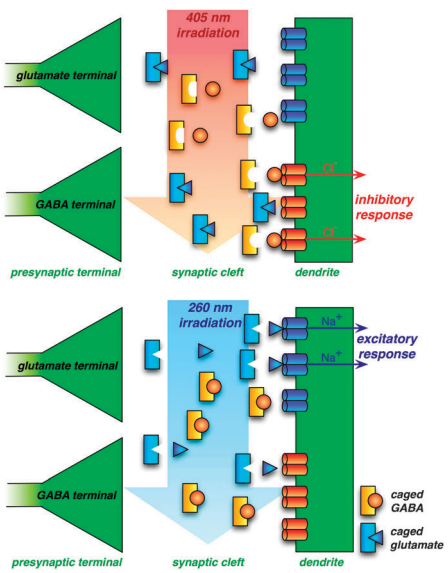

Fig. 1 Wavelength-orthogonal photolysis of caged glutamate and GABA. Irradiation at 405 nm activates GABA receptors whereas irradiation at 250–260 nm activates glutamate receptors.

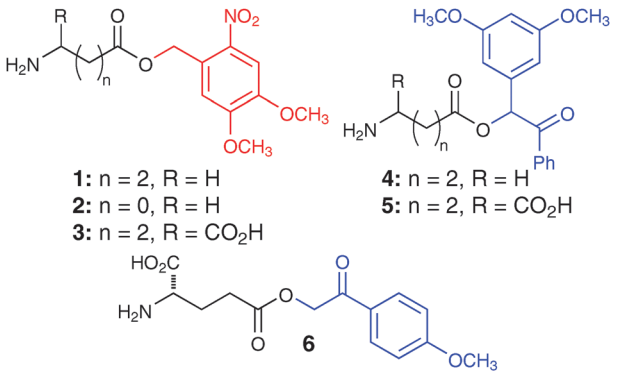

Fig. 2 DMNB-caged GABA (**1**),[27] glycine (**2**) and Glu (**3**), 3,5-dimethoxybenzoin-caged GABA (**4**), Glu (**5**), and MPA-caged Glu (**6**).

signalling pathways.[12] Schäfer *et al.* have demonstrated the wavelength-selective photorelease of two oligonucleotides.[13] Recently, Goguen *et al.* have reported the synthesis of wavelength-selectively-caged phosphopeptides and used them to study Wip 1 phosphatase.[14] Kantevari *et al.* have published an example of 2-photon, 2-color photolysis of GABA and Glu, in which they show optically independent photolysis.[15] Herein, we demonstrate wavelength-orthogonal photolysis of Glu and GABA across a heterologous population of receptors in hippocampal neurons.

Initially, the 3,5-dimethoxybenzoin and DMNB groups employed by Bochet were used in the synthesis of caged pairs of Glu and GABA (**1–5**, Fig. 2 and ESI†). However, despite successful demonstration of wavelength-orthogonal photolysis during ^{1}H NMR studies, the 3,5-dimethoxybenzoin caging group was found to be toxic to neurons. Although 3,5-dimethoxybenzoin-caged ATP has been used *in vitro* previously without toxicity being reported,[16] we were unable to continue using this caging group. Therefore it was necessary to identify another biocompatible caging group that could be photolysed in an orthogonal manner to the DMNB group. Previously, during the development of caged TRPV1 agonists,[17,18] we investigated the use of the 4-methoxyphenacyl (MPA) caging group.[19] This group and its derivatives have been employed by Givens and co-workers to cage phosphates,[20] neurotransmitters[21,22] and protein kinase A.[23] The MPA group typically displays a λ_{max} of shorter wavelength than the DMNB group and hence was likely to be wavelength orthogonal to the DMNB group. Compounds similar to the photolysis by-products from the MPA group (4-hydroxyacetophenone, 4-methoxyacetophenone and 4-hydroxyphenylacetic acid) were applied to the neurons and were found to be non-toxic. Therefore, MPA-caged Glu was synthesised (**6**, Fig. 2 and ESI†) and was observed to have a λ_{max} = 283 nm and an extinction coefficient (ε) of 14 000. The UV/vis spectra of **1** and **6** show that MPA-Glu does not absorb at 405 nm in contrast to DMNB-GABA, which has significant absorption at this wavelength (Fig. S1, ESI†). This observation indicates that it should be possible to remove the DMNB group in the presence of the MPA group by irradiation at 405 nm. Although both compounds absorb in the 250–285 nm region, the higher extinction coefficient, relatively fast release and good quantum yield of the 4-methoxyphenacyl group might contribute to successful wavelength-orthogonal photolysis.[24–26]

When a mixture of MPA-Glu and DMNB-GABA was irradiated using a 405 nm diode, photolysis of DMNB-GABA was observed by ^{1}H NMR analysis (Fig. S2A and B, ESI†), but MPA-Glu was unaffected. When an identical mixture was irradiated using a UV lamp equipped with a filter having maximum transmission at 285 nm (Fig. S1, ESI†), only MPA-Glu was photolysed (Fig. S2C and D, Tables S1–S2 and Fig. S3–4, ESI†). These results demonstrate that MPA-Glu is a suitable orthogonal partner for DMNB-GABA. The relatively long times required for photolysis in these experiments simply reflect the need to photolyse sufficient material for NMR analysis and do not reflect the *in vitro* kinetics of photorelease. The *in vitro* photolysis of these groups occurs in the millisecond timeframe (Fig. 3).

With the potential for wavelength-orthogonal photolysis established, MPA-Glu (**6**) and DMNB-GABA (**1**) were applied separately to hippocampal pyramidal neurons. MPA-Glu had no effect when applied to the bath solution, indicated by a lack of discernable increase in neuronal excitability, action potential firing or change in holding potential. Additionally, when bath-applied MPA-Glu was irradiated at 405 nm, no electrophysiological response was observed (Fig. 3C, bottom panel), showing that no Glu was released and that 405 nm light does not affect the neurons. However, irradiation using a UV flash lamp equipped with a 250–260 nm bandpass filter evoked a cation current consistent with activation of Glu receptors (Fig. 3C, top panel; MPA-Glu [25–125 μM] was photolysed at 250–260 nm in 17 different cells a total of 30 times). DMNB-GABA showed no activity on cells when applied to the bath solution. Irradiation at 250–260 nm did not produce a response (Fig. 3D, bottom panel), showing that no GABA was released and that 250–260 nm light does not affect the neurons for the timescale of the experiment. However, irradiation at 405 nm evoked an anion current consistent with activation of GABA receptors (Fig. 3D, top panel; DMNB-GABA [25–100 μM] was photolysed at 405 nm in 20 different cells a total of 30 times). To establish that the currents observed were evoked by activation of either Glu or GABA receptors, photolysis was conducted in the presence or absence of either Glu or GABA receptor antagonists. DNQX is an antagonist of AMPA receptors, through which fast Glu currents are mediated. Picrotoxin is an antagonist at the ionotropic $GABA_A$ receptors, which mediate the fast GABA response. It was shown that photolysis of MPA-Glu at 250–260 nm evoked a current, which was abolished in the presence of DNQX (Fig. 3A, n = 5). Similarly, DMNB-GABA was shown to produce a current when irradiated at 405 nm. This current was abrogated in the presence of picrotoxin (Fig. 3B, n = 6).

With the potential for wavelength-orthogonal photolysis *in vitro* established, both MPA-Glu (**6**) and DMNB-GABA (**1**) were applied simultaneously to the bath solution of hippocampal pyramidal neurons. The compounds showed no effect until photolysed (Fig. 3E). Irradiation of this mixture, using a UV flash lamp equipped with a 250–260 nm bandpass filter, evoked a cation current consistent with activation of Glu receptors (Fig. 3E). Subsequent exposure of the mixture to light of 405 nm wavelength caused an anion current, consistent with activation of GABA receptors (Fig. 3E, n = 6). To confirm that wavelength orthogonality rather than wavelength selectivity was being observed, the order of irradiation was reversed.

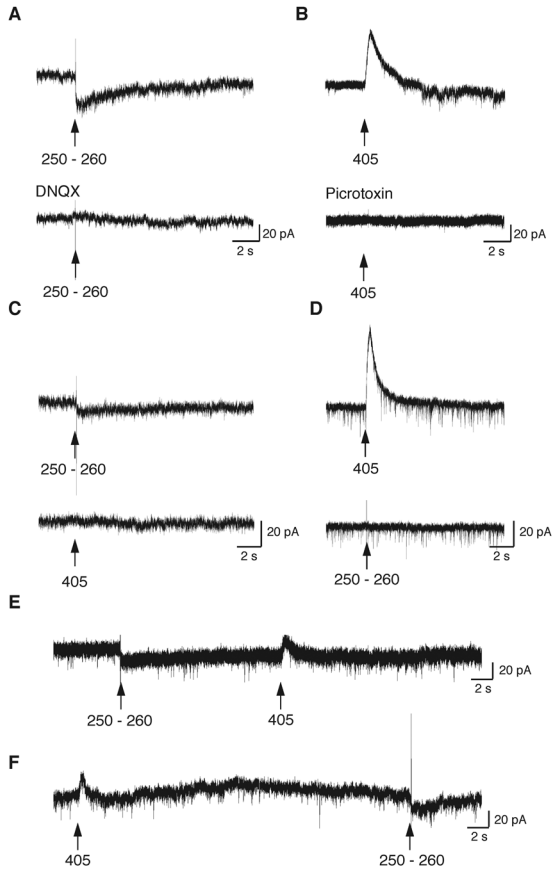

Fig. 3 Wavelength-orthogonal photolysis of MPA-Glu (**6**) and DMNB-GABA (**1**) on neurons. (A) Representative voltage clamp experiment showing that illumination of 50 μM MPA-Glu by light at 250–260 nm (arrow) gives activation of a glutamatergic current (top) that is suppressed by 50 μM DNQX. (B) In the presence of 50 μM DMNB-GABA, illumination by light at 405 nm results in activation of a GABAergic current that is suppressed by 100 μM picrotoxin (bottom). (C) A glutamatergic current is elicited in response to light at 250–260 nm (top), but not in response to light at 405 nm (bottom) indicating that photolysis of 125 μM MPA-Glu is wavelength specific. (D) Light irradiation at 405 nm (top panel), but not light at 250–260 nm (bottom panel) elicits a GABAergic current in the presence of 50 μM DMNB-GABA. (E) Demonstration of wavelength-orthogonal photolysis. In the presence of 125 μM MPA-Glu and 50 μM DMNB-GABA, irradiation at 250–260 nm results in a glutmatergic current, while subsequent irradiation at 405 nm evokes a GABAergic current. (F) Wavelength-orthogonal photolysis occurs with either order of wavelength presentation.

It was demonstrated that photolysis first at 405 nm led to activation of GABA receptors, and subsequent photolysis at 250–260 nm led to activation of Glu receptors (Fig. 3F, $n = 3$). Hence, application of both compounds to the bath solution allows rapid and interchangeable control of both Glu and GABA channels, modulated only by the wavelength of irradiating light. No toxicity associated with irradiation of neurons at 250–260 nm was observed in the time course of our experiments, as assessed by physiological and visual means. The control photolyses of the individual caged neurotransmitters *in vitro* were conducted at the same concentration, and were irradiated with the same power and for the same length of time, as the experiments with the mixture of caged compounds. Consequently, we are confident that we are observing pure anion and cation currents in the orthogonal experiments. A significant advantage of our compounds is that they can be used across multiple neurons, with areas of different receptor expression, without the need for manipulation of irradiating power or multiple control experiments.

In conclusion, we have synthesised MPA-Glu (**6**) and DMNB-GABA (**1**) and shown that these two compounds can be photoreleased in a wavelength-orthogonal manner. These compounds were employed in the wavelength-orthogonal activation of Glu and GABA receptors in hippocampal neurons. These data represent the first example of 1-photon wavelength-orthogonal photolysis *in vitro*, giving exquisite control over the activation of Glu and GABA receptors. Our results prove the principle that wavelength-orthogonal photolysis can be applied to a variety of neurotransmitters and other biological systems.

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