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RuBiGABA-2: a hydrophilic caged GABA with long wavelength sensitivity†

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We have devised a new caged GABA based on ruthenium bipyridyl coordination chemistry. This phototrigger delivers GABA upon irradiation with wavelengths up to 532 nm undergoing heterolytic photocleavage, in a clean and very fast (a few nanoseconds) photoreaction. With an absorptivity coefficient ε_{MAX} = 5300 M^{-1} cm^{-1} at 447 nm and a quantum efficiency $\phi \sim 0.09$, RuBiGABA-2 is among the most active caged-GABAs, especially at long wavelengths. This highly hydrophilic caged GABA can be synthesized in a simple one-pot reaction. The synthesis, chemical characterization and photochemical properties are presented. Finally, the usefulness of this caged compound is demonstrated by photodelivering free GABA on leech motoneurons.

Introduction

Caged compounds are profusely used in biological research. They are the perfect tool for delivering a precise amount of a bioactive chemical with very high spatial and temporal resolution. A caged compound, also called a phototrigger, is composed of two parts: the relevant biomolecule being "caged" and the "cageing fragment". It is important to note that in this case the term "caged" does not refer to actual topological enclosure but rather to "caged" functionality, as the steric hindrance imposed by the cageing fragment inhibits the normal biological interactions of the caged molecule.[1]

Phototriggers are routinely used to release neurochemicals and other bioactive small molecules in a variety of formulations,[2] and to non-invasively manipulate the chemistry of a variety of systems. In the case of ruthenium bipyridine-based technology the caged molecule is released with nanosecond kinetics upon absorption of one visible or two IR photons.[3] This unparalleled uncageing speed allows the study of protein conformational changes.[4] Spatial precision is achieved by focusing this uncageing light, usually from a visible laser source, to submicrometric dimensions. Gamma-aminobutyric acid (GABA) is perhaps the most important inhibitory neurotransmitter in mammals, and it is present not only in vertebrates as it is also ubiquitous in other phyla, like nematodes, insects, molluscs and annelids. Elucidating its role as an excitatory neurotransmitter during development has also gained importance.[5] The precise delivery of known amounts of GABA in a spatiotemporal controlled way is a very valuable tool in neurobiology research. Caged GABA has been used to inhibit single action potentials in firing neurons,[6] and has potential biomedical applications such as preventing epilepsy by releasing GABA in a way analogous to intermittent vagal electrical stimulation (IVS).[7] The most popular GABA phototriggers in use today are based on organic protecting groups,[8] and require harmful ultraviolet radiation,[9,10] and expensive light guides. Conversely, our previously devised ruthenium bipyridyl based caged GABA phototrigger[11] $[Ru(bpy)_2(PPh_3)(GABA)]^+$ (bpy = 2,2′ bipyridine, PPh_3 = triphenylphosphine), which is now commercially available (RuBiGABA), can be used with visible light and conventional optics.[12] However, its three phenyl rings make this compound somewhat hydrophobic, and although its water solubility is very high, it does interact somewhat with the lipid bilayers resulting in side-effects such as a decrease of the cell membrane resistance. Although this unwanted behaviour can almost completely be avoided by using dilute solutions and/or topical instead of bulk applications, we wanted to improve the biocompatibility of this GABA phototrigger.

The $Ru(bpy)_2(PMe_3)$ cageing group (PMe_3 = trimethylphosphine) displays great performance as a glutamate protecting group.[13] With an absorptivity maximum at visible wavelengths, possible photodamage caused by uncageing light is minimized.[14] This kind of ruthenium complex undergoes a very fast heterolytic photocleavage and releases the caged fragment in a very clean way, within nanoseconds of capturing light,[15] under one- or two-photon regimes. They are very stable in the dark at room temperature, both as a solid or in aqueous solutions and physiological media. Using a design similar to that

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of our RuBi-Glutamate phototrigger we now extend the chemical family of caged compounds based on this technology to include the complex $[Ru(bpy)_2(PMe_3)(GABA)]^+$ (RuBiGABA-2).

Experimental

Syntheses

The precursor complex $[Ru(bpy)_2(PMe_3)Cl)]PF_6$ was obtained as described elsewere.[15] Further synthesis steps took place using diminished or orange-filtered light to avoid photouncaging of the complexes, mainly in the last steps. The PF_6 salt of the acid form of the photoactive complex, *cis*-$[Ru(bpy)_2(PMe_3)(GABAH)](PF_6)_2$ (bpy = 2,2′bipyridine and PMe_3 = trimethylphosphine), was obtained as follows:

100 mg $[Ru(bpy)_2(PMe_3)Cl]PF_6$ were dissolved in 5 mL acetone. A suspension of 400 mg of a chloride-loaded anionic exchange resin (DOWEX 2 × 8) in 5 mL water was added, and stirred for 15 minutes. The resulting $[Ru(bpy)_2(PMe_3)Cl]Cl$ solution was filtered to remove the resin. 300 mg of γ-aminobutyric acid (Sigma-Aldrich) and 2.9 mL of 1 M NaOH were then added, and the resulting mixture was heated at 80 °C for 90 minutes. 1 mL of saturated KPF_6 was added, and the resulting precipitate was discarded. The solution was then cooled to 0 °C and acidified by the dropwise addition of 1 M HCl until pH = 2 and a solid orange precipitate was evident. The solid was then washed three times with ice-cold water and dried. Yield: 67%. To obtain a more soluble salt, an ion exchange step was performed as follows: the obtained PF_6 salt was dissolved in 1 mL of acetone at 0 °C and 5 mL of water. 400 mg of chloride-loaded anionic exchange resin (DOWEX 2 × 8) was added, and the mixture stirred for 20 minutes. The suspension was filtered and the obtained solution was basified until pH = 7, rotoevaporated to remove any remaining acetone, frozen under liquid N_2 and lyophilized. The obtained *cis*-$[Ru(bpy)_2(PMe_3)(GABA)]Cl{\cdot}NaCl$ salt was stored in a dry and dark environment. Yield: 94%. ^{1}H-NMR (500 MHz, Bruker) in D_2O, δ = 1.08 (d, 9H, J = 8.55 Hz), 1.54 (m, 2H), 1.67 (m, 1H), 1.93 (m, 1H), 2.03 (m, 2H), 3.58 (t, 1H, J = 11.54 Hz), 3.89 (t, 1H, J = 11.54 Hz), 7.12 (t, 1H, J = 6 Hz), 7.28 (t, 1H, J = 6.41 Hz), 7.49 (d, 1H, J = 5.56 Hz), 7.55 (d, 1H, J = 5.56 Hz), 7.73 (t, 1H, J = 6 Hz), 7.79 (t, 1H, J = 6 Hz), 7.81 (t, 1H, J = 7.69 Hz), 7.97 (t, 1H, J = 7.69 Hz), 8.17 (t, 1H, J = 7.69 Hz), 8.22 (t, 1H, J = 7.69 Hz), 8.28 (d, 1H, J = 7.69 Hz), 8.42 (d, 1H, J = 7.69 Hz), 8.48 (d, 1H, J = 8.12 Hz), 8.53 (d, 1H, J = 8.12 Hz), 8.91 (d, 1H, J = 5.56 Hz), 9.10 (d, 1H, J = 5.56 Hz). Anal. calcd: C, 36.74; H, 3.88; N, 7.94. Found: C, 34.6; H, 4.01; N, 7.7.

Electrochemistry, UV-Vis spectra and NMR

Redox potentials were measured in $CH_3CN/TBAPF_6$ (0.1 M) using a three-electrode potentiostat based on an operational amplifier (TL071) in a current-to-voltage configuration, with acquisition software written in QB 4.5. A 1 cm Pt wire of 500 μm diameter was used as a working electrode. The reference was an Ag/AgCl electrode, and redox potentials were obtained using a Ferrocene/Ferricinium redox couple as a standard. The counter electrode was a 10 cm long Pt wire, coiled around the 2 mL cell.

Photolysis

In situ photolysis of the NMR samples was performed without opening the NMR tubes, by using an array of eight high power Luxeon LXHLPM01 LEDs at a wavelength of 525 ± 20 nm. UV-Vis spectra were measured with an HP8453 diode-array spectrometer. Photolysis for quantum yield calculations was performed using an optical bench in which three individual solid state lasers at 405 nm (Lasever LSR405ML, China), 473 nm (Crystalaser, USA) and 532 nm (Z-Laser H8-M18, Germany) were aligned perpendicularly to the spectrometer light path. Total irradiation power was determined directly with a calibrated luximeter. The chosen wavelength was directed to the sample while complete absorbance spectra were taken using the OceanOptics spectrophotometer.

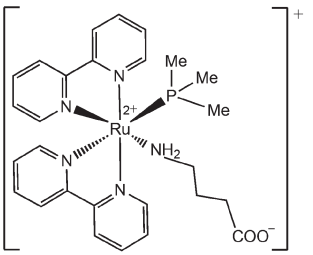
Electrophysiology

Individual leech ganglia (*H. medicinalis*) from body segments 10 to 20 were isolated, pinned down dorsal side up in a small-volume Sylgard(™)-coated recording petri dish, and desheathed under recording saline (7 : 1 Mg^{2+} : Ca^{2+}).[16] Motoneurons DE-3 or DE-5 were impaled with ~20 MΩ microelectrodes obtained with a WPI vertical pipette puller and filled with 3 M KAc solution. The preparation was kept in the dark. Cell identification and impalement was aided by infrared illumination (980 nm) and a CCD camera with its IR filter removed. Our opto-electrophysiological setup software allows us to define and automate the sequence of irradiation targets. Recording was started before cell impalement. A low power (7 mW) laser beam, 405 nm wavelength focused on a 20 μm spot, was directed during 800 ms onto each of a set of predefined targets over the ganglion, one spot being the impaled cell's soma. After this, 100 μL, 0.4 mM $[Ru(bpy)_2(PMe_3)(GABA)]Cl$ were added to the recording solution, yielding a final concentration of about 40 μM caged-GABA in the recording chamber. After recording a few minutes baseline, the laser irradiation sequence was repeated. The preparation was washed with normal recording saline solution for at least 15 minutes before repeating the irradiation sequence over the same ganglion for a third time. The recording chamber was thoroughly rinsed between ganglia.

Results

The complex $[Ru(bpy)_2(PMe_3)(GABA)]Cl$ (RuBiGABA-2 chloride form) presents a deep orange color and has a very high water solubility, being hygroscopic. We detected no decomposition after months stored at RT in containers protected from actinic light (λ < 550 nm). Its water solutions are also stable for months if exposure to light is avoided.

At physiological pH, the complex exists as a monopositive species $[Ru(bpy)_2(PMe_3)(GABA)]^+$ bearing a deprotonated GABA ligand. The structure is depicted in Scheme 1. Its solutions



Scheme 1 Structure of the complex $[Ru(bpy)_2(PMe_3)(GABA)]^+$ at physiological pH.

present a strong metal to ligand charge transfer (MLCT) band centered at 450 nm, and the weak reddish emission characteristic of similar complexes.[15] Cyclic voltammetry of the compound dissolved in acetonitrile shows three irreversible oxidation processes at 1.25 V, 1.57 V and 1.72 V *vs.* NHE, where the first process corresponds to the Ru(III)/Ru(II) couple of the GABA complex, and the latter two to those complexes bearing further oxidation products of the coordinated amine.

Fig. 1(A) shows the ^{1}H-NMR spectrum of the complex (aliphatic part). The characteristic $-NH_2$ protons a_1 and a_2 appear at 3.57 and 3.87 ppm. The fact that these signals can be seen even in a D_2O solution is evidence of the absence of isotopic exchange in the complex and indicates that the coordination of GABA is done *via* the amine nitrogen, hindering further protonation. This behaviour differs from that of free GABA which, under the same conditions, exchanges rapidly H^+ for D^+. The signals of the backbone protons in the coordinated GABA (b–d) appear between 1.54 and 2.03 ppm. The methyl hydrogens of coordinated PMe_3 are apparent at 1.08 ppm as a doublet.

Fig. 1(B) and (C) show the ^{1}H-NMR spectrum of the same, unopened NMR tube after being irradiated during 40 s and 200 s, respectively, with 525 ± 20 nm light. The three characteristic triplets of free GABA (b′, c′ and d′) at 1.89, 2.32 and 3.00 ppm appear. The new signal of PMe_3 protons now shifted to higher fields corresponds to that of the newly formed aquo complex. No sign of free PMe_3 as a decomposition product is present. The photoprocess is very clean, showing no sign of other photoproducts besides free GABA, after complete photolysis, as is expected from the known photochemistry of this kind of compound. The aromatic part of the spectra are available as ESI.†

Fig. 2 shows the UV-Vis spectrum of the complex as it undergoes photolysis while irradiating with a 473 nm DPSS Nd-YAG laser in water at pH = 7 and T = 24 °C. The photoreaction proceeds to completion in around 4 minutes, yielding just one absorbing species, as is deduced from the presence of isosbestic points at 337 nm and 429 nm. The spectrum of the photoproduct corresponds to that of the complex $[Ru(bpy)_2(PMe_3)(H_2O)]^{2+}$ which is the product of all similar

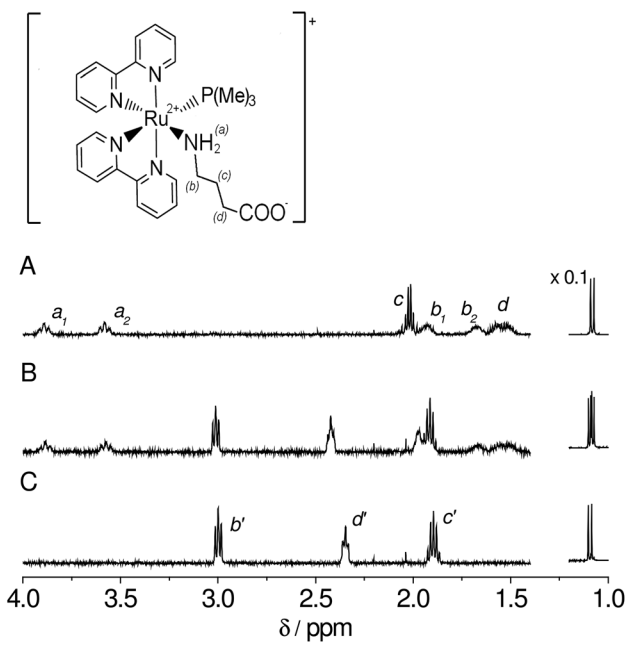


Fig. 1 Aliphatic part of the ^{1}H-NMR spectrum of RuBiGABA-2 in D_2O before irradiation (A), after 40 s photolysis inside the NMR tube with 525 nm green light (B) and after 3 minutes irradiation, achieving full photolysis (C). The signals at 1.89, 2.32 and 3.00 ppm correspond to free GABA.

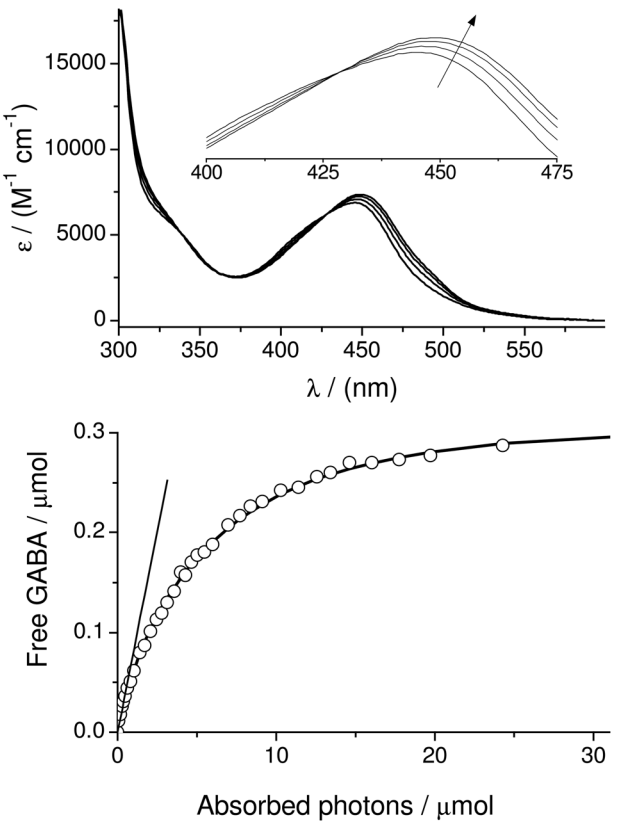


Fig. 2 Top: UV-Vis spectra of $[Ru(bpy)_2(PMe_3)(GABA)]^+$ acquired in aqueous solution at pH = 7 while irradiating with a 473 nm Nd-YAG laser. The inset shows one isosbestic point and the visible range maximum. Arrow indicates photolysis progress. Bottom: progression of the spectra during a photolysis reaction (circles) and the theoretical fit according to eqn (1) (continuous line). The initial slope is ϕ = 0.088 (straight line).

caged compounds when photolyzed in water. At pH > 12, a similar behaviour was observed, although in this case the product is the hydroxo complex $[Ru(bpy)_2(PMe_3)(OH)]^+$ which presents a broader and red-shifted absorption spectrum.

The progression of the photoreaction is shown as the amount of released GABA *versus* the total number of photons absorbed by the sample. The initial slope of this graph is the quantum efficiency of the phototrigger, although it is better to fit the complete curve, as follows: given the power of the irradiation beam, its optical path, the volume and concentration of the complex solution it is possible to calculate the differential amount of the product as:

$$\frac{dn_P}{dt} = I_{beam} \cdot \left(1 - 10^{-Abs_T}\right) \cdot \frac{Abs_R}{Abs_T} \cdot \phi_{PC} \qquad (1)$$

where n_P are the moles of the uncaged product, I_{beam} is the intensity of the incident light in Einsteins per second, Abs_T and Abs_R are the total solution's absorbance and the reactant's absorbance, respectively, and ϕ_{PC} is the quantum yield of photouncaging. Although the expression has nonlinear factors, it is easy to obtain the values of ϕ_{PC} iterating a finite differences algorithm using Excel solver.

Fig. 3 shows the number of moles of free GABA generated by photolysis at 405, 473 and 532 nm. The symbols correspond to the experimental data, while the solid lines correspond to the fitting of the integrated eqn (1). The differences in the slopes of these three curves are fully explained by the different absorptivities at these three wavelengths. The quantum yield of photouncaging, however, remains nearly constant in the wavelength range studied. Complete results are given in Table 1.

For a quick comparison, a $t_{1/2}$ of uncageing a 1 mM RuBi-GABA-2 solution was calculated on a model sample of 8 fL, a cube having a size of 2 × 2 × 2 μm^3, typical of a less-than-perfect focus in a biological sample which is to be irradiated with different wavelengths at 1 mW.

The physiological response of neurons to RuBiGABA-2 was demonstrated in the leech isolated ganglion preparation. It is known that Dorsal Excitor 3 (DE-3) and Dorsal Excitor 5 (DE-5) motoneurons response to somatic GABA application is a tonic hyperpolarization and a lower action potential firing frecuency.[17] DE motoneurons are located at the dorsal side of the ganglion, posterior glial packet, towards the center of the ganglion. They are easily recognized by their firing pattern, relative position and size, even after the glial sheath has been removed.

Fig. 4 shows a motoneurons response to GABA released by an 800 ms, 405 nm uncageing laser pulse delivered to the cell soma. The onset has a very short delay and the offset closely follows the end of the light pulse, indicating that the neurotransmitter is delivered closer to the soma and in a lower absolute amount than with conventional pressure-ejection

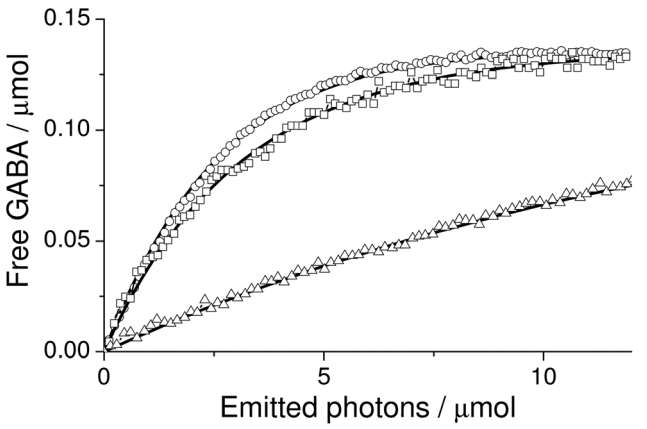


Fig. 3 Photodecomposition of RuBiGABA-2 solutions at pH = 7 using 405 nm (circles), 473 nm (squares) and 532 nm (triangles) lasers. The much shallower slope of the curve at 532 nm is a direct consequence of the lower absorptivity coefficient at that wavelength. The quantum yield remains unchanged.

Table 1 Molar absorptivities, quantum yields of photouncageing, and photoactivities of RuBiGABA-2 for three visible wavelengths

λ/nm	ε/M^{-1} cm^{-1}	ϕ_{PC}	$\varepsilon \times \phi_{PC}$/$M^{-1}$ cm^{-1}	$t_{1/2}$/μs (see the text)
405	4580	0.086	394	9.0
473	3470	0.088	306	9.8
532	575	0.085	49	64.8

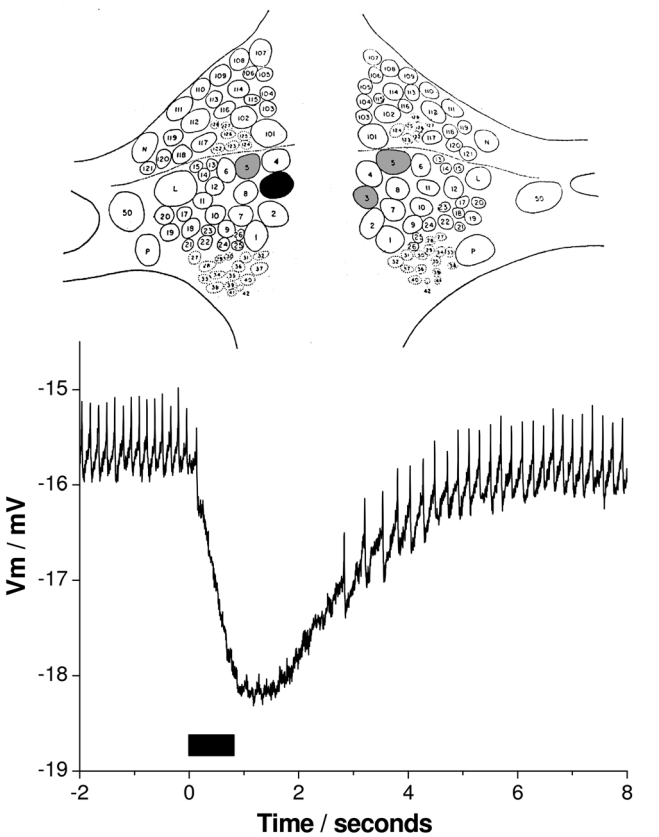


Fig. 4 Top: diagram of the dorsal view of a leech ganglion. Motoneurons DE-3 and DE-5 are filled gray. This particular recording is from cell DE-3 marked black. Bottom: effect of a brief 800 ms, 405 nm light-triggered GABA application (dark bar) onto the targeted neuron.

Table 2 Comparison of the photoactivities ($\varepsilon \times \phi$/M^{-1} cm^{-1}) of several GABA phototriggers

λ/nm	CNB-GABA	DPNI-GABA	CNI-GABA	CDNI-GABA	Coum-GABA	RuBi-GABA	RuBi-GABA2
350	60	38	420[a]	3400	1520	767	370
405	0	3	15	207	1866	1281	394
445	0	0	0	0	103	1107	563
473	0	0	0	0	0	447	305
532	0	0	0	0	0	0	49

[a] Products $\varepsilon \times \phi$ for CNI are given at 330 nm.[20]

devices. Cells known not to respond to GABA do not display any change upon GABA photorelease, and there was no response to laser light pulses before caged-GABA application, or after washing it out. Shown is an extract of a much longer recording in which this stimulus was repeated several times with the same response.

Discussion

These results show that the quantum yield under physiological conditions is independent of the irradiation wavelength between 405 and 532 nm. This allows the use of a wide range of light sources with similar efficiency. This range not only includes the violet 405 nm laser diodes, popularized due to their use in Blue-ray® devices, but also the new InGaN 445 nm true blue laser diode, being compact, inexpensive and easily modulated. Some commercially available caged GABAs are active mainly in UV, as CNB-GABA,[18] while others, as DPNI-GABA[19] extend to the violet region. The chromophores CNI and CDNI are even more sensitive at these wavelengths.[20] The latter presents a very high quantum yield (0.5–0.6) but the studies suggest that cage compounds based on this chromophore can lead to more than one photoproduct.[21] A new generation of GABA organic phototriggers as the coumarin based Coum-GABA[22] extends the active range up to blue light. Although the Ru-based compounds present an $\varepsilon \times \phi$ value at short wavelengths which is lower than some new caged-GABAs, it outperforms all the other phototriggers at long wavelengths. Having an $\varepsilon \times \phi$ value of about 50 at a wavelength as long as 532 nm, uncageing with green Nd-YAG lasers becomes possible. Together with the analog phototrigger RuBi-Glutamate we have extended the irradiation window of caged neurotransmitters, both excitatory and inhibitory, to the green region of the visible spectrum.

A comparison with other caged GABAs is shown in Table 2. The data of the photoactivity $\varepsilon \times \phi$ were obtained from the reported spectra and quantum yields.

Shifting the useful wavelength range to long wavelengths is the most straightforward strategy to expand the application universe of caged compounds. However, there is another way to achieve long wavelength excitation: two-photon regime (2P) irradiation. Although this technique implies the use of expensive equipment, it allows a very precise spatial control of the photodelivery, not only in the x and y axes, but also in the z (depth) axis, which cannot be achieved by means of conventional 1P irradiation. Many caged compounds cannot be used in the 2P regime due to their negligible 2P cross section, but experiments on the analog ruthenium phototrigger have shown 2P absorption,[15] and a similar behaviour is expected for RuBiGABA-2. Further research on this subject is being performed.

The lack of toxicity or any side effects evidenced in these experiments opens the possibility of using this caged GABA in *in vivo* preparations. This possibility could turn up useful in preventing epilepsy episodes using noninvasive visible light pulses, instead of electrodes. Preliminary experiments indicate that no deleterious effects are present when solutions of up to 1 mM RuBiGABA-2 are topically applied directly over the brain cortex of anesthetized mice, while GABA effects on the pattern of neuronal firing and single unit firing behaviour due to irradiation can be detected using extracellular electrodes. These experiments have recently been published.[23]

Conclusions

We have synthesized a new caged phototrigger, RuBiGABA-2, capable of releasing the inhibitory neurotransmitter GABA with good photoactivity when irradiated with visible light between 405 nm and 532 nm. The new compound presents an extended absorption allowing the use of green Nd-YAG lasers that guarantee high penetrability and absence of cellular damage using very low cost equipment. Its improved water solubility facilitates the use in biological environments. This compound does not present any deleterious effect on invertebrate ganglia, nor in cortical mouse brain slices or in whole brains of living mice.

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