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## *In vivo* photorelease of GABA in the mouse cortex

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# *In vivo* photorelease of GABA in the mouse cortex

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## Abstract

Electrical stimulation has been used for more than 100 years in neuroscientific and biomedical research as a powerful tool for controlled perturbations of neural activity. Despite quickly driving neuronal activity, this technique presents some important limitations, such as the impossibility to activate or deactivate specific neuronal populations within a single stimulation site. This problem can be avoided by pharmacological methods based on the administration of receptor ligands able to cause specific changes in neuronal activity. However, intracerebral injections of neuroactive molecules inherently confound the dynamics of drug diffusion with receptor activation. Caged compounds have been proposed to circumvent this problem, for spatially and temporally controlled release of molecules. Caged compounds consist of a protecting group and a ligand made inactive by the bond between the two parts. By breaking this bond with light of an appropriate wavelength, the ligand recovers its activity within milliseconds. To test these compounds *in vivo*, we recorded local field potentials (LFPs) from the cerebral cortex of anesthetized female mice (CF1, 60-70 days, 20-30 g) before and after infusion with caged  $\gamma$ -amino-butyric-acid (GABA). After 30 min, we irradiated the cortical surface with pulses of blue light in order to photorelease the caged GABA and measure its effect on global brain activity. Laser pulses significantly and consistently decreased LFP power in four different frequency bands with a precision of few milliseconds ( $P < 0.000001$ ); however, the inhibitory effects lasted several minutes ( $P < 0.0043$ ). The technical difficulties and limitations of neurotransmitter photorelease are presented, and perspectives for future *in vivo* applications of the method are discussed.

Key words: Caged compounds; Neural stimulation; GABA; Cerebral cortex; Photorelease

## Introduction

Since Luigi Galvani discovered that static electricity applied to nerves could evoke muscle contractions, bioelectricity has been a major topic in biology and subsequently in neuroscience. Several studies in the past century demonstrated how nerve cells exchange information in the form of ionic currents and biochemical processes (1,2). The search for a better understanding of these phenomena has made extensive use of direct brain stimulation. Electrical microstimulation, for instance, led to fundamental discoveries regarding perception, motor activity, memory consolidation, and the treatment of brain-related illnesses (3-6). In spite of the many successes of electrical stimulation, criticism has been raised due to the simple fact that this kind of electrical current is not a natural phenomenon in the brain. In addition, the spatial resolution is very difficult to control

because dendrites, soma and axons are all sensitive to this kind of stimulation, making it impossible to establish precisely which neurons are affected. Zemelman et al. (7), based on the biochemistry of retinal phototransducers, developed a method capable of controlling the firing rates of genetically designated neurons *in vitro* using white light. Thus, it was possible to target functional populations rather than anatomical locations (8). Despite its many exquisite advantages, such optogenetical methods are still unavailable to most researchers. A simpler and more affordable alternative for the photostimulation of neural circuits is the use of caged compounds. A caged compound is the union of a compound of interest with a chemical cage, which renders the compound ineffectual. The term "caging" was first used by Kaplan et al. (9) with reference to a

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protected compound, so called "caged ATP", able to release adenosine 5'-triphosphate upon illumination. By irradiating the complex with light of appropriate wavelength, the compound is irreversibly released from the cage, becoming free to interact with the environment. Thus, the use of caged neurotransmitters may bypass the limitations of the electrical stimulation techniques cited above, making this new kind of stimulation a promising alternative to the use of electrical stimulation.

The first caged agonists for neurotransmitter receptors were activators of acetylcholine receptors released by UV irradiation (10). Some years later the first caged glutamate was developed, and its application to brain slices led to a better understanding of the connection patterns in neurons of the visual cortex (11). Spatial resolution, however, was still a problem due to the scattering properties of UV light. Another solution came from the development of compounds that could be uncaged by visible wavelengths, allowing greater tissue penetration, less light scattering, and better spatiotemporal resolution, while at the same time providing greater simplicity and lower cost (12). Further advantages are presented by the development of caged molecules that could be photolyzed by two-photon irradiation (13).

Caged  $\gamma$ -amino-butyric-acid (GABA) compounds have also been introduced, being used to suppress experimental seizures in brain slices (14) and to investigate GABA receptors in patch clamp recordings of rat hippocampal slices (15). Visible light-sensitive inorganic cages using a ruthenium complex (ruthenium bipyridines) as a photosensor for visible light (16,17) have been recently developed for both GABA (RuBiGABA) (18) and glutamate (RuBiGlutamate) (16) with no apparent toxicity and with faster dynamics than organic approaches. In the present study, we demonstrate for the first time that the photorelease of RuBiGABA-PMe<sub>3</sub>, a modified version of RuBiGABA, with a visible light pulse successfully suppresses cortical local field potential (LFP) activity in anesthetized animals with high temporal resolution. Our experiments, however, did not address caveats related to the spatial resolution of *in vivo* photostimulation by caged compounds. In this sense, we discuss the technical limitations of the caged compound method and propose new experiments to explore its advantages.

## Material and Methods

### Animals and surgical procedures

Five adult female CF1 mice (FUNDACAL, Buenos Aires, Argentina; age: 60-70 days, weight: 20-30 g) were anesthetized with 100 mg/kg ketamine and 8.0 mg/kg xylazine, *ip*. A craniotomy with a ~3-mm<sup>2</sup> circular area was drilled in the skull bone over the primary somatosensory cortex of each mouse (stereotaxic coordinates of the craniotomy center: ML = 1.7 mm, AP = -0.3 mm). The experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals

(NIH publication 80-23/96) and Argentinian regulations and were approved by the Ethics Committee of Animal Usage in Research of the Alberto Santos Dumont Research Support Association, Brazil (protocol #3/2009). All efforts were made to minimize animal suffering and to reduce the number of animals used. Due to physicochemical artifacts, data from 2 animals were not utilized.

### RuBiGABA-PMe<sub>3</sub> synthesis

The precursor complex *cis*-[Ru(bpy)<sub>2</sub>(PMe<sub>3</sub>)Cl]PF<sub>6</sub> (bpy = 2,2'-bipyridine and PMe<sub>3</sub> = trimethylphosphine) was obtained by the following procedure: 520 mg Ru(bpy)<sub>2</sub>Cl<sub>2</sub> (19) was suspended in 20 mL of a 1:1 mixture of methanol and water, and refluxed under N<sub>2</sub>. A 1.2-mL aliquot of 1 M trimethylphosphine in tetrahydrofuran (THF; 324108, Aldrich, USA) was added with a syringe. The reaction was monitored by UV-Vis spectroscopy. In some cases, additional phosphine solution was added. Once the UV-Vis spectrum was stable, methanol and excess phosphine were removed by vacuum distillation. The resulting aqueous solution was filtered to remove any solids, and precipitated by the addition of excess potassium hexafluorophosphate (KPF<sub>6</sub>) at 0°C. The dark orange solid was washed three times with cold water and dried. The yield was 93%. The next part of the synthesis took place under filtered orange light at  $\lambda > 580$  nm.

The photoactive complex, *cis*-[Ru(bpy)<sub>2</sub>(PMe<sub>3</sub>)(GABAH)](PF<sub>6</sub>)<sub>2</sub> was obtained as follows: 110 mg [Ru(bpy)<sub>2</sub>(PMe<sub>3</sub>)Cl]PF<sub>6</sub> was dissolved in 2 mL acetone. A suspension of 2 mL water with 200 mg of a chloride-containing anionic exchange resin (DOWEX 2 x 8) was added and stirred for 10 min. The resulting [Ru(bpy)<sub>2</sub>(PMe<sub>3</sub>)Cl] Cl solution was filtered to remove the resin, 500 mg GABA and 1.8 mL 1 M NaOH were then added, and the resulting mixture was heated for 5 h to 80°C. One milliliter saturated KPF<sub>6</sub> was added and the resulting precipitate was discarded. The solution was then cooled to 0°C and acidified by the addition of 5 M HCl to pH ~2.0. The final compound, -[Ru(bpy)<sub>2</sub>(PMe<sub>3</sub>)(GABAH)](PF<sub>6</sub>)<sub>2</sub>, precipitated upon the addition of excess KPF<sub>6</sub>. The yellow-orange solid was then washed with cold water and dried. The yield was 46%. Full chemical characterization of the compound obtained will be published elsewhere.

### Experimental procedures

After surgery, each mouse was placed in a dark box and the LFPs were recorded with a sharp microelectrode (~10 M $\Omega$  at 1 kHz; A-M Systems Inc., USA) connected to a Neuroprobe 1600 (A-M Systems Inc.) amplifier and an A/D signal acquisition board (RTX-03B(II), Costronic Co. Ltd., Taiwan) operating at a 250-Hz sampling rate and running in the custom-made QBasic software. Throughout each experiment, images of the mouse brain were continuously recorded by an infrared camera. Laser pulses were delivered by a 4-mW 473-nm wavelength DPSS laser (Shangai

Laser & Optics Century Co., China) to release the caged compound in the mouse's brain. Pulse timing was controlled by the computer. A lens fixed to a remote control motor allowed the experimenter to target the light stimulus on the area of interest. The recordings were divided in ~30-min sessions: i) baseline pre-compound application: saline was applied to the exposed area and 1-s laser pulses were delivered; ii) baseline post-compound application: 50  $\mu$ L 250  $\mu$ M RuBiGABA- $\text{PMe}_3$  diluted in saline was applied over the exposed brain area using an air displacement pipette of a 100- $\mu$ L volume; iii) post-light stimulus session: a 1-s laser pulse was delivered on the region of interest in order to release the caged GABA.

### Signal analysis

In order to evaluate how compound infusion and light-stimulus photorelease affect LFP power, we first decomposed the signals into 4 non-redundant components using biorthogonal spline wavelets. The resulting components were analyzed in 4 frequency bands of interest: delta (0.5 to 3.9 Hz), theta (3.9 to 7.8 Hz), alpha (7.8 to 15.6 Hz), and beta (15.6 to 31.25 Hz). Since calculation of the root mean square would require binning and therefore would decrease the temporal resolution, we calculated the amplitude envelope of the signals using a Hilbert transform, as shown in Figure 1A. The square of the amplitude envelope signal can be understood to be the instantaneous energy of the signal. Projecting the signals onto continuous Morlet wavelets and again calculating the amplitude envelope using a Hilbert transform, we plotted the wavelet spectrogram of the LFPs.

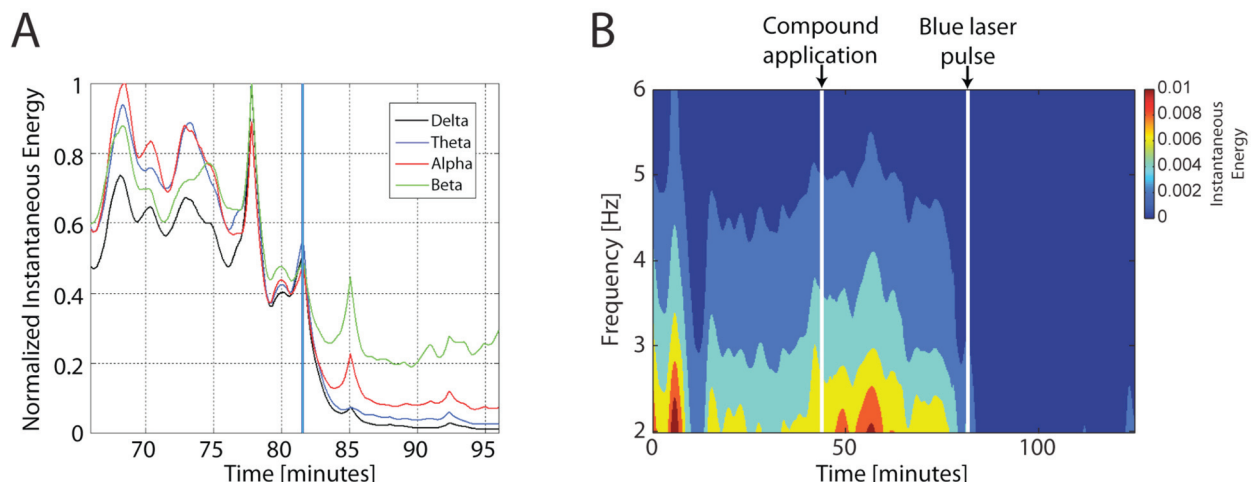
The use of the continuous wavelet transform improved the temporal resolution in spectrogram calculations, an essential feature for evaluating transitions. After calculation, the data were subjected to non-parametric statistical testing (Kruskal-Wallis test followed by Dunn-Sidak correction for the number of comparisons).

### Results

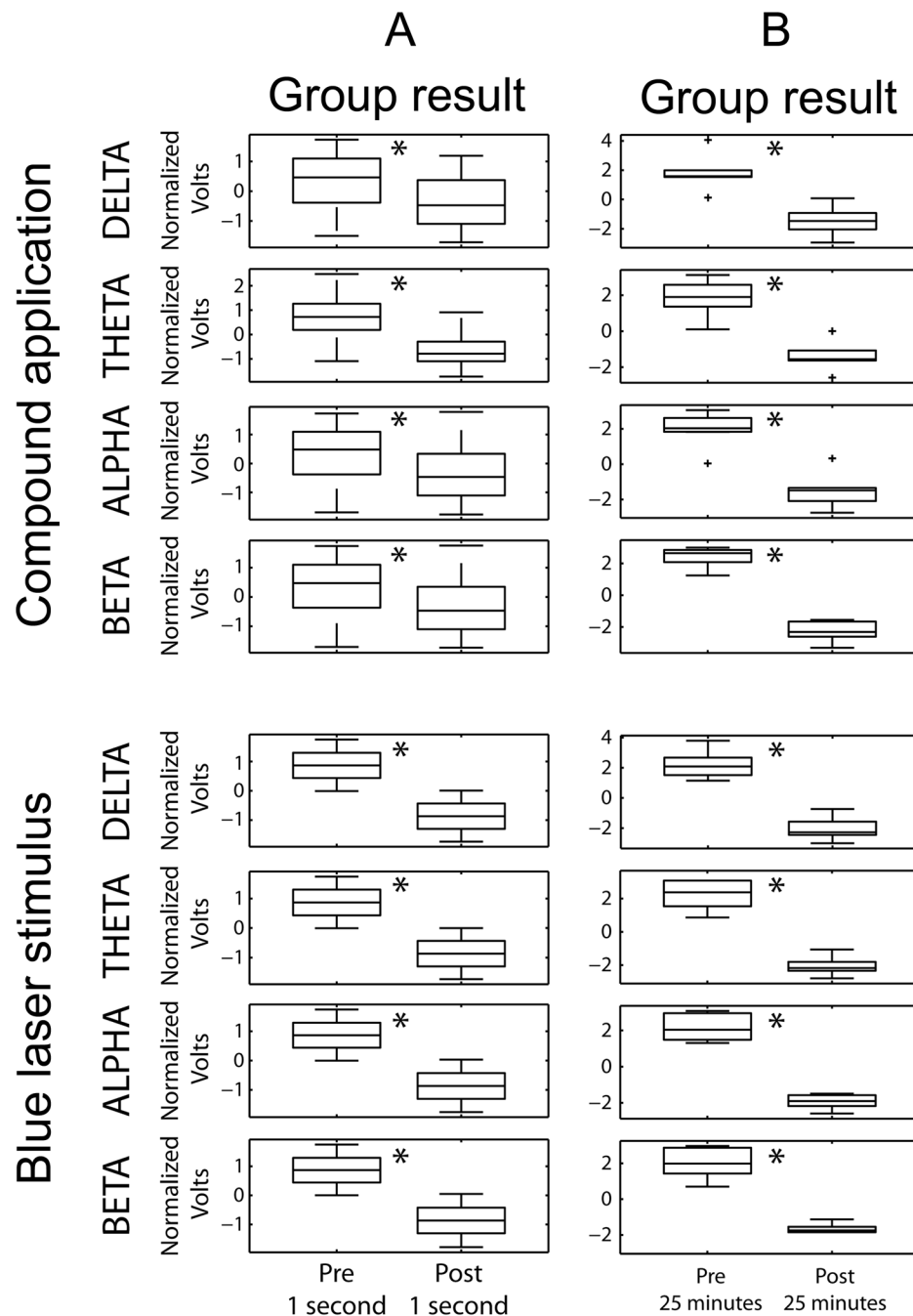
Figure 1B shows the wavelet spectrogram for mouse 1, which was the best case. The first vertical line indicates the moment of RuBiGABA- $\text{PMe}_3$  application and the second line indicates the time when the laser stimulus was flashed. Note that neural activity drops abruptly after the light pulse. Changes in the statistical stationarity of the LFP instantaneous amplitude within 4 different frequency bands were calculated at the boundaries of compound application and laser stimulation.

In order to evaluate short-term effects, we compared 1-s windows pre- and post-transition using amplitude envelopes of the signal wavelet filtered for the frequency bands of interest. The group result was calculated by adding up the distributions of three mice after z-score normalization (Figure 2A). LFP power at all frequency bands differed significantly across transitions ( $P < 0.000001$ , Mann-Whitney rank-sum test).

In order to evaluate the long-term effects of compound application and light stimulus in different frequency bands, we applied fast Fourier transform to 10 non-overlapping 2.5-min windows before and after each transition. The pairs of



**Figure 1.** The photorelease of  $\gamma$ -amino-butyric-acid (GABA) decreases local field potentials (LFP). A, Instantaneous energy calculated by the amplitude envelope of the analytical representation (AR) of the LFP signals for four frequency bands. The AR of a signal was computed using the Hilbert transform, which provides high temporal resolution for analyses at transitions. The blue vertical line indicates the exact time when the laser pulse caused inhibition of neural activity. A clear reduction of all frequency bands was noted following the laser pulse. B, A wavelet spectrogram for Mouse 1 showing neural inhibition at the time of the laser pulse, which lasted at least 40 min.



**Figure 2.** Short- and long-term effects of the infusion and photorelease of caged  $\gamma$ -amino-butyric-acid (GABA). **A**, Boxplots evaluating short-term effects of compound application and light stimulus ( $N = 3$  mice). Calculation of the amplitude envelope in wavelet-filtered bands allows for high temporal resolution. We show that local field potential (LFP) amplitude distribution of windows of 1 s pre- and post-transitions are statistically different (pre vs post,  $*P < 0.000001$ , Mann-Whitney-Wilcoxon test). **B**, Boxplots show the long-term changes in the LFP voltage amplitudes in 4 frequency bands for 3 mice at transition times. Each boxplot represents voltage levels within a specific frequency band calculated by fast Fourier transform in ten non-overlapping 2.5-min windows. Thus, 25-min pre- and post-compound application and light stimulus are evaluated. All transitions differed significantly, indicating long-term effects of both compound application and laser pulse stimuli on LFP power ( $*P < 0.0043$ , Mann-Whitney-Wilcoxon test).



10 measurements before and after each transition in every frequency band were compared by the Wilcoxon-Mann-Whitney test. Group results in Figure 2B were calculated as in Figure 2A. For long-term effects, mouse 1 showed a non-significant trend to an increase of the LFP energy after compound application (delta:  $P = 0.065$ ; theta:  $P = 0.065$ ; alpha:  $P = 0.442$ , and beta:  $P = 0.130$ ). Accordingly, a significant decrease of activity was observed following the light stimulus ( $P = 0.0001$  for all frequency bands). Mouse 2 presented a decrease in LFP amplitudes for nearly all frequency bands, both at compound application (delta:  $P = 0.0019$ ; theta:  $P = 0.0002$ ; alpha:  $P = 0.0002$ , and beta:  $P = 0.0002$ ) and light stimulus (delta:  $P = 0.0011$ ; theta:  $P = 0.0006$ ; alpha:  $P = 0.0011$ , and beta:  $P = 0.0019$ ). For mouse 3, the energy dropped significantly after compound application (delta:  $P = 0.0022$ ; theta:  $P = 0.0022$ ; alpha:  $P = 0.0022$ , and beta:  $P = 0.0022$ ) but not after the light stimulus (delta:  $P = 0.8182$ ; theta:  $P = 0.6991$ ; alpha:  $P = 0.6991$ , and beta:  $P = 0.9372$ ), indicating that most of the caged GABA was already released before the laser pulse was applied. Group results presented statistically significant differences for both transitions ( $P = 0.0022$  for all frequency bands).

## Discussion

In this study, we describe how GABA photorelease with visible light affects different frequency bands of the LFP recorded from the cerebral cortex of anesthetized mice. We show that a blue laser pulse cast over the area infused with RuBiGABA- $\text{PMe}_3$  can significantly inhibit neural activity.

Some technical problems are worth mentioning. First, there is a concern regarding the sensitivity of the caged complex to visible light. Since we have chosen to use very high concentrations in order to attempt complete inhibition of cortical activity, it is very difficult to avoid residual photorelease by environmental light. For this reason it is strongly recommended that the compound be stored as a solid, and be dissolved to proper concentration as close as possible to the application time. It is also important to ensure that the experimental environment is completely dark. Some undesired release of GABA prior to light stimulation can be seen in the data from two mice (as commented above), which show a decrease of LFP power following compound application. On the other hand, in mouse 1 the LFP power slightly increased in all frequency bands after compound application, a result that can be explained by a previously reported GABA antagonism by RuBiGABA (17).

The intrinsic disturbance of neural activity caused by the application of the caged compound, whether from GABA antagonism effects of the caged compound or from the GABA unintended release, is in fact objectionable. It is clear from our results that when the compound application causes a marked drop in neural activity by itself, the subsequent light

pulse is less effective. The strongest inhibition elicited by the laser stimulus occurred when application of the compound did not significantly alter LFP energy levels.

These problems can be diminished with further refinement of the method, including the determination of best concentration levels for *in vivo* applications. In addition, since RuBiGABA has been shown not to be toxic (17), it is in principle possible to inject low concentrations into the blood stream of mice, although we have no clear evidence that RuBiGABA can cross the blood-brain barrier. Another alternative would be to inject the caged compound into the brain ventricles. The most convenient compound concentration and the best duration of the light stimuli will vary according to the experiment. We have used both high concentrations and long stimulus durations in order to release a large quantity of GABA at once.

It must be noted that our results do not show that this kind of neural stimulation can be used in applications that require high spatial resolution. In order to explore the spatiotemporal limitations of our approach, one may need to use single unit recordings in order to evaluate how the observed effects affect individual cells immersed in the cerebral cortex. By using more refined electrophysiological techniques it would be possible to record single neuronal cells spatially distributed in the cortex, and therefore, to evaluate how the compound diffusion and the scattering of laser pulses influence the spatial precision of the stimulation. In addition, since the stimulation effects we observed were in fact long-lasting, we believe that single unit recordings may answer the question of whether the limited release of caged GABA can be used to produce short-term inhibitory effects.

Although the use of caged neurotransmitters for *in vivo* applications must still overcome some hurdles and be more rigorously tested, our results indicate that neural perturbation by the photorelease of caged compounds may soon be widely used in experimental and medical applications. Since light is employed to affect the release, one can temporally separate the injection of the compound from the photorelease, making it possible to activate the compound when it is well diffused, homogeneously and simultaneously affecting a larger area of the brain, with greater temporal resolution. RuBiGABA- $\text{PMe}_3$  may be used as a substitute for GABA agonists such as muscimol. The use of other neurotransmitters with different functions, such as glutamate or serotonin (20), opens a wide range of possibilities. The possibility in controlling brain activity by laser pulses is potentially a very important application in behavioral studies, since light pulses do not disturb behavior. For instance, this approach could be used to inhibit brain areas during memory retention, or to stimulate the brain during specific wake-sleep stages. Although we have not yet mastered the use of caged compounds for applications to behaving animals, this approach can probably be used as a complementary methodology for these kinds of experiments.

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