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Application of High Efficiency Enzymatic Compensated Two-Photon Photodissociation Material

Theses of the Ph.D Dissertation

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I. Introduction

In the latest decade neuroscience went through an enormous change with respect to the aspect of technology applied. The two-photon (2P) microscopy has revolutionized the whole neuroscience field of live imaging. In the past, confocal microscopy was applied for functional live imaging, but nowadays due to its obvious disadvantages, its application is mostly limited for anatomical studies. Nowadays, 2P imaging have replaced the confocal technique in functional, real time imaging field, and thanks to its high efficacy, the researchers could redefine their scientific questions. They could examine the phenomenon directly in living organism, deep in the tissue and analyze the outcome in parallel with the electrophysiology for instance. Not only the electrophysiology but a series of techniques can be also combined with nonlinear microscopy (chemical and viral dying, focal and optogenetic stimulation, behavior tools for *in vivo* studies, etc.). Nonlinear, 2P microscopy became the leading tool in functional neuroscience. These thoughts are also true for two photon uncaging. After the millennium some research groups have published different kind of uncaging compounds, which were capable of two-photon absorption, but with quite low two-photon absorption cross section (TPσ) and efficacy. The common character of these compounds is the relative low cross-section and their low-

moderate hydrolytic stability. According to a general conclusion, the larger the cross-section the more decreased hydrolytic stability of the compound. In the first section of the results, I show a novel series of compounds which were aimed to design to gap this efficacy/stability dichotomy, by means of a complex quantum chemical modeling. I validated the results of these calculations by detailed neurobiological characterization. I also developed a novel enzymatic method to provide an increased stability for the caged-molecule. In the second part of the result, I demonstrate an example about the usability of the new caged-compound via a neuroscientific question, namely how can generate calcium spikes in neuronal dendritic segments. This novel two-photon uncaging tool can help to understand better the dendritic processes during ongoing brain activities.

II. Scientific Aims

In the 21st century neuroscience, the greatest aim of the science community is to overcome the problem of data volume. The estimated numbers of neuronal connections are higher than we thought before, and it is not limited to neuronal ensembles or single cell level, but in dendritic level. The smallest unit for neuronal calculations is a dendritic segment. Therefore, if we ought to understand the mechanism better we have to try to image in sub micrometer range in a living tissue during ongoing brain activity. The modern imaging technique such as two-photon microscopy allows us not just collect information from the brain with high spatial and temporal resolution but controlling and changing the neuronal environment with the help of two-photon uncaging. This technique can mimic physiological release of neurotransmitters. One of the possible caged compound candidate is the widely used MNI-Glu. However, it has certain disadvantages, like smaller two-photon quantum efficacy (TPQE) and other side effects. In the case of low TPQE, higher laser intensity would be necessary to reach threshold levels for dendritic integration processes, which is very undesirable due to the higher phototoxicity. The one-photon uncaging cannot also be an option, because of its low spatial resolution and tissue penetration depth. Therefore, we

started to design a novel uncaging compound, using quantum chemical modeling, to increase the TPQE which allows more efficient measurements on living tissue.
Along these perspectives, I addressed the following scientific questions:

Q1: Using theoretical modeling, is it possible to apply higher TPQE compound without any undesirable side effects?

Q2: Can it be used for extended, sensitive and complex biological experiments such as neuronal network modulation and dendritic integration during brain oscillation?

III. Material and methods

III.1. Slice preparation and electrophysiology

Acute hippocampal slices were prepared from 15-20-day-old rats and mice using isoflurane anesthesia followed by swift decapitation. Horizontal (300 µm) brain slices were cut with a vibratome and stored at room temperature in artificial cerebrospinal fluid (ACSF) (in mM: 126 NaCl, 2.5 KCl, 2 CaCl2, 2 MgCl2, 1.25 NaH2PO4, 26 NaHCO3 and 10 glucose). Whole-cell recordings were made at 32°C (MultiClamp 700B, Digidata 1440; Molecular Devices, Sunnyvale, CA, USA) with glass electrodes (current-clamp: 6–9 MΩ; voltage clamp: 3-5 MΩ) filled with (in mM): 125 K-gluconate, 20 KCl, 10 HEPES, 10 di-tris-salt phosphocreatine, 0.3 Na-GTP, 4 Mg-ATP, 10 NaCl, 0.1 Fluo-4, 0.1 ALEXA 594, and 0.008 biocytin. Cells with a resting membrane potential more negative than –50 mV were accepted. The recorded cells were classified as hippocampal interneurons and pyramidal cells according to their electrophysiological properties. GABA IPSCs were induced by focal synaptic stimulation in the presence of AP5 (60 µM) and CNQX (10 µM). Glass electrodes (6-9 MΩ) filled with ACSF were placed at a distance of 10-15 µm from the soma (stimulation: 0.1 ms, 10-50 V, 10 ms pulse interval, 1 stimulus; BioStim, Supertech). All evoked IPSCs were verified for synaptic delay.

III.2. Measurement of free glutamate concentration

Spontaneous hydrolysis of DNI-Glu•TFA was detected by direct measurement of glutamate concentration. Standard solutions of the glutamic acid were prepared with distilled water at concentrations of ~ 1.9□2.5□10-3 M (weighed with analytical precision) and further diluted before use. Stock solution of o-phthalaldehyde (OPA) contained 0.20 g (weighed with analytical precision) in 10 ml methanol (referred to as methanolic OPA solution). DNI-Glu•TFA (~0.01□0.014 g) was weighed with analytical precision and dissolved in 10 □ 50 ml distilled water or in 12 ml ACSF (final concentration 2.5 mM for DNI-Glu•TFA). Borate buffer was mixed in 50/50 (v/v)

ratios from 0.4 M boric acid (dissolved in 0.4 M potassium chloride) – 0.4 M sodium hydroxide. OPA/MPA reagents were obtained by mixing, in the order of listing, 500 µl methanolic OPA with 4 ml buffer solution and 20 µl MPA. 12 µl NADP solution (0.2 M) was added to the ACSF solution of DNI-Glu•TFA with or without the glutamate dehydrogenase enzyme. Different amounts of enzyme were added (200, 520, 1040, 2000 and 5200 units/L) to the 12 mL ACSF solution saturated with 95 % O2 and 5 % CO2 gas. Derivatizations of blank, standard, and sample solutions were performed with reagent solutions stored in the refrigerator no longer than < 9 days, at ~4 C°. 400 µl reagent solutions were mixed with 60 µl glutamic acid or DNI-Glu•TFA solutions and let react for 5 min before injection. The analytical setup consisted of a Waters HPLC instrument (Waters, Milford, MA, USA), made of a Waters 996 PDA Detector and a Waters 474 Detector, a Waters 616 Controller quaternary pump with a thermostattable column area, and a Waters 717 Autosampler, operating with the Millennium Software (version 2010, 1992□95, validated by ISO 9002). The analytical columns were a BST Hypersil ODS, 15 cm × 4.0 mm, 5 µm; a Thermo Hypersil ODS 15 cm × 4.6 mm, 5 µm; and a Thermo Hypersil Gold 20 cm × 4.6 mm, 5 µm, all three used with guard columns (BST Hypersil ODS 20 mm × 4 mm, 5 µm or Thermo Hypersil ODS 10 mm × 4 mm, 5 µm).

III.3. Two-photon imaging

Two-photon imaging started 15–20 min after attaining the whole-cell configuration, on a two-photon laser-scanning system (Femto2D, Femtonics Ltd.) using femtosecond lasers (830-850 nm), (Mai Tai HP, SpectraPhysics). The Multiple Line Scanning Method was used to image long dendritic segments. At the end of each experiment, a series of images were taken across the depth of the volume encompassing the imaged neuron. Measurement control, real-time data acquisition and analysis were performed with a MATLAB-based program (MES, Femtonics Ltd.) and using a custom-written software.

III.4. Two-photon uncaging

After achieving whole-cell mode and filling pyramidal cells or interneurons with 100 μM Fluo-4, the bath solution was changed to ACSF containing 2.5 mM MNI-Glu•TFA (1), DNI-Glu•TFA (2), MNI-Ulg•TFA (3), or DNI-Ulg•TFA (4). Photolysis of caged glutamate was performed with 690-830 nm ultrafast, pulsed laser light (Mai Tai HP Deep See, SpectraPhysics or Cameleon Ultra II, Coherent). The intensity of the uncaging laser beam was controlled with an electro-optical modulator (Model 350-80 LA, Conoptics). Dispersion compensation was set to have maximal response at the depth of uncaging (50-80 μm from surface). The uncaging laser beam was coupled to the imaging optical pathway with a dichroic mirror (custom laser combiner, z750bcm; Chroma Technology Corp). Imaging was interleaved with two-photon glutamate uncaging periods when galvanometers jumped to 15-25 selected locations (within <60 μs jump time) and returned back to the imaging trajectory thereafter. Positions of uncaging sites were finely adjusted according to background images taken. Line scan data were also used to avoid overlapping between uncaging locations and the dendrite. Photolysis of caged glutamates was performed in "clustered" patterns (0.8±0.1 μm distance between inputs) along the dendrite. Small drifts of the sample (approximately 0.1-0.2 μm/min) were compensated manually according to regularly taken background images and fluorescent pixel intensities in uncaging locations during photo-stimulation. The same uncaging pattern in the same dendritic location was used during comparison of different uncaging materials. Perfusion rate was set to 6 ml/min in order to increase the exchange rate of ACSF containing different uncaging materials. L-glutamic dehydrogenase (Sigma-Aldrich) was applied to the bath. β-nicotinamide adenine dinucleotide phosphate hydrate (200 μM, Sigma-Aldrich) was also applied to the bath, or alternatively, was injected with 1 μM/min with an initial concentration of 50-100 μM in order to compensate for consumption during the enzymatic reaction. Unless otherwise indicated, data are presented as mean±s.e.m. Statistical comparisons were performed using the Student's paired t-test.

IV. Results

The purpose is to understand the relationship between spontaneous hydrolysis, two-photon photochemical cross-section, two photon spectrum and chemical structure. For this reason, we synthesized the MNI-Glu (**1**) and its three novel chemical analogues and compared their biological characteristics to data predicted by the quantum chemical modeling. The experimental data, in good agreement with the modeling results, showed that DNI-Glu (**2**) has indeed higher two-photon photochemical cross-section but its spontaneous hydrolysis is also higher compared to MNI-Glu (**1**). To overcome this drawback, or at least compensate it, but preferably eliminated the high spontaneous hydrolysis to MNI-Glu (**1**) level with a new enzymatic, biocompatible method.

IV. Theses

1.1 **The DNI-Glu•TFA has an increased 2PQE compare with MNI-Glu.** Quantum chemical modelling is an excellent tool to select the proper caged glutamate candidate. I concluded that the 2PQE of DNI-Glu•TFA is 7 times higher (according to the calcium responses) and it allows to measure more precisely active regenerative dendritic processes.

Related publications: Pálfi et al., 2018.; Chiovini et al., 2014.

1.2 **With enzymatic correction, spontaneous hydrolysis level was decreased for DNI-Glu•TFA.** It is known that the most undesired process is the spontaneous hydrolysis for high QE compounds, which may result in dendritic swelling, intensive precipitation of the compound, robust changes in electrophysiological properties, or even toxicity. This process could prevent long-term measurements. Spontaneous activity was significantly decreased during enzymatic correction which allowed us long-term dendritic calcium measurements. I showed in different experiments that enzymatic correction can stabilize the ambient glutamate level.

Related publications: Pálfi et al., 2018.; Vasanits-Zsigrai et al., 2015.

1.3 **GABA-A receptor blocking effect is significantly less in case of DNI-Glu•TFA compare to MNI-Glu.** The caged compounds must be ineffective during experiments without any light irradiation, however caged compound has ion channel antagonist effect. MNI-Glu has a significant antagonist effect on endogenous GABAergic currents (~80% at 300 μM). I isolated the GABA current from other current components with pharmacology to quantify the GABA antagonist effect of DNI-Glu•TFA. I showed that there is no significant change

(~5% at 350 μM) in inhibition current amplitude in the presence of DNI-Glu•TFA.

Related publication: Pálfi et al., 2018.

1.4 **No significant effect on the amplitude of potassium currents in the presence of DNI-Glu•TFA.** Potassium channels are responsible for maintaining the resting membrane potential of a neuron as well as its repolarization following an action potential. Therefore, in physiological experiment it is crucial caged material must not affect on K^+ channels. I showed experimentally, that the DNI-Glu•TFA did not change the potassium currents during long time-scale measurements.

Related publications: Bywalez et al., 2015.; Pálfi et al., 2018.

2.1 Localized physiological dendritic Ca^{2+} transients could be simulate only with DNI-Glu•TFA uncaging. In order to validate precise physiological dendritic activity patterns on PV INs, we had to introduce a more efficient caged compound. The commercially available MNI-Glu is not enough sensitive to induce well localized 'hot-spot' region with the accompanying regenerative Ca transients. It is crucial for long timescale measurements to keep laser intensity on a moderate level during frequent glutamate uncaging. Tuft regions of the dendrites are very sensitive to phototoxicity, therefore the key step is to define the TP efficacy dependent threshold of spike initiation. I showed that dendritic Ca^{2+} spikes could be simulate only with DNI-Glu•TFA uncaging.

Related publications: Chiovini et al. 2014., Pálfi et al., 2018.

2.2 DNI-Glu•TFA uncaging could be used for pharmacological experiment to quantify the ion channel contributions in the generation of dendritic Ca^{2+} transients. Ongoing network activities cause multiple and heterogeneous calcium patterns along the dendrites. In one hand, pharmacological experiment is a widely accepted method to validate the contributions of ion channel in dendritic calcium

transients. On the other hand, dynamic of the network could be blocked after the application of different pharmacons and result a distorted input pattern. To avoid this issue, I had to use glutamate uncaging techniques to simulate physiological dendritic activities. To precisely quantify the distribution of ion channels along dendrites it is necessary to repeat the photoactivation in well-defined uncaging localizations. In physiological experiments DNI-Glu•TFA is capable for the generation and long-term repetition of this precise and stable dendritic pattern. The result of our biological experiment showed that the uncaging evoked Ca^{2+} spikes are mainly mediated by L-type voltage gated calcium channels on apical and basal dendritic part too.

Related publications: Pálfi et al., 2018.; Chiovini et al. 2014.

V. Potential application of the results

In modern neuroscience, the potential two-photon uncaging is significant. To synthetize the perfect antenna molecule structure is very challenging and expensive, because approximately 15 million possible molecule candidate existing. Using quantumchemical modelling we could synthetize the best molecule for uncaging. The two-photon uncaging creates an opportunity to quantify ion channel distribution along dendrites with high spatial (<1μm) resolution and modify dendritic and network processes during brain oscillations or even neurologic diseases (e.g. epilepsy). We synthesized the MNI-Glu and its three novel chemical analogues and compared their biological characteristics to data predicted by the quantum chemical modeling. From the scientific side this novel uncaging application could help understand better different neuronal processes and from the other side (e.g. commercial) these novel compounds can be sold on the market. The widely used MNI-Glu is quite expensive. Therefore, DNI-Glu•TFA may replace MNI-Glu in the laboratories worldwide.

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High efficiency two-photon uncaging coupled by the correction of spontaneous hydrolysis.
Pálfi D, Chiovini B, Szalay G, Kaszás A, Turi GF, Katona G, Ábrányi-Balogh P, Szőri M, Potor A, Frigyesi O, Lukácsné Haveland C, Szadai Z, Madarász M, Vasanits-Zsigrai A, Molnár-Perl I, Viskolcz B, Csizmadia IG, Mucsi Z, Rózsa B.
Org Biomol Chem. 2018 Mar 2. doi: 10.1039/c8ob00025e.
Impact factor: *3.564*

Dendritic spikes induce ripples in parvalbumin interneurons during hippocampal sharp waves.
Chiovini B, Turi GF, Katona G, Kaszás A, Pálfi D, Maák P, Szalay G, Szabó MF, Szabó G, Szadai Z, Káli S,
Rózsa B.
Neuron. 2014 May 21;82(4):908-24.
doi: 10.1016/j.neuron.2014.04.004.
Impact Factor: *15.054*

Local postsynaptic voltage-gated sodium channel activation in dendritic spines of olfactory bulb granule cells.
Bywalez WG, Patirniche D, Rupprecht V, Stemmler M, Herz AV, Pálfi D, Rózsa B, Egger V.
Neuron. 2015 Feb 4;85(3):590-601. doi: 10.1016/j.neuron.2014.12.051. Epub 2015 Jan 22.
Impact Factor: *15.054*

Quantitation of various indolinyl caged glutamates as their o-phthalaldehyde derivatives by high performance liquid chromatography coupled with tandem spectroscopic detections: derivatization, stoichiometry and stability studies.
Vasanits-Zsigrai A, Majercsik O, Tóth G, Csámpai A, Haveland-Lukács C, Pálfi D, Szadai Z, Rózsa B, Molnár-Perl I.
J Chromatogr A. 2015 May 15;1394:81-8. doi: 10.1016/j.chroma.2015.03.039. Epub 2015 Mar 25.
Impact Factor: *4.169*

Fast three-dimensional two-photon scanning methods for studying neuronal physiology on cellular and network level
Szalay G, Judák L, Szadai Z, Chiovini B, Mezey D, Pálfi D, Madarász M, Ócsai K, Csikor F, Veress M, Maák P, Katona G.
Orv Hetil. 2015 Dec 27;156(52):2120-6. doi: 10.1556/650.2015.30329. Hungarian.
Impact Factor: *0.291*

Other Publications

Combined two-photon imaging, electrophysiological, and anatomical investigation of the human neocortex in vitro
Bálint Péter Kerekes, Kinga Tóth, Attila Kaszás, Balázs Chiovini, Zoltán Szadai, Gergely Szalay, Dénes Pálfi, Attila Bagó, Klaudia Spitzer, Balázs Rózsa, István Ulbert, and Lucia Wittner
Neurophoton. 2014; 1(1):011013. doi: 10.1117/1.NPh.1.1.011013

Silicon carbide quantum dots for bioimaging
D. Beke, Zs. Szekrenyes, D. Palfi, G. Rona, I. Balogh, P. Maak, G. Katona, Zs. Czigany, K. Kamaras, B. Rozsa, L. Buday, B. Vertessy, and A. Gali
Journal of Materials Research Vol. 28, Issue 02, 2012, pp 205-209

Oral presentations

New caged compounds
Dénes Pálfi, Balázs Chiovini, Gergely Szalay, Gergely Katona, Balázs Rózsa
KOKI Days 2013, Poroszló, Hungary

Three-dimensional two-photon functional imaging with millimeter z-scanning range and sub-millisecond temporal resolution in vivo and in vitro.
Pálfi D., Katona G., Szalay G., Maák P., Kaszás A., Veress M., Hillier D., Chiovini B., Vizi ES., Roska B., Rózsa B.
MMT 2012, Siófok, Hungary

Local calcium events investigating in hippocampal CA1 interneurons and pyramidal cells with real-time multi-photon scanning technic.

Dénes Pálfi, Balázs Chiovini, Gergely F. Turi, Gergely Katona, Gábor Tamás, Attila Kaszás, Gergely Szalay, B. Rózsa
MMT 2011, Siófok, Hungary

Poster presentations

Denes Palfi, Balázs Chiovini, Linda Judak, Gergely Szalay, Gábor Juhász, Gergely Katona, Balázs Rozsa

Three-dimensional calcium imaging of mouse hippocampal neuronal ensembles during sharp wave-ripple complexes. SFN 2016, San Diego, USA

Dénes Pálfi, Gergely F. Turi, Balázs Chiovini, Attila Kaszás, Pál Maák, Gergely Katona, Gábor Szabó, Gergely Szalay, Zoltán Szadai, Miklós Madarász, Szabolcs Káli, Balázs Rózsa

Dentritic integration in fast-spiking, parvalbumin-expressing interneurons during sharp wave-ripple activity. FENS 2014, Milano, Italy

Dénes Pálfi, Balázs Chiovini, Gergely Katona, Zoltán Szadai, Attila Kaszás, Gergely Turi, Balázs Rózsa

Hippocampal sharp waves associated dendritic calcium transients revealed by three dimension acousto-optic imaging in parvalbumin positive interneurons. SFN 2013, San Diego, USA

Dénes Pálfi, Gergely Szalay, Klaudia Spitzer, Gergely Katona, Attila Kaszás, Balázs Chiovini, Miklós Madarász, Imre Csizmadia, Balázs Rózsa

More effective caged compounds for two photon uncaging, MITT 2013, Budapest, Hungary

Dénes Pálfi, Gergely Katona, Balázs Chiovini, Gergely Szalay, Klaudia Spitzer, Attila Kaszás, Balázs Rózsa

Roller Coaster Scanning Method for high-resolution 3D two photon laser scanning, FENS 2012, Barcelona, Spain

Dénes Pálfi, Balázs Chiovini, Klaudia Spitzer, Gergely Katona, Attila Kaszás, Gergely Szalay, Zoltán Szadai,Csilla Haveland Lukácsné, Orsolya Majercsik, Zoltán Mucsi, Imre Csizmadia, Balázs Rózsa

Development of new GABA and more effective glutamate uncaging materials for two-photon microscopy. IBRO Workshop 2012, Szeged, Hungary.

Dénes Pálfi, Balázs Chiovini, Klaudia Spitzer, Gergely Katona, Attila Kaszás, Gergely Szalay, Zoltán Szadai,Csilla Haveland Lukácsné, Orsolya Majercsik, Zoltán Mucsi, Imre Csizmadia, Balázs Rózsa

Development of new GABA and more effective glutamate uncaging materials for two-photon microscopy. 3rd European Synapse Meeting, 13th – 15th October 2011, Balatonfüred, Hungary

Patent

Dr. Csizmadia Imre Gyula, Mucsi Zoltán, Lukácsné Haveland Csilla, Katona Gergely, Dr. Rózsa József Balázs, Majercsik Orsolya, Potor Attila, Kaszás Attila, Gündisch Dorina, Chiovini Balázs, Szalay Gergely, **Pálfi Dénes**, *Fotoaktív vegyületek alkalmazása*, **P1100550**