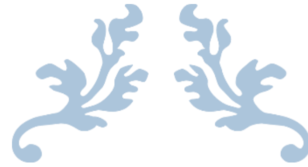

Antagonistic properties of caged GABA compounds used for activation of $GABA_A$ receptors in neocortical pyramidal neurons

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Caged photolysable compounds have served to be pivotal to neuroscientific investigations; allowing the cognizing of molecular kinetics and properties of neuronal micro-machinery such as neurotransmitter receptors. Precision in terms of temporal and spatial resolution of neurotransmitter release endowed by photolysis has multitudinal applicabilities in the realm of GABAA receptors (GABAARs), their neuronal niche and effects on neuronal and network activity. Caged compounds, in their caged form, may display certain unideal traits such as undesired interactions with the system and antagonistic activity on the target receptor. This study aims to reevaluate the GABAAR antagonistic actions of caged Rubi-GABA, which was found to antagonize these receptors at significantly lower concentrations than those reported in the literature. Furthermore, this study electrophysiologically characterizes the possible antagonistic properties of a novel quinoline-derived UV-photolysable caged GABA compound, 8 DMAQ GABA, whose activity, in its caged form appears to have a much more favorable antagonism profile compared to the widely used RuBi-GABA. To assess the antagonistic effects of these compounds on GABAAR-mediated miniature inhibitory postsynaptic currents (mIPSCs) patch-clamp recordings were carried out in the whole-cell voltage clamp configuration on cortical layer 2/3 cortical pyramidal neurons in acute neocortical slices prepared from 16-18 day-old rat rats. The results of this study indicate a revised antagonism profile for caged Rubi-GABA, with marked GABAAR toxicity in the low micromolar range. The study also scrutinizes the photo-kinetic properties of both caged GABA compounds and reveals that the rate of GABA release from 8-DMAQ is slower than from RuBi-GABA.

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1. Abstract

Caged photolysable compounds have served to be pivotal to neuroscientific investigations; allowing the cognizing of molecular kinetics and properties of neuronal micro-machinery such as neurotransmitter receptors. Precision in terms of temporal and spatial resolution of neurotransmitter release endowed by photolysis has multitudinal applicabilities in the realm of $GABA_A$ receptors ($GABA_ARs$), their neuronal niche and effects on neuronal and network activity. Caged compounds, in their caged form, may display certain unideal traits such as undesired interactions with the system and antagonistic activity on the target receptor. This study aims to reevaluate the $GABA_AR$ antagonistic actions of caged Rubi-GABA, which was found to antagonize these receptors at significantly lower concentrations than those reported in the literature. Furthermore, this study electrophysiologically characterizes the possible antagonistic properties of a novel quinoline-derived UV-photolysable caged GABA compound, 8-DMAQ-GABA, whose activity, in its caged form appears to have a much more favorable antagonism profile compared to the widely used RuBi-GABA. To assess the antagonistic effects of these compounds on $GABA_AR$-mediated miniature inhibitory postsynaptic currents (mIPSCs) patch-clamp recordings were carried out in the whole-cell voltage clamp configuration on cortical layer 2/3 cortical pyramidal neurons in acute neocortical slices prepared from 16-18 day-old rat rats. The results of this study indicate a revised antagonism profile for caged Rubi-GABA, with marked $GABA_AR$ toxicity in the low micromolar range. The study also scrutinizes the photo-kinetic properties of both caged GABA compounds and reveals that the rate of GABA release from 8-DMAQ is slower than from RuBi-GABA.

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2. Introduction

2.1 Caged compounds

A vast assemblage of diverse compounds stratified as “caged compounds” are essentially photosensitive molecular tools, comprising of a biologically active molecule or particles, that have been rendered inert by a sheathing cage group (Furuta & Noguchi, 2004; (Adams & Tsien, 1993). The ensnared bio-active component of this conjugate is liberated upon illumination, thus facilitating intended interference with cellular processes and cascades; steered specifically and with precision(Ellis-Davies, 2007)(Furuta & Noguchi, 2004). These photo-chemically propelled molecular apparatuses have been described as complemental to, yet, well demarcated from optical methods such as fluorescence microscopy, as these optical techniques serve discrete purposes. Caged compounds confer control of cellular processes while fluorescence microscopy serves as an observational tool for intracellular morphology and mechanisms(Ellis-Davies, 2007).

It is pertinent to emphasize that the term “caging”, (Jack H. Kaplan, Forbush, & Hoffman, 1978), is used merely for the ease of description and not for its accuracy in this context (Adams & Tsien, 1993). Though scientific methods have indeed enabled encapsulation of a molecule in its entirety by another(Cram, 1992)(Fagan, Calabrese, & Malone, 1992); disintegration of these shells via photolysis is extremely arduous if not unfeasible. Hence, the fundamental edifice of all efficacious caged compounds is held together simply by a covalent bond that veils an essential facet implicated in the compound’s recognition by the biological system. The consequent breakage of that particular bond results in the discharge of the biologically active chemical(Adams & Tsien, 1993).

From ions to massive bio macromolecules, practically all classes of signaling entities have been rendered into caged compounds (Mayer & Heckel, 2006)(Ellis-Davies, 2007). When employed in concert with other investigative methods such as genetics or electrophysiology, manipulation via photons allows experimental manipulation a specific cellular target with high temporal and spatial resolution (Mayer & Heckel, 2006). Photolytic caged compounds are useful in biological investigations as a light source can be finely tuned spatially and, temporally, thus endowing the ability to alter concentration of the given bio-active chemical in an instantaneous and specified manner (Adams & Tsien, 1993).

The capacity of manipulation via caged compounds is especially pivotal in cases where mechanical interferences are unfeasible, such as in the case of an integral cellular entity or tissue; or when spatial gradients of miniscule nature are necessitated (Adams & Tsien, 1993). Furthermore, since photons are able to traverse membranes and membranal compartments, active molecules can be precipitously liberated and conveyed to intracellular regions via uncaging. This is not achievable via extracellular application of second messengers such as ATP, IP3 cAMP and calcium ions for instance, as these charged entities are unable to penetrate the membrane (Ellis-Davies, 2007).

Photolytic intervention via caged compounds is a useful method for investigation of rapid kinetics and spatial disparities of biochemical processes (Adams & Tsien, 1993). Confined illumination of the beam can be used to specifically alter biochemistry of the cell in one region, and alternatively, a global light source liberates the species throughout the cell. It is also possible to alter extracellular biochemistry, thereby influencing a population of cells synchronously, to monitor network dynamics (Mayer & Heckel, 2006)(Ellis-Davies, 2007). Thus, due to their diverse applications, the use of photo-active compounds has become preponderant in numerous arenas of biology, especially in neuroscience, in the recent decades (Jerome & Heck, 2011).

Caged species

Significant caged second messengers and caged bio active chemicals include caged ions, notably caged calcium (Tsien & Zucker, 1986) (Adams, Kao, Grynkiewicz, Minta, & Tsien, 1988) (Ellis-Davies, 1998), caged inositols (J W Walker, Feeney, & Trentham, 1989); caged nucleic acids (DNA and mRNA) (J H Kaplan, Forbush, & Hoffman, 1978)(Jeffery W. Walker, Reid, McCray, & Trentham, 1988) (Monroe, McQuain, Chang, Alexander, & Haselton, 1999) (Ando, Furuta, Tsien, & Okamoto, 2001); and even caged macromolecules such as peptides and enzymes (Ghosh et al., 2004)(Mendel, Ellman, & Schultz, 1991)(Rothman et al., 2005)(Jeffery W. Walker et al., 1998); and caged neurotransmitters (Bartels, Wassermann, & Erlanger, 1971)(Wieboldt et al., 1994a)(J W Walker, McCray, & Hess, 1986a)(M Matsuzaki et al., 2001)(A. M. Gurney & Lester, 1987).

2.2 Optical electrophysiology

The field of optical electrophysiology and its expansive milieu employs energy in the form of light to probe the cellular, neuronal, genetic and molecular machinery, thereby influencing and

comprehending their function. Caged neurotransmitters, optogenetic techniques and voltage-sensitive dyes are currently the most persuasive methods in this context, offering discrete facilities to study and control neural systems. Here, these photochemically delineated techniques will be briefly overviewed to thereby demarcate the applicability of caged neurotransmitters.

Caged neurotransmitters

Specifically in the field of electrophysiology, caged neurotransmitters impart the experimenter the ability to stimulate and perturb neuronal activity devoid of electrodes, among other mechanically invasive probes (Shi, Trigo, Semmelhack, & Wang, 2014b). This offers the possibility of interfering with the activity of singular neurons to more global contexts such as circuits and neural networks. Upon application to intact neuronal tissues and brain slices, these compounds activate discrete transmitter networks and pathways with high spatiotemporal resolution and control down to micrometer and millisecond scales (Furuta & Noguchi, 2004). As explained earlier, as all caged molecules, photolabile neurotransmitters are essentially neurotransmitters conjugated with a photolytic sheathing cage group. In its caged form the neurotransmitter is inactive and is not able to modulate its target receptor in a fashion it normally would without the cage. With suitable illumination, the covalent bond is broken, liberating the neurotransmitter and allowing it to bind its receptor and exert its usual functions (Adams & Tsien, 1993).

Using caged neurotransmitter molecules, methodical and periodic light activation is achievable, controlling multiple neuronal sites synchronously (Shoham, O'Connor, Sarkisov, & Wang, 2005)(Katz & Dalva, 1994). This is not feasible with a comparable degree of control or precision when using microelectrode techniques(Shi et al., 2014b).

Photo-labile caged neurotransmitters are an expedient substitute to methods that require genetic alterations and interferences such as optogenetics. This is because caged molecules do not necessitate any form of recombination, genetic targeting or delivery—thus leaving the system genetically unperturbed and in its intrinsic state. This means there are no off target gene alterations, and laborious gene work related breeding/culturing is circumvented. Furthermore, in comparison to other photo-stimulatory methods, there is a good degree of freedom with respect to the parameters such as wavelengths of light sources used in uncaging techniques (Shi et al., 2014b).

Optogenetics

Amalgamating technologies of optical and genetic nature, optogenetics is a photostimulatory method that enables manipulation of distinct and demarcated cellular functions (Deisseroth, 2011). Neuronal cells and other cellular entities may be genetically altered and made to express photo-sensitive elements such as receptors or ion channels (Deubner, Coulon, & Diester, 2019). The methods confer modulatory control over cellular functions and capacity to monitor biological phenomena in real time, and in live systems. These techniques may be employed in vitro and in vivo, propounding an extensive range of prospects to understand neuronal, genetic and cellular mechanisms in conjunction with behavioral manifestations. The effects of the photostimulatory events, from biological to behavioral contexts, may be observed and computed in instantaneously (Deisseroth, 2011). The principal players of this method are photosensitive peptides and protein macromolecules that exert their functions in response to encountering light (Mancuso et al., 2011)(Pastrana, 2011)(Fenno, Yizhar, & Deisseroth, 2011).

In the neuroscientific paradigms, optogenetic tools are frequently categorized into one of the two classes of “actuators” and “sensors” (Mancuso et al., 2011). Implementing optogenetic actuator proteins permits alteration of neuronal activity by triggering or inhibiting their firing. High spatiotemporal regulation and resolution is a concomitant feature of this technique; and photosensitive actuators may be genetically incorporated to distinct neuronal types and networks. Interference and regulation of neuronal activity is conferred by employing proteins such as channelrhodopsins and halorhodopsins (Mancuso et al., 2011). The other class of optogenetic proteins are the sensors, who are necessitated in the process of mapping neuronal networks and cellular activity. These proteins are essentially altered genetically so their photophysical attributes are modified in concursion with neural functions. They are employed to distinguish minute events and alterations of the membrane voltage, and also bigger events such are action potential firing (Pastrana, 2011).

Despite serving as powerful apparatuses for manipulating and computing neuronal events, there are several predicaments associated with optogenetic tools (Packer, Roska, & Häusser, 2013)(Mancuso et al., 2011). Some of these issues include unspecific and inaccurate targeting of neuronal populations and inaccuracies with respect to numerical and spatial control of neuronal manipulation (Packer et al., 2013). Furthermore, there is also a marked degree of inconsistency in

the extent to which a group of cells are optogenetically modulated, in given a site of neural tissue. These issues in essence reflect erroneous targeting, from unpredictable and imprecise expression of optically modified genes to the overall photophysical scope of manipulating (Packer et al., 2013)(Mancuso et al., 2011).

Voltage sensitive dyes

Voltage sensitive dyes are comparable to optogenetic sensors in their functions; they are essentially potentiometric dyes that alter their optical properties as a consequence of fluctuations or variations in the membrane potential (Cohen & Salzberg, 1978)(Antic, Empson, & Knöpfel, 2016)(Miller, 2016). They possess a range of applications, from the quantification of action potentials elicited by distinct neuronal cells, to activity seen in vast neuronal networks. They are also used to study the electrical activity of non-neuronal cells such as muscle and cardiac cells (Miller, 2016)(Herron, Lee, & Jalife, 2012).

Potentiometric dyes are employed to distinguish or ascertain the location where an action potential is initiated; and kinetic parameters, such as rate, velocity and course, concomitant to neuronal firing may be computed, elucidating morphological information about neurons (Baker et al., 2005)(Antic et al., 2016)(Cohen & Salzberg, 1978). They can be employed to synchronously monitor the activity of numerous locations, to comprehend networks (Baker et al., 2005). Their application is especially pertinent when electrophysiological techniques necessitating electrodes is not feasible to measure and scrutinize activity within a cell. They can be used even to detect membrane potential changes within cellular organelles, such as the mitochondria (Perry, Norman, Barbieri, Brown, & Gelbard, 2011), and fine neuronal structures such as dendritic spines (Popovic, Carnevale, Rozsa, & Zecevic, 2015), where traditional microelectrodes cannot be used. Studies have even demonstrated their applications *in vivo,* allowing scrutiny and quantification of neuronal activity in live animals (Ferezou, Matyas, & Petersen, 2009) permitting real time assessments, conjugating behavioural phenomena with encephalic functions.

Regardless of their vast applications, these optical apparatuses are associated with certain limitations. In *in vitro* studies, potentiometric dyes appear to display inconsistency with respect to how they perform in different experiments (Baker et al., 2005). They also exhibit inadequacies with respect to properly permeating samples, especially in regions with connective tissue. Thus, proper staining is not achieved, and is accompanied by several predicaments (Preuss & Stein,

2013). Additionally, undesired, off target effects may be elicited due to the pharmacological attributes of the dyes, instigating perpetual effects on neuronal cells subjected to staining (Preuss & Stein, 2013). Other concomitant predicaments include poor signal to noise ratio, photobleaching and inadequate staining intensity (Preuss & Stein, 2013)(Baker et al., 2005). In *in vivo* studies, voltage-sensitive dyes have also displayed deficiencies—such as small amplitudes, lack of specificity with respect to staining membranes, restrained permeation within certain cortical layers, and inability to distinguish between different types of neuronal populations (Ferezou et al., 2009)(Canepari, Willadt, Zecevic, & Vogt, 2010)(Chemla & Chavane, 2009).

Thus, the varying forms of photo-stimulatory and optical electrophysiological methods have intrinsic benefits and limitations, as briefly addressed by this section. Employment of these techniques for neuroscientific investigations is based on choices delineated by their inherent attributes, the limitations they levy, the query that is being pursued and the pertinent experimental model (Kramer, Fortin, & Trauner, 2009). Having illustrated a background of comparable techniques, I will now focus on elucidating the properties of photoactivatable caged neurotransmitters and their applications.

2.3 Photoactivatable neurotransmitters

Photoactivatable caged neurotransmitters have been a robust tool to comprehend neural function. The caged precursor molecule liberates the neurotransmitter upon illumination, following the disintegration of the covalent bond (Ellis-Davies, 2007)(Kramer et al., 2009)(Adams & Tsien, 1993). In the neuroscientific paradigms, neurotransmitters and neurotransmitter receptor agonists have been the most preponderantly used photoactivatable molecules (Kramer et al., 2009). Their spatiotemporal resolution with respect to altering neurotransmitter, agonist, and antagonist levels elicited by illumination offers numerous facilities to scrutinize and explicate kinetic, structural, pharmacological and mechanical properties of neuronal molecular machinery (Mayer & Heckel, 2006).

The first transmitter receptor agonists to be employed in caging techniques were UV-sensitive o-nitrobenzyl carbomoylcholine molecules. They were pivotal in the comprehending kinetic properties of acetylcholine receptors and neuronal activation mechanisms (Milburn et al., 1989)(J W Walker, McCray, & Hess, 1986b)(Kramer et al., 2009). Caged glutamate however, was the first

truly effective caged neurotransmitter and still persists as the most extensively used (Wieboldt et al., 1994b)(Kramer et al., 2009)(Ellis-Davies, 2007). Notably, glutamate uncaging led to discovery of visual cortex network and synaptic alterations over the course of neurodevelopment (Dalva & Katz, 1994). Currently, various forms of photolabile caged glutamate exist and have been formulated using different caging tactics (Ellis-Davies, 2007).

The caged photolabile counterparts of various neurotransmitters and neuroactive agents have been produced including single photon and two photon-sensitive varieties of photolabile GABA, glycine, dopamine, serotonin, anandamides and endocannabinoid receptor agonists (Mayer & Heckel, 2006)(Ellis-Davies, 2007)(Rial Verde, Zayat, Etchenique, & Yuste, 2008)(Heinbockel et al., 2005)(Shembekar, Chen, Carpenter, & Hess, 2007)(Kramer et al., 2009). Furthermore, caging has been employed with receptor antagonists, such as a photolabile azide conjugated AMPA receptor antagonist ANQX, which, notably, illumined AMPA receptor trafficking rates during LTP (Adesnik, Nicoll, & England, 2005).

Ideal traits of caged neurotransmitters

To be efficaciously employed in research of biological nature, caged photolabile compounds such as neurotransmitters must fulfill several requisites. The caging conjugate must make the bioactive neurotransmitter completely inert when in the caged form. The caged compound needs to be sufficiently soluble in solutions of aqueous nature and must be stable in physiological conditions, temperatures and pH. The cage molecule or products following photolysis should not pose toxic effects in the cell, and must not elicit any off-target effect or interferences with the cellular mechanisms, except for the intended effect of the uncaged neurotransmitter. With respect to the photolysis reaction, the products must be generated with high quantum yield, and the reaction must take place at an adequate velocity. Thus, both the quantum yield (ceiling limit of quantity of neurotransmitter liberated) and rate of photolysis (velocity of reaction) must be quantifiable and useful. Kinetics of cellular and molecular response events can be appreciated only if the photolysis reaction is rapid compared to the investigated mechanism (J H Kaplan & Somlyo, 1989).(Mayer & Heckel, 2006)(Adams & Tsien, 1993)(Ellis-Davies, 2007)(Kramer et al., 2009)(Hess, Lewis, & Chen, 2014)(Lester & Nerbonne, 1982).

Light eliciting the photolysis must pose minimal phototoxicity; and hence wavelengths not shorter than long-wave UV, which has sufficiently low photon energy, are are used. Control experiments

must be performed to ensure that the flash itself, independent of the caged neurotransmitter does not actuate a response. Stability of the caged compound and consequent photolytic products is crucial; so there is indeed a veritable concentration elevation of the neurotransmitter following the triggered light source (Mayer & Heckel, 2006)(Adams & Tsien, 1993)(Ellis-Davies, 2007)(Kramer et al., 2009)(Hess et al., 2014)(Lester & Nerbonne, 1982)(J H Kaplan & Somlyo, 1989).

2.3.1 Advantages

The use of caged photoactivatable neurotransmitters has several intrinsic benefits in comparison to alternative techniques for modifying intra and extra-neuronal concentrations of neurotransmitters. Their use enables tremendously quick intracellular release with high spatiotemporal resolution and reproducibility (Ellis-Davies, 2007).

Temporal resolution

Neurotransmitter release as a consequence of photolysis of the caged antecedent molecule is usually more rapid in comparison to other methods such as rapid perfusion change and pressurized application of the neurotransmitter by 'puffing' or 'spritzing'. In the former case a lag is certainly present as the solution with no bioactive molecules needs to be replaced with a solution with the bioactive constituents. Pressurized application is not devoid of lag due to diffusion of the neurotransmitter to its cognate receptor. The employment of photoactivatable compounds circumvents these predicaments by placing the caged transmitter in close proximity to the receptor (Ellis-Davies, 2007).

Spatial resolution

A primary potent feature of photoactivatable neurotransmitter release is that it is specific to the location of illumination, with minimal spot size being limited by diffraction. Caged compounds can be profusely present in the cytoplasm of neurons and photolysis can be specified by choosing between illuminating globally or at precise intracellular compartments (Ellis-Davies, 2007)(Rial Verde et al., 2008), a feature not often within the scope of other manipulative methods such as puffing and iontophoresis whose spatial resolution is not comparable (Rial Verde et al., 2008).

Juxtaposing uncaging with alternative approaches

Various methods have been employed to scrutinize and comprehend neurotransmitter and synaptic mechanisms such as stimulation with electrodes (Connors, Malenka, & Silva, 1988) (Alger &

Nicoll, 1979), application of neurotransmitters via iontophoresis (Andersen, Dingledine, Gjerstad, Langmoen, & Laursen, 1980), puffing (Feng, Tang, Chen, & Yang, 2017), and pressure injection (Connors et al., 1988). These aforementioned methods while holding their applications, also display several restrictions. Stimulating with electrodes has been critised for being a method with poor yield, and localization of receptors is not feasible with this method. Iontophoresis on the other hand may offer spatiotemporal regulation but this is limited. It is also a very invasive method and hence unfavorable for repeated or continuous investigation of a single neuron or local network (Rial Verde et al., 2008). Optogenetics, which offers comparable spatiotemporal resolution jeopardizes the genetic integrity of the tissue—and genetic alterations make the intrinsic attributes of the preparation questionable (Malyshev, Goz, LoTurco, & Volgushev, 2015).

Methods such as exchanging solution, rapid profusion and puffing though permitting transmitter application, do not evoke swift responses nor access to intra-neuronal sites. They also offer inadequate spatiotemporal control (Amatrudo, Olson, Agarwal, & Ellis-Davies, 2015). Photoactivatable neurotransmitters hence offer superior spatiotemporal release, and are non-invasive, and do not necessitate genetic alterations – offering optimal control with minimal perturbation.

2.3.2 Limitations

A predominant number of erroneous methodologies related to caged neurotransmitters are primarily due to cellular or pharmacological interactions exhibited by the caged precursor or its non-target by-products; reactions triggered by the light source on its own, and the rate-limiting nature of the relatively slower dark reactions of photolysis, whose speed is specific to the chemistries of both the cage molecule and the molecule being caged (Gurney, 2008)(Kaplan & Somlyo, 1989). Therefore, it is crucial that the caged neurotransmitter is thoroughly evaluated, its parameters quantified and validated prior to its employment in biological research(Hess et al., 2014).

A notorious predicament associated with caged neurotransmitters is the antagonistic behavior of some caged compounds on target receptors when in the un-photolysed form (Masanori Matsuzaki, Hayama, Kasai, & Ellis-Davies, 2010a)(Rial Verde et al., 2008)(Molnár & Nadler, 2000)(Amatrudo et al., 2015)(Shi, Trigo, Semmelhack, & Wang, 2014a) setting limitations on the

ceiling concentrations that may be used. This is often much lower than the true concentrations of neurotransmitters present in the cleft during synaptic neurotransmission(Shi et al., 2014a).

Studies have illumined that the caged precursor also has several off-target and undesired interactions with the cellular and neuronal systems(Adams & Tsien, 1993)(A. Gurney, 2008). Certain photoactivatable compounds also appear to display activity in target receptors even prior to illumination. Effects of photolysed by-products on cellular processes is also a concomitant issue(A. Gurney, 2008).

2.4. Brief glance at GABA receptors in the CNS

Gamma-aminobutyric acid (GABA) is the principal inhibitory neurotransmitter in the supraspinal CNS the adult CNS, GABA exerts its inhibitory function by diminishing neuronal excitability. The receptor targets of this neurotransmitter are ligand-gated ion channel $GABA_A$ receptors ($GABA_A$Rs) and metabotropic $GABA_B$ receptors ($GABA_B$Rs), in the synaptic, extra synaptic and presynaptic forms (Kaila, 1994)(Buzsáki, Kaila, & Raichle, 2007)(Farrant & Nusser, 2005)(Watanabe, Maemura, Kanbara, Tamayama, & Hayasaki, 2002a)(Farrant & Kaila, 2007). The fundamental subunit constitution determines their molecular edifice, isoform; and consequently their functions and affinities to GABA.

2.4.1 $GABA_A$ receptors

Under physiological conditions $GABA_A$Rs are permeable to chloride and to a lesser extent bicarbonate ions (Kaila & Voipio, 1987)(Kaila, 1994)(Farrant & Nusser, 2005). In most mature neuron, with low intracellular chloride concentrations, $GABA_A$R activation mediates inward flow of negative ions and an inhibitory post synaptic response. The electrochemical potential gradient across the neuronal membrane for chloride, is set by secondary-active chloride transporters, and ultimately determines the action on the membrane voltage of GABA binding to $GABA_A$Rs. For instance secondary active uptake of chloride raising its concentration in the neuron enables $GABA_A$Rs to mediate depolarizing currents as chloride ions are expelled down what is now an outward electrochemical gradient. Under such circumstances, GABA may function as an excitatory transmitter, as seen in some adult neuronal populations and more typically in immature neurons (Farrant & Kaila, 2007)(W Sieghart, 1995)(Watanabe, Maemura, Kanbara, Tamayama, & Hayasaki, 2002b)(Bureau et al., 1999)(Kai Kaila, Price, Payne, Puskarjov, & Voipio, 2014).

$GABA_A$Rs are heteropentameric structures incorporating five subunits circumscribing a central ligand-gated, anion-permeant pore (Sigel & Steinmann, 2012). All isoforms of this class of receptors adhere to this structural blueprint and their subunit configuration determines their neuronal functions, location, attributes and pharmacological pertinence.

The subunits of this receptors are stratified into several classes; presently 16 subunits including α1–6, β1–3, γ1–3, δ, ε, θ, π and 3 ρ subunits have been distinguished, cloned and sequenced from the mammalian encephalic systems (W Sieghart & Sperk, 2002). Moreover, several splice variants for certain subunits have been sequestered and examined (Barnard et al., 1998)(Simon, Wakimoto, Fujita, Lalande, & Barnard, 2004). The preponderant subunit composition of native receptors encompasses alpha, beta and gamma subunits (α, β, γ). In less predominant isoforms, delta (δ), epsilon (ε), and pi (π) subunits replace the gamma subunit, and theta (θ) subunit replaces the beta (Werner Sieghart, 2006).

$GABA_A$Rs distinguished both by subunits and neuronal niche mediate fast inhibition in discrete forms. They elicit fast inhibition in the millisecond time frame when sited synaptically; and long-term, slow inhibition as a consequence of ambient GABA (tonic) when located peri- and extra-synaptically (Farrant & Kaila, 2007)(Farrant & Nusser, 2005).

$GABA_A$Rs were primarily recognized pharmacologically by their agonistic activation by GABA, and selective agonist muscimol; their modulation by CNS depressant drug classes such as benzodiazepines, barbiturates, alcohol, and their obstruction by bicuculline and picrotoxin. These pharmacological agents exert their effect through binding with discrete allosteric sites on $GABA_A$Rs (Macdonald & Olsen, 1994)(Smith & Olsen, 1995)(Scimemi, 2014).

These inhibitory receptors are indispensable to CNS functions, playing pivotal roles in various phenomena — from fundamentally regulating excitation, shaping CNS development to learning and memory (Davies, 2003; Scimemi, 2014)(Olsen & Avoli, 1997)(Möhler, 2012)(Pratt, 1992)(Wagner, Castel, Gainer, & Yarom, 1997)(Izquierdo & Medina, 1991)(Berridge & Peciña, 1995)(Sarter, Schneider, & Stephens, 1988)(Paulsen & Moser, 1998)(Farrant & Kaila, 2007). As a consequence of their preponderance in the CNS, this class of inhibitory receptors serve as

pharmacologically manipulated targets for a multitude of drugs in research and clinical domains (Hevers & Lüddens, 1998)(Szczot, Kisiel, Czyzewska, & Mozrzymas, 2014).

2.4.1.1 Synaptic (phasic) $GABA_A$ receptors

Fast GABAergic synaptic transmission is mediated by phasic $GABA_A$Rs, whose subunit composition (in the mature neocortex most commonly α1β2γ2) enables them to facilitate synaptic shunting or voltage inhibition with high temporal precision (Kaila et al. 2014 CurrOpNeurobiol; Farrant and Nusser, 2005 NRN). Upon the advent of neuronal action potential firing in a GABAergic neuron, there is a localized influx of calcium ions which subsequently elicits the fusion of presynaptic neurotransmitter vesicles. This educes a discharge of thousands of GABA molecules into the synaptic cleft. They then diffuse and traverse the cleft and consequently bind synaptic $GABA_A$Rs, eliciting a contemporaneous activation of ion pores. Membrane permeability for chloride and bicarbonate ions is thereby intensified (Farrant & Nusser, 2005).

$GABA_A$Rs located in the synapse sites of neurons orchestrate a form of inhibition referred to as phasic inhibition. They aspect pre-synapse neurotransmitter release sites and are consequently triggered by being ephemerally subjected to an elevated level of GABA liberated by exocytic vesicular elements (Farrant & Nusser, 2005). In an extremely transitory timeframe of hundred microseconds, the GABA transmitter molecules are rapidly disseminated from the original site of liberation (Mozrzymas, Zarnowska, Pytel, Mercik, & Zarmowska, 2003). On the other hand they may be cleared via uptake by GABA transporters (Scimemi, 2014) such as GAT, and hence depleting the activation of the channels. This is principally phasic inhibition, concomitant with a tremendously transitory interval of GABAergic currents and its spatiotemporal facets highly tuned by vesicular liberation of the neurotransmitter GABA (Istvan Mody, 2001).

With respect to synaptic cleft concentrations of GABA, even a singular synaptic vesicle fusing unfetters up to thousands of neurotransmitter molecules in a 20nm wide cleft (Farrant & Kaila, 2007), eliciting ceiling concentrations of millimolar GABA (Mody, De Koninck, Otis, & Soltesz, 1994)(Mozrzymas, 2004)(Mozrzymas et al., 2003).

Hence, an elevated concentration of neurotransmitter is a pivotal to phasic receptors and the timeframe of the persisting transmitter is crucial to molding the currents. Studies have unveiled

the peak GABAergic phasic concentration to be around 3-5mM, which is dispelled in the timeframe of 300 to 500 microseconds (Cherubini, 2012).

2.4.1.2 Extrasynaptic (tonic) $GABA_A$ receptors

Extrasynaptic, high-affinity $GABA_A$Rs (with a subunit composition consisting of α5βγ2, α4βγ2 or α1βδ) orchestrate a tonic form of inhibition (Kaila, Ruusuvuori, Seja, Voipio, Puskarjov, 2014). They are sited away from synaptic regions and perpetually subjected to exposure of ambient nano-micromolar concentrations of GABA (Wu et al., 2013)(Farrant & Kaila, 2007)(Farrant & Nusser, 2005). Tonic forms of inhibition, contrariwise to the precipitous phasic inhibition, are spatiotemporally disconnected from presynaptic GABAergic liberation. Tonic inhibition is characterized by a perpetual and tenacious chloride conductance indicating steady state extrasynaptic $GABA_A$ receptor activation(Farrant & Nusser, 2005). The fundament of this "paracrine" activation is the profuse localization of $GABA_A$ receptors in the neuronal membranes of somatic, dendritic and axonal regions that are distally sited from the original location of neurotransmitter release(Kullmann et al., 2005a)(Semyanov, Walker, Kullmann, & Silver, 2004).

Concentrations of GABA present ambiently and necessitated to activate the extrasynaptic $GABA_A$ receptor population has been approximated to be in the tens of nanomolar to micromolar range (Farrant & Nusser, 2005), upto 0.2- 2.5 µM (Glykys & Mody, 2007). Though various sources have been postulated for tonic GABA, it is evident that vescicular release is a crucial determinant of tonic GABA presence (Farrant & Nusser, 2005)(Glykys & Mody, 2007), and phasic and tonic inhibition mechanisms are elicited with a mutualistic source and relationship.

2.4.1.3 Presynaptic $GABA_A$ receptors

Recent research has indicated, that counterintuitive to the traditional perspective of post-synaptic receptor localization; an extensive range of synaptic receptors are located pre-synaptically. This means they are in intimate proximity to neurotransmitter release site and thereby potently impact the processes such as fusion of transmitter vesicles. In this manner, $GABA_A$Rs have also been found to be located in the presynapse (Engelman & MacDermott, 2004)(Kullmann et al., 2005b). These receptors exert their functions by modulating and influencing neurotransmitter release. Such populations of presynaptic $GABA_A$Rs have been indicated to be located in various encephalic and

CNS regions such as the hippocampus, pituitary, and spinal cord. They are even implicated in action potential generation and may underpin events of neuropathology (Kullmann et al., 2005b).

2.4.2 $GABA_B$ receptors

A structurally divergent class of GABA receptors, $GABA_B$ receptors are inhibitory metabotropic G-protein coupled receptors (GPCRs). In comparison to $GABA_A$Rs, they elicit slow and persistent forms of inhibition upon GABA binding (Scimemi, 2014). This distinct class of receptors is expressed presynaptically and postsynaptically in various neuronal populations, serving discrete functions. As GPCRs, they modulate potassium and calcium channel activity, consequently eliciting slow post and presynaptic inhibition (Benarroch, 2012)(Waxham, 2013)(Pinard, Seddik, & Bettler, 2010).

In the presynaptic context, these receptors exert their influences by functioning as autoreceptors; consequently triggering potassium conductance and waning calcium conductance. In the postsynaptic niche, they trigger a distinctively slow inhibitory post synaptic potential, via activating potassium currents (Benarroch, 2012)(Pinard et al., 2010).

This group of receptors are ubiquitous in the several regions of the encephalon and CNS. They play diverse roles including modulation of excitation and synaptic plasticity in the neuronal populations of the cerebrum and cortex; eliciting rhythmic cortico-thalamic activation; altering dopamine function and conveying primary afferent inputs to distal regions such as the brainstem and spinal cord (Benarroch, 2012).

2.5. Caged GABA compounds as $GABA_A$ receptor agonists and antagonists

Photoactivatable caged GABA compounds have been novel implements for the investigation of the vast inhibitory GABAergic networks of the CNS; and the spatiotemporal and kinetic properties of the receptors intrinsic to these systems (Rial Verde et al., 2008)(Shi et al., 2014a)(Molnár & Nadler, 2000). Caged GABA compounds have a broad scope of applications for neuroscientific investigations, which have been addressed and enumerated in various concomitant studies. Notable, examples of caged forms of GABA that are more commonly used include CNB-GABA, DPNI-GABA, CNI-GABA, CDNI-GABA, Coum-GABA a RuBi-GABA (Molnár & Nadler, 2000)(Hess et al., 2014)(Ellis-Davies, 2007)(Rial Verde et al., 2008)(Amatrudo et al., 2014)(Masanori Matsuzaki, Hayama, Kasai, & Ellis-Davies, 2010b)(Richers, Amatrudo, Olson,

& Ellis-Davies, 2017)(Fan, Lewis, Hess, & Ganem, 2009)(Shi et al., 2014a)(Jerome & Heck, 2011)(Kramer et al., 2009)(Amatrudo et al., 2015)(Trigo, Papageorgiou, Corrie, & Ogden, 2009)(Trigo et al., 2010). A comparison of the most commonly used caged GABA photoactivities is illustrated in Table 2.5.1.

Table 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
350	60	38	420[a]	3400	1520	767
405	0	3	15	207	1866	1281
445	0	0	0	0	103	1107
473	0	0	0	0	0	447
532	0	0	0	0	0	0

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

Table 2.5.1: Most commonly used caged GABA compounds for single photon photolysis have a photosensitive covalent bond that is broken by photons with wavelengths corresponding to ultraviolet to violet. The sensitivity of RuBi-GABA is extended into longer wavelengths, making it useful for photolysis with blue LEDs. Modified from Filevich & Etchenique, 2013.

The spatiotemporal manipulation endowed by these photo-labile tools enables investigations of the kinetic properties of GABA receptors, mapping of GABAergic networks, neuronal and network related localizing of GABA receptor populations. Synaptic release can be evoked, and neuronal populations may be activated or optically silenced. Thus, caged GABA compounds have been particularly useful for investigation as a consequence of their role as $GABA_AR$ agonists (Rial Verde et al., 2008)(Shi et al., 2014a).

An undesired trait of several caged GABA compounds is their antagonistic actions on $GABA_ARs$ populations when in the unphotolysed precursor form. This imposes a predicament of the ceiling concentrations that may be used to elicit quantifiable evoked responses (Richers et al., 2017)(Shi et al., 2014a). This is an important to consider as synaptic $GABA_AR$-mediated transmission necessitates close to millimolar range local concentration (Farrant & Kaila, 2007; Farrant & Nusser, 2005). Caged GABA compounds are usually non-detrimental with respect to pre-photolytic interactions only at a concentration lower than 200µM (Shi et al., 2014a).

Design of caged compounds for GABA receptors must therefore must be propelled with an object of optimizing the antagonistic profile of compound and minimizing all such off-target reactions. Another sagacious tactic to circumvent these predicaments would be proper quantification of the antagonism elicited by these compounds. This would enable us to obtain valid results, and even use the antagonistic facet of these compounds in experiments that necessitate such features(Molnár & Nadler, 2000)Thus caged GABA compounds serve as both $GABA_A$ receptor agonists and "inadvertent" antagonists and proper quantification or examination of all their properties would enable a comprehensive, validated exploitation of these implements. This would yield accurate and reliable empirical results.

caged compound	IC_{50}	stability
mono-CNB-GABA[a]	28 μM	$t_{1/2}$ = 138 days
RuBi-glutamate, GABA[b]	0.1–0.3 mM	stable in dark
DPNI-GABA[c]	0.5 mM	stable in dark
CDNI-GABA[d]	0.6 mM	stable in dark
bis-CNB-GABA[e]	≥2.5 mM	$t_{1/2}$ = 98 days

Table 2.5.2 IC_{50} values of some caged GABA and glutamate compounds, including RuBi-GABA, indicating their half maximal concentration of antagonism for $GABA_AR$ and stability. From Shi et al., 2014.

RuBi-GABA

Ruthenium-bipyridine-triphenylphosphine- GABA, RuBi-GABA, is an inorganic, ruthenium based photoactivatable GABA compound (Nikolenko, Yuste, Zayat, Baraldo, & Etchenique, 2005; Leonardo Zayat, Marcelo Salierno, & Etchenique*, 2006; Zayat, Calero, Alborés, Baraldo, & Etchenique, 2003; Zayat et al., 2007)(Rial Verde et al., 2008). It has been employed for spatiotemporal investigation of $GABA_A$ receptors and GABAergic network populations. RuBi-GABA has been endorsed for several of its expedient attributes, such as high quantum yield, its photo-activation velocity due to the inorganic caging group, and particularly for its photolytic properties in the visible light spectrum, enabling GABA uncaging without the need for relatively more expensive UV lasers (Rial Verde et al., 2008).The compound is able to produce GABAergic responses mimicking endogenous event kinetics, such as rise time kinetics of inhibitory post-

synaptic currents (IPSCs) (Rial Verde et al., 2008). With respect to its optical parameters, RuBi-GABA has an absorption coefficient of εMAX = 5300 M−1 cm−1 at 447 nm and a quantum yield of ϕ ~ 0.09; hence endorsed for its high activity in the arena of caged GABA compounds (Filevich & Etchenique, 2013).

Thus it has been used in studies on receptor kinetic analysis, $GABA_AR$ spatiotemporal mapping and silencing of neurons (Rial Verde et al., 2008).The consensus based on current literature has been that RuBi-GABA antagonizes $GABA_ARs$ only at millimolar concentrations (Rial Verde; Shi et al. 2014). However, no systematic data supporting this claim has been provided (see Rial Verde et al. 2008).

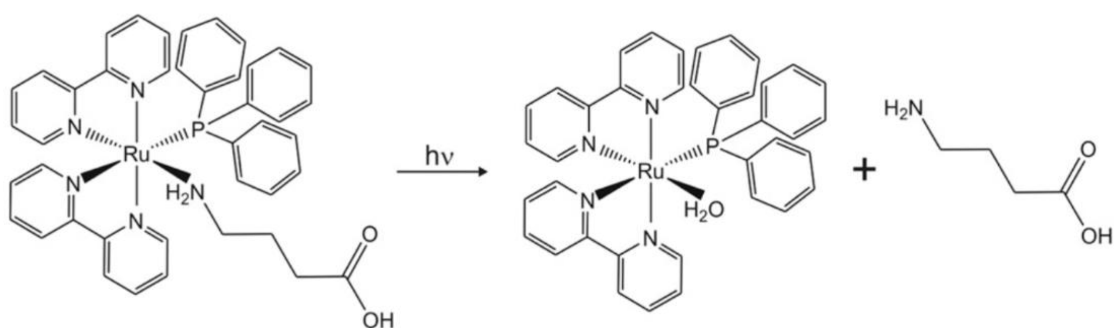

Figure 2.5.1: RuBi-GABA structure and photolysis. From Rial Verde et al., 2008

Table | **Comparison of kinetics of physiological and uncaging responses.**

Event type	**Number of events (cells)**	**Time to peak (ms)**		**20–80% Rise speed (pA/ms)**		**Decay time constant* (ms)**	
		Median	**IQR†**	**Median**	**IQR†**	**Median**	**IQR†**
Somatic mIPSCs	1099 (11)	6.6	4.7–9	2.4	1.49–4.54	22.44	17.84–27.57
mIPSCs	380 (3)	6.8	5.25–9.15	2.14	1.36–3.83	33.37	27.62–38.79
Uncaging responses							
All events	157 (7)	15.5	12.38–20.08	2.17	1.59–3.02	70.2	61.97–77.1
0.5 ms Pulse	25 (2)	16.9	13.68–19.65	1.49	0.98–2.59	76.87	70.61–89.31
1 ms Pulse	100 (2)	13.95	11.85–16.7	2.28	1.68–3.01	67.18	60.27–75
5 ms Pulse	32 (3)	28.55	19.4–35	2.39	1.93–3.86	70.61	63.87–79.46

**Decay time constant measured using single exponential fit, †Inter-quartile range.*

Table 2.5.2: Kinetics of GABAAR currents evoked by RuBi-GABA uncaging. From Rial Verde et al., 2008.

8-DMAQ-GABA

8-dimethylamino hydroxymethyl quinolone (8-DMAQ) caging groups show excellent applicability in both one-photon and two-photon set ups, are highly soluble and possess expeditious kinetic properties thus making them good candidates for neurotransmitter uncaging in electrophysiological experiments. Furthermore, their absorption peaks at a red-shifted spectrum making them highly alluring for neuroscientific and biological investigations. They have been used previously to cage kainate and glutamate for glutamatergic experiments where they displayed great efficacy, especially in comparison to MNI-glutamate(Dunkel et al., 2014)(Tran, Dunkel, Dhimane, Ogden, & Dalko, 2016)(Petit et al., 2012).

The 8-DMAQ caging group has now been utilized in the context of caged GABA, (8-DMAQ-GABA (Amit Kumar Pundir, Petra Dunkel, Hamid Dhimane, David Ogden, Peter I. Dalko, unpublished results), however its $GABA_AR$ antagonism profile is unknown.The varients of the cage 8-DMAQ were photolysed at 365nm UV and displayed quantum yields of 9.3 and 6.6%(Dunkel et al., 2014). The molar extinction for 8-DMAQ is εmax=1.5x103(M−1cm−1).

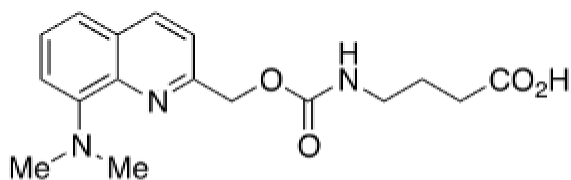

Figure 2.5.2: 8-DMAQ GABA Structure from Dunkel et al, 2014

3. Aims

The introductory chapters have elucidated the pivotal properties, intrinsic advantages, applicability and limitations of caged neurotransmitters and photo-labile GABA compounds; setting a pertinent background for the rationale for this study. The primary motivations of the study are to comprehend the intrinsic properties of two caged GABA compounds targeting $GABA_A$ receptors. This study introduces a novel caged GABA compound 8-DMAQ- GABA and aspires to revise the intrinsic properties of RuBi-GABA. The primary motivations of this study are:

1) To examine the $GABA_AR$ antagonism profile of a novel caged compound, 8-DMAQ-GABA, in its caged form, comparing it to that of the widely used RuBi-GABA.

2) To assess whether 8-DMAQ-GABA is useful for mimicking the kinetics of endogenous $GABA_AR$-mediated inhibitory post-synaptic currents, IPSCs; while examining the rise time kinetics of RuBi-GABA and 8-DMAQ.

3) To examine the utility of either caged compound at low micromolar concentrations (which are least likely to be antagonistic) for inducing photolysis-evoked $GABA_AR$ currents.

4. Methods and Materials

4.1 Cortical slice preparation

Postnatal day 16-18 male and female Han Wistar rats were used to obtain coronal brain slices of 400 micron thickness. Animals were brought from the animal housing 5- 10 minutes prior to decapitation to minimize time of maternal separation, and anaesthetized with halothane (2-bromo-2-chloro-1,1,1-trifluroethane obtained from Sigma Aldrich, St. Louis MO). The depth of anaesthesia was verified using tail and toe pinches before decapitation on ice cold sectioning solution bubbled with 95% oxygen and 5% carbon dioxide carbogen gas. Following decapitation, rapid craniotomy was performed and the brain (with olfactory bulb, brainstem and cerebellum dissected out) was removed and placed into the slicing chamber with ice-cold sectioning solution (temperature -0.4 to 0 °C). The brain was glued with cyanoacrylate to the vibratome stage and coronal slices were cut using a vibrating microtome (7000smz, Campden Instruments, Leicester, UK) equipped with a stainless steel blade. The entire sequence of procedures leading up-to the slicing were all executed within 3-4 mins after decapitation, and the brain was constantly immersed in the ice-cold sectioning solution at all times.

The entirety of the slicing procedure exposed the brain tissue to sectioning solution temperatures below 4°C, where the solution was consistently bubbled with carbogen gas. After each slice was obtained from the vibratome it was dissected into two parts (to enable easy placement in the recording chamber) and promptly transferred to ACSF standard solution bubbled with carbogen and preheated to ~ 34 °C. All of the slices obtained from each brain were recovered in the ACSF standard solution, at 34°C with consistent carbogen bubbling (pH 7.4) for a period of 1 hour.

The solution compositions were as follows:

Sectioning solution: 87 mM NaCl, 2.5 mM KCl, 0.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 1.25 mM NaH_2PO_4, 7 mM $MgSO_4$, 75 mM sucrose and 25 mM D-glucose. Osmolarity ~ 310 mOsm

ACSF Standard solution : 124 mM NaCl, 3 mM KCl, 2 mM $CaCl_2$, 25 mM $NaHCO_3$, 1.1 mM NaH_2PO_4, 2 mM $MgSO_4$, and 10 mM D-glucose. Osmolarity ~ 300 mOsm

4.2 Electrophysiology

Techniques which fall under the vast umbrella of electrophysiology are implemented for various forms of neuroscientific investigation. They enable elucidation of functions ranging from minute macromolecular machinery to more comprehensive milieus such as network activity. Electrophysiological techniques such as the patch-clamp methods can be utilized to monitor and comprehend intrinsic electrical properties of neurons, neuronal events and synaptic functions. Here, to analyze the antagonistic effects on $GABA_A$Rs of caged GABA compounds in their caged form, whole-cell voltage-clamp was employed to record miniature inhibitory postsynaptic currents (mIPSCs), which are an integral current of single $GABA_A$R channel mediated events triggered by spontaneous release of a single quanta of GABA into the synaptic cleft.

4.2.1 Whole-cell voltage clamp recordings of mIPSCs

The whole-cell voltage clamp technique has become the most common way of implementing the powerful voltage-clamp method in work on mammalian neurons. The less than 5 μm-diameter tip of a glass micropipette is gradually brought close the neuronal membrane and a mild suction force is applied; creating a tight seal between the membrane and pipette tip. The seal is characterized by an electrical resistance of gigaohms and hence referred to as the gigaseal. In this stage, cell-attached recordings may be obtained. For the whole-cell configuration however, an additional step of rupturing the membrane (within the ambit of the micropipette seal) is necessary.

This rupturing is accomplished by the application of an extremely brief but robust suction pulse, which efficaciously eradicates the intra-pipette section of the membrane. The neuron's cytosolic solutions are effectively replaced with the pipette solution within the matter of tens of seconds to minutes, thus offering a myriad of opportunities to manipulate the intracellular contents. The goal is however, to mostly mimic the intrinsic environment of the neuron's cytosol, barring a few minor alterations to aid the experiment's objectives (the most common alteration is substitution of cesium for potassium in the pipette solution, in order to improve space-clamp, i.e. electrical control of the neurons membrane in compartments beyond the spherical soma). In voltage-clamp, membrane potential is controlled by currents that are automatically generated by the negative feedback loop of the voltage-clamp circuitry, effectively mirroring and canceling out membrane currents, which makes the membrane potential follow the "command" potential. In other words the recorded

membrane currents are the opposite in polarity to those generated by the amplifier in attempt to maintain membrane voltage at the command potential.

Slices were placed post recovery in the submerged recording chamber, which was consistently perfused with ACSF standard solution (composition in previous section). To provide the necessary mechanical stability necessary for patch-clamp recordings, the slices were anchored in the chamber using a Lycra- threaded stainless steel harp (Warner Instruments, USA). The perfusion rate for the recordings was 3.5 ml/minute. In most cases, the ACSF was recycled in baseline recordings for economical use of chemicals in long-duration experiments. In the whole-cell recordings of mIPSCs, the ASCF extracellular solution also contained TTX (0.5 µM), D-AP5 (20 µM) and CNQX (10 µM; all obtained from Tocris) for blocking voltage-gated sodium channels, and glutamatergic NMDAR- and AMPAR-mediated currents, respectively. The recording ACSF was constantly bubbled with 95% O_2/5% CO_2 carbogen gas (pH 7.4) and the recording chamber temperature was maintained at 32±1 °C.

Cortical pyramidal neurons were patched using fire polished borosilicate micropipettes with a resistance of around 4-6 MΩ, and a cesium-based intracellular/intrapipette solution was used with the following composition: 140 mM Cs-methanesulfonate, 2 mM MgCl2, 10 mM Hepes [with pH adjusted to 7.2 with CsOH (280 ± 5 mOsm) (Spoljaric et al., 2017)

For the recording of mIPSCs with the specified solutions, the membrane voltage was clamped at 0 mV after whole cell configuration was achieved during patching. The liquid junction potential generated (with the Cs-based pipette solution) in the first steps of patching was around -13mV which was corrected for online. The access resistance was monitored at 120 s intervals throughout the recording to ensure that any changes in mIPSC frequency and amplitude were not due to voltage clamp deterioration. Only recording with less than 30% change of access resistance were processed for further analysis and integrated in the results.

The mIPSCs generated by $GABA_A$Rs were recorded and monitored using HEKA Patchmaster software and also with Win-EDR (Strathclyde Electrophysiology), with an EPC 10 patch-clamp amplifier at a sampling frequency of 50 kHz (HEKA Elektronik, Germany).

Spontaneous mIPSCs were recorded at 0 mV for at least 5 min in ACSF with blockers before bath application of the caged GABA compound (either 5, 10 or 100 µM RuBi-GABA, or 500 µM

8-DMAQ). Recordings were continued in the presence of the caged GABA compound for a minimum of 10 minutes. As the experiments aspired to elucidate the effects of the caged, unphotolysed forms of the caged GABAs and their GABA receptor antagonism, uncaging was not performed and all experiments were conducted in darkness. Special precautions were taken with RuBi-GABA, which is photolyzed also with visible light, hence all potential sources of light were minimized.

Data were low pass filtered at 1.5 kHz and manually analysed using Mini-Analysis software (Synaptosoft). For illustrations, the full-length recordings were high pass filtered at 5.3 Hz

4.2.2 Flash photolysis experiments

Flash photolysis experiments were conducted to compare the rise time kinetics of the $GABA_AR$ currents evoked by the two caged GABA compounds. To this end, the two caged compounds were employed in different concentrations via the ACSF extracellularly and uncaged at the soma of pyramidal neuron with a UV laser (372 nm; 2 mW; ~10 µm spot diameter, 0.1 – 10.0 ms flash duration). Evoked responses were recorded along with the spontaneously occurring mIPSCs. The recordings were then analysed using MiniAnalysis software (Synaptosoft) and the rise-time and decay kinetics of the evoked responses were analysed.

RuBi-GABA was used at the concentration of 5 µM and uncaged with a laser duration of 0.1ms after testing at 10 ms and 1 ms laser flashes, which elicited currents much larger in amplitude than even the largest mIPSCs. The 5 µM RuBi-GABA with 0.1ms laser duration elicited evoked responses that appeared reasonably similar to endogenous mIPSCs.

8-DMAQ was employed at the concentrations of 1, 5 and 35 µM. For the concentrations of 1 and 5 µM a laser duration of 10 ms was necessitated to evoke a response a distinguishable from the baseline noise. For the 35 µM concentration, a 0.7 ms laser duration was sufficient to see a large response. Control experiments were performed to ascertain that the laser stimulation even at 10 ms flash duration evoked no response in the absence of a cage compound in the bath.

Gabazine ($GABA_AR$ antagonist) application was titrated at 0.2-0.8 µM with all events (spontaneous mIPSCs and the RuBi-evoked responses) abolished at 0.6 µM of Gabazine.

5. Results

5.1 UV-photolysis of RuBi-GABA and 8-DMAQ-GABA

While RuBi-GABA has been reported to generate uncaging current similar to synaptic $GABA_A$Rs mediated events (Rial Verde et al. 2008), whether the recently synthesized 8-DMAQ-GABA is useful for mimicking IPSCs is not known.

RuBi-GABA uncaged at a 0.1 ms flash duration at the concentration of 5 µM used by Yuste and colleagues (Rial Verde et al. 2008), elicited events similar in amplitude to mIPSC, with the rise times of the evoked events qualitatively similar to those previously reported by these authors (Table 2.1.1). Though having marginally slower rise times, in line with the above previous report, these events to some degree, mimicked endogenous mIPSCs (Figure 5.1.3).

Reproducibly evoked currents with 8-DMAQ-GABA uncaging at a comparable concentration (1-5 µM) necessitated increasing the flash duration up-to 10 ms. Event rise times obtained under these conditions were comparable. Increasing the concentration of the caged GABA to 35 µM permitted decreasing the flash duration to 0.7 ms, while still retaining reproducibility in the amplitude of the evoked responses.

As mentioned in the methods section, 8-DMAQ GABA was used in the concentrations of 1, 5 and 35 µM. Uncaging was done using 10 ms laser duration for the 1 and 5 µM 8-DMAQ GABA and at 35 µM concentration uncaged at 0.7 ms laser duration to achieve a reasonably sized (amplitude-wise) response. Typical responses elicited by RuBi-GABA and 8-DMAQ GABA are illustrated in Figure 5.1.1 A-D.

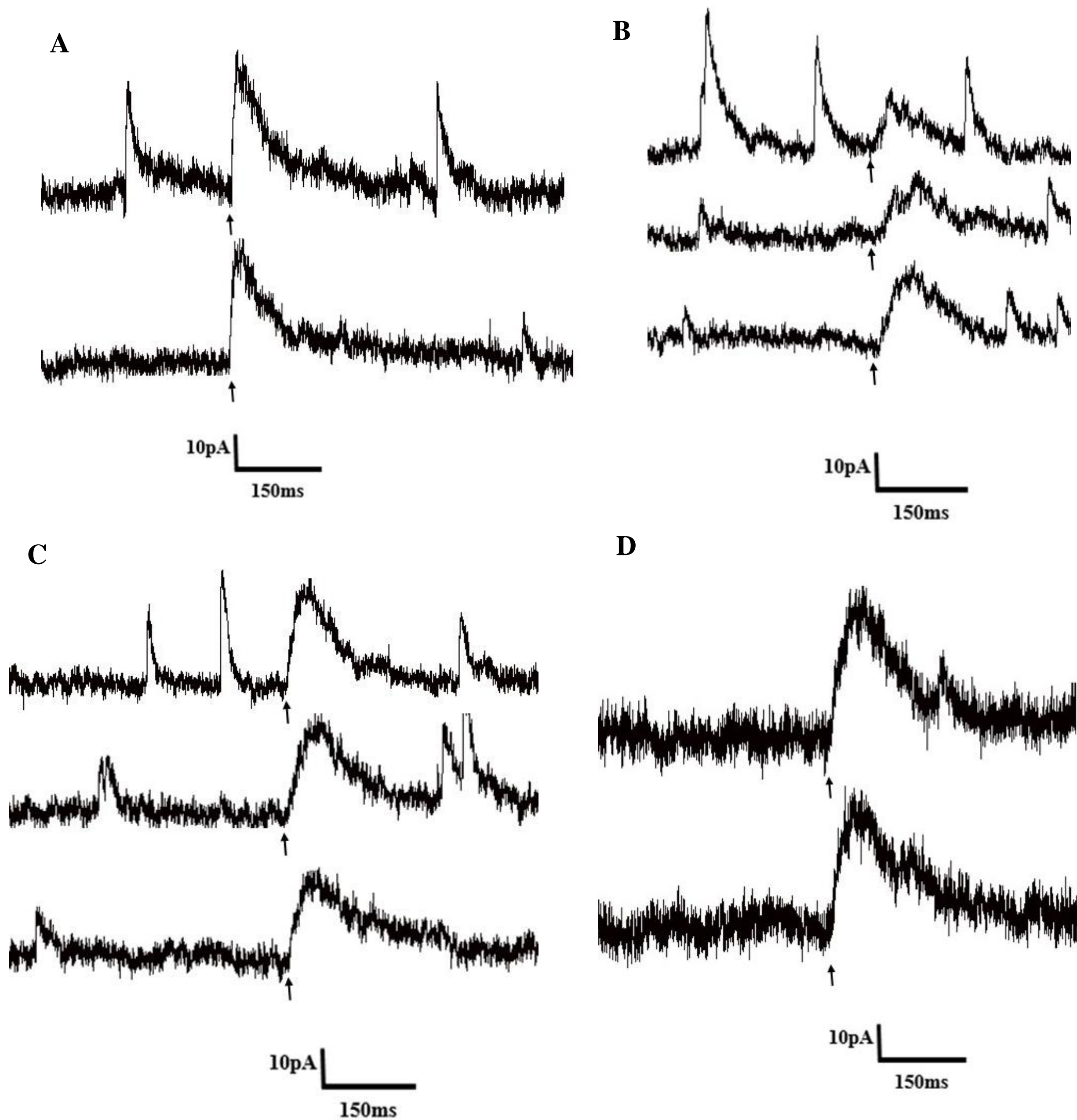

Figure 5.1.2: (A) Evoked responses elicited by 5 μM RuBi-GABA at 0.1 ms laser durations. The arrow indicates the timing of the laser flash of 0.1 ms. The other responses shown in the figure are endogenous mIPSCs. Evoked responses elicited by 8-DMAQ GABA at the concentrations 1 μM with 10 ms laser duration **(B),** 5 μM with 10 ms laser duration **(C)** and 35 μM with 0.7 ms laser duration **(D).**

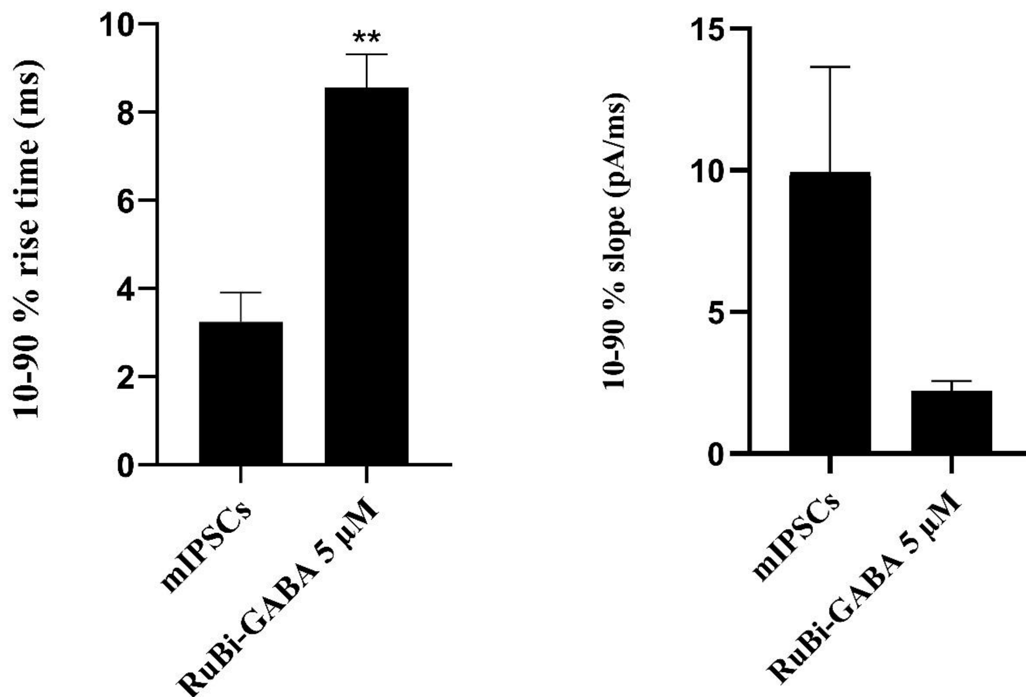

Figure 5.1.3: Comparison of 10-90% rise time of RuBi-GABA evoked responses (5 µM uncaged with 0.1 ms laser duration) to that of endogenous mIPSCs. Values used were the mean 10-90 rise time and slope (for endogenous mIPSCs and the evoked response and t-tests were used for statistical analysis. Double asterisks indicates $p < 0.005$. Here the p value for rise times was 0.0018. The slope values did not display statistically significant difference between the two groups. n=6 for evoked responses and a total of 29 mIPSC events.

The evoked responses elicited by 8-DMAQ GABA were much slower with respect to rise time kinetics (when compared to endogenous events and also RuBi-elicited responses). 8-DMAQ GABA evoked responses did not display (as indicated in Figure 5.1.2) the distinctive "sharp" rise seen in mIPSCs (symptomatic of fast rise times and high slope values), even to the degree of RuBi-GABA.

The 10-90% rise time and 10-90% slope of the 8-DMAQ GABA elicited events were analyzed and compared with the endogenous mIPSCs of each recording, revealing that the 8-DMAQ evoked responses rose more slowly than the mIPSCs, and 8-DMAQ slope values were significantly lower (Figure 5.1.4).

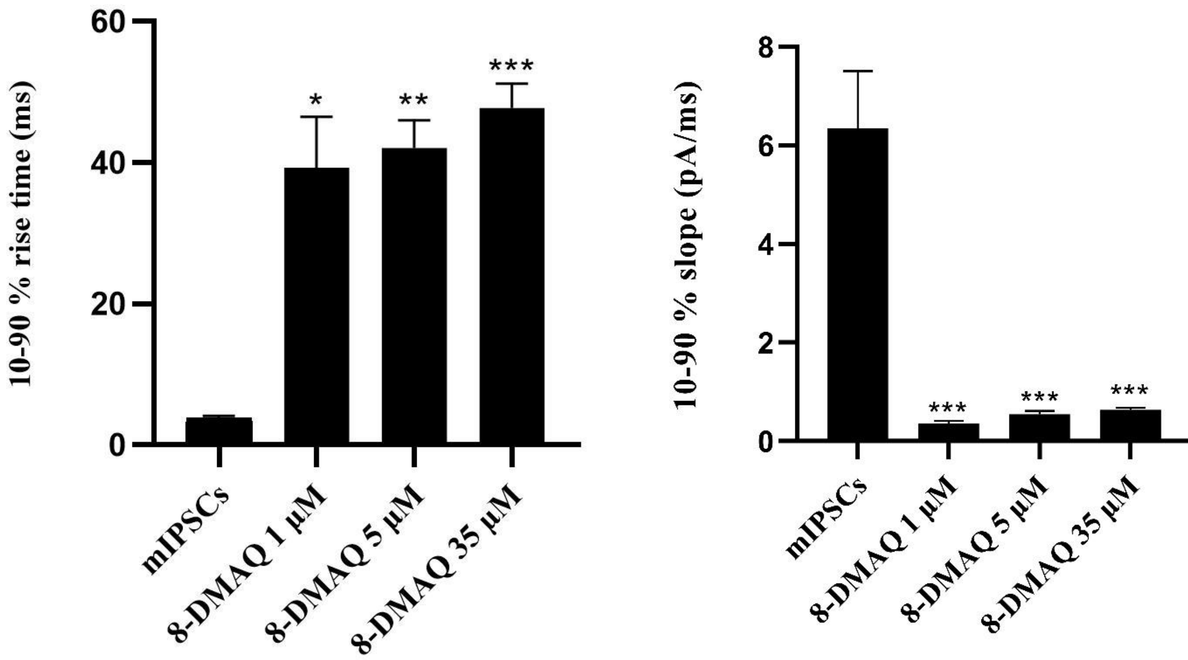

Figure 5.1.4: Comparison of 10-90% (A) rise time and 10-90% slope (B) of 8-DMAQ GABA elicited responses and endogenous mIPSCs. Values used were the mean 10-90 rise time and slope in each case (for the evoked response and mIPSCs) and *post hoc* Anova analysis was used for statistical analysis. Here, an asterisk indicates $p < 0.05$, two indicate $p < 0.005$ three asterisks indicate a significance of $p < 0.0005$. n= 5-6 for evoked responses at each concentration and a total of 56 mIPSCs events.

5.2. GABA$_A$ receptor antagonism by micromolar RuBi-GABA

RuBi-GABA effectively abolishes mIPSCs at 100 µM

Cortical pyramidal neurons were patched and after recording the baseline with spontaneous mIPSCs for a minimum of 5 minutes; RuBi-GABA was applied via the extracellular solution (ACSF) perfusion system for a minimum of 10 minutes (see Methods and Materials, voltage clamp). Access resistance was constantly monitored to ensure that any changes in mIPSC frequency and amplitude were not due to access resistance change. In a surprising manner, RuBi-GABA consistently and almost completely abolished mIPSCs, dramatically reducing both the amplitude and frequency of mIPSCs (Figures 5.2.1; 5.2.4).

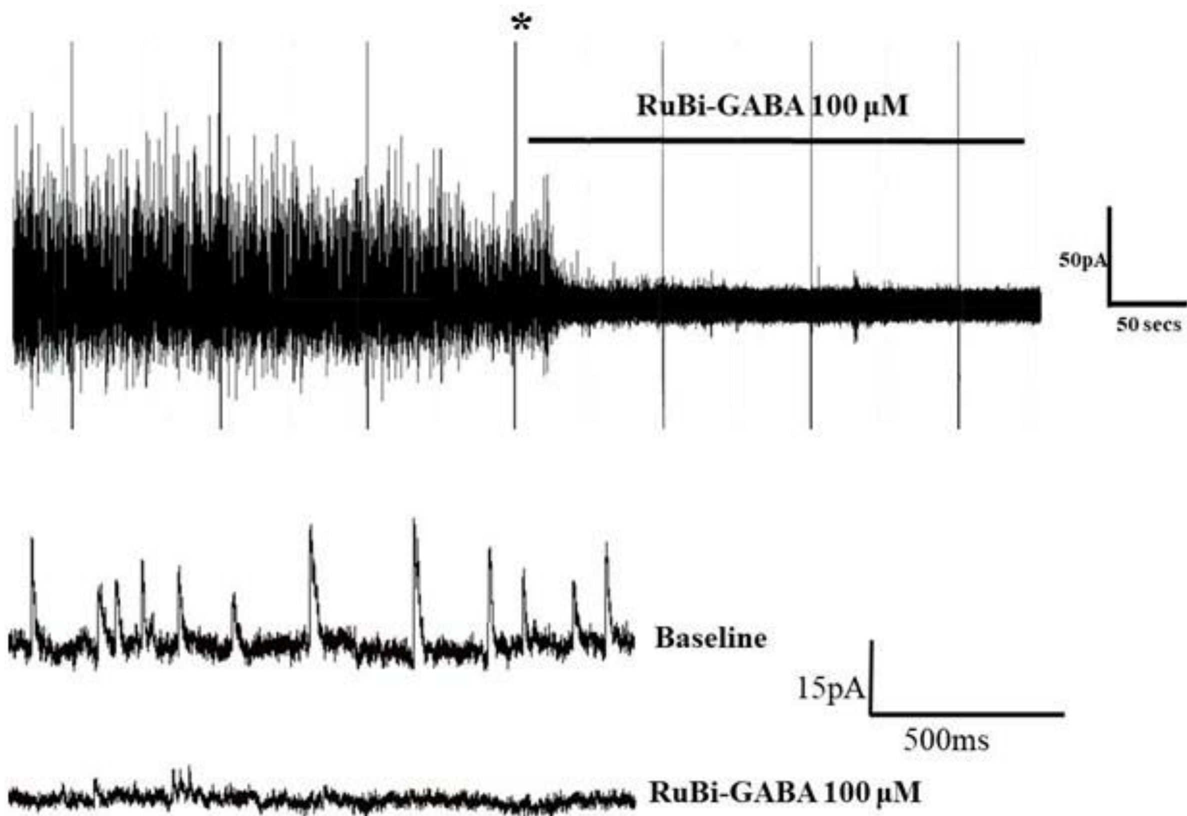

Figure 5.2.1: RuBi-GABA effectively abolishes mIPSCs at 100 µM concentration. This is indicative of severe GABAAR antagonism. Upper trace depicting baseline mIPSCs and their amplitudes after RuBi-GABA application in a contracted time scale. The transient full-amplitude deflections that are seen with a constant interval in the top trace are caused by access resistance monitoring. The inset lower traces depict basline mIPSCs and mIPSCs post RuBi-GABA application in an expanded time scale. *Test pulse current transients elicited every two minutes to monitor access resistance were truncated.

RuBi-GABA exerts marked GABA$_A$R antagonistic effects at low micromolar concentrations

Motivated by the striking antagonistic effect achieved RuBi-GABA at 100 µM we next sought to examine whether this effect extends to the low micromolar concentrations 5-10 µM, that have been used in patch clamp experiments (e.g. Chamma et al. 2013 JNS), and reported not to impart GABA$_A$R antagonism (Rial Verde et al. 2008). Contrary to this report, under the present conditions, RuBi GABA, both at 5 (Figures 5.2.2) and 10 µM (Figure 5.2.3) RuBi-GABA continued to significantly suppress both the amplitude and frequency of mIPSCs in a dose dependent manner (Figure 5.2.4).

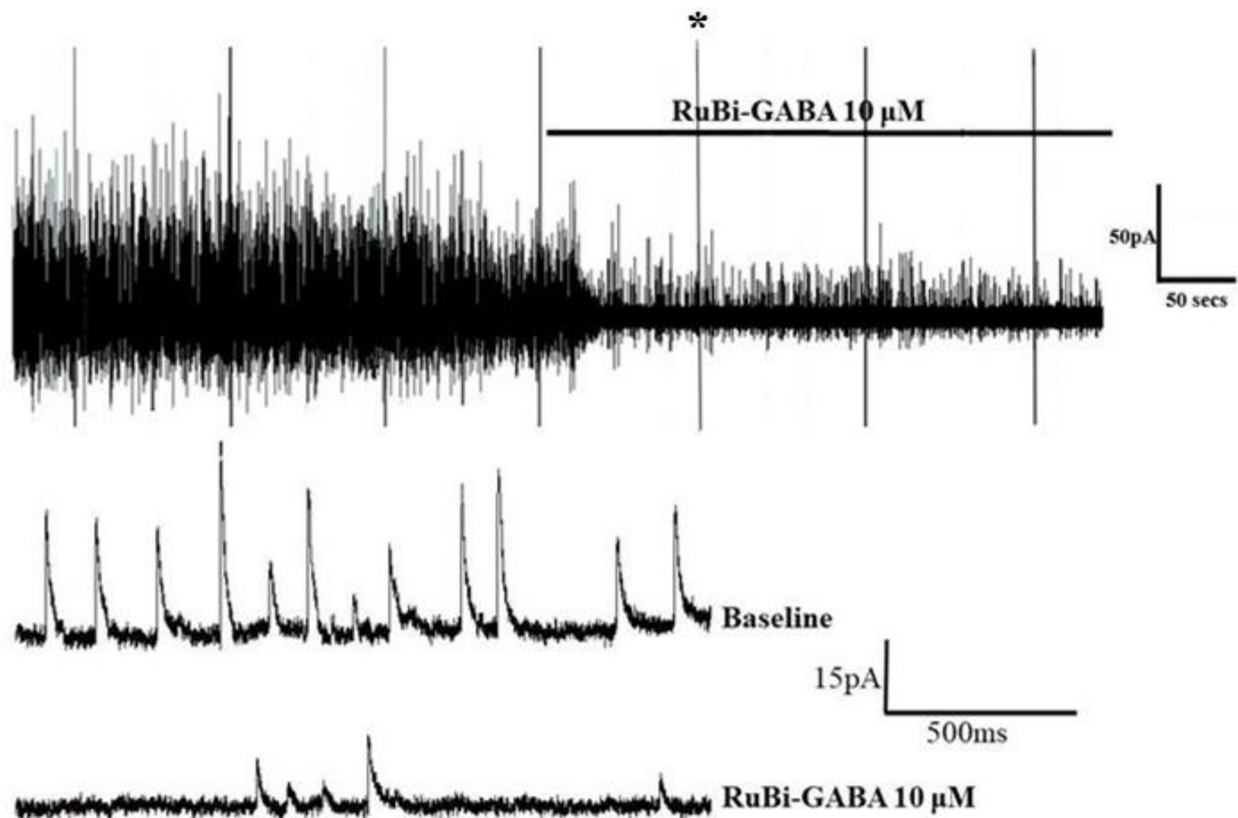

Figure 5.2.2: RuBi-GABA has a marked antagonistic effect even at 10 µM concentration. Upper trace depicting baseline mIPSCs and their amplitudes after RuBi-GABA application. The transient full-amplitude deflections that are seen with a constant interval in the top trace are caused by access resistance monitoring. The inset lower traces depict baseline mIPSCs and mIPSCs post RuBi-GABA application in an expanded time scale. The markedly diminished amplitude and frequency are visible in all traces. *Test pulse current transients elicited every two minutes to monitor access resistance were truncated.

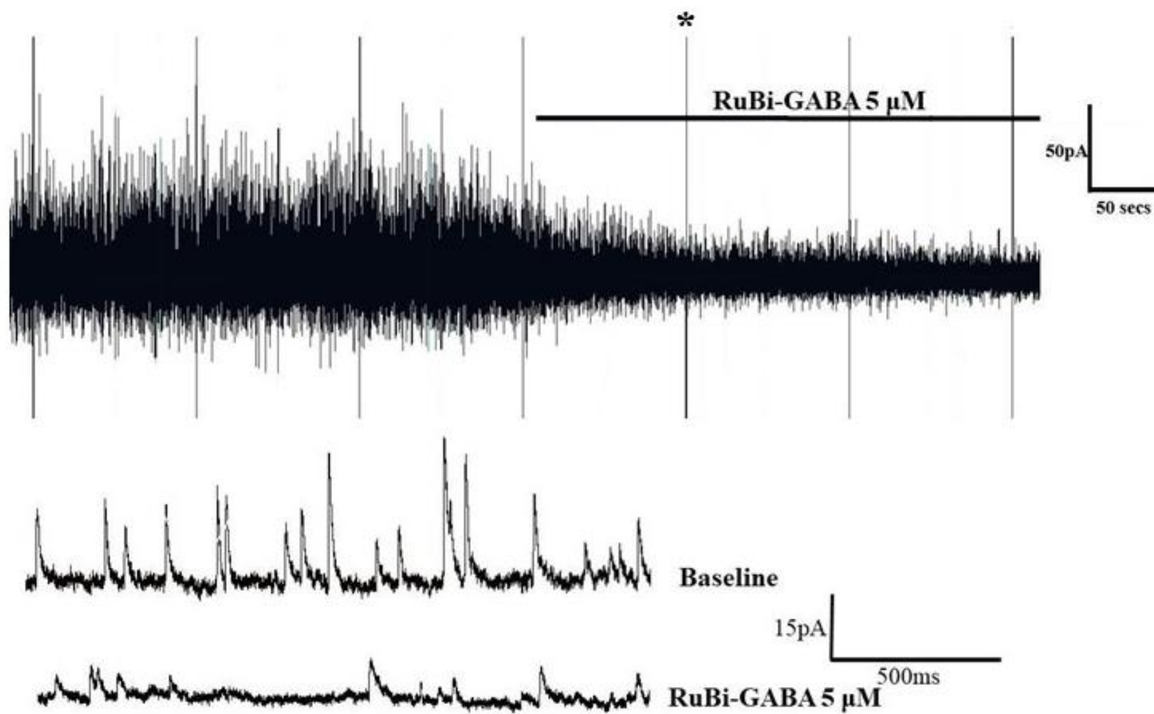

Figure 5.2.3: RuBi-GABA antagonism persists and is distinguishable at 5 µM concentration. Upper trace depicting baseline mIPSCs and their amplitudes after RuBi-GABA application .The transient full-amplitude deflections that are seen with a constant interval in the top trace are caused by access resistance monitoring. The inset lower traces depict baseline mIPSCs and mIPSCs post RuBi-GABA application in an expanded time scale. The $GABA_AR$ antagonism posed by RuBi-GABA is evidently distinguishable in all traces even at such a low concentration. *Test pulse current transients elicited every two minutes to monitor access resistance were truncated.

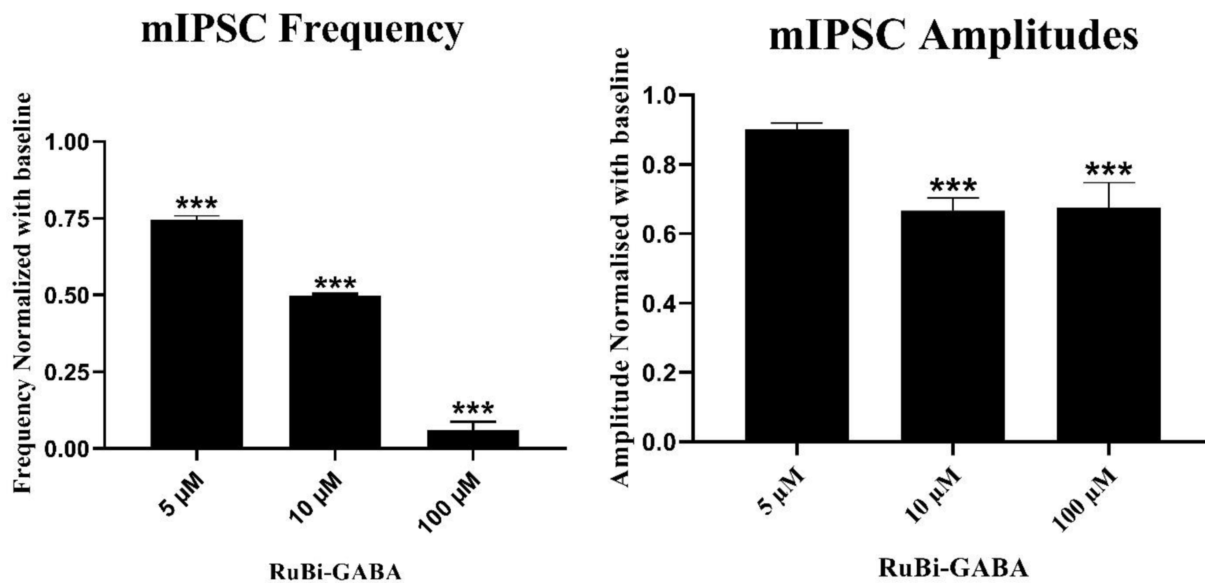

Figure 5.2.4: The effect of RuBi-GABA on mIPSC frequencies and amplitudes. All numbers were normalized with the baseline values. n = 3 to 4 recorded neurons for each RuBi-GABA concentration and a total of 10 baseline controls. Statistical significance was observed in each case (paired t test with *post hoc* mixed-effects analysis); ***p < 0.0001.

5.3 GABA$_A$ receptor antagonism profile of 8-DMAQ-GABA

8-DMAQ-GABA does not display GABAAR antagonism even at 500 µM

The novel caged GABA compound 8-DMAQ GABA (provided by Dr. Peter Dalko, Paris Descartes University) was employed to examine its possible antagonistic actions on GABA$_A$Rs and the mIPSCs mediated by the receptors. Based on previous pilot experiments on spontaneous IPSCs (TTX-free recording conditions) in our laboratory this compound was presently used at a concentration of 500 µM and similar experiments were conducted in darkness, where the caged 8-DMAQ GABA was applied for a minimum of 10 minute after a 5 minute baseline recording. The caged compound was applied via the extracellular ACSF perfusion.

The novel caged GABA 8-DMAQ did not have any antagonistic actions nor did it diminish mIPSCs (Figure 5.3.1), even at the high concentration of 500 µM. (Here too, access resistance was constantly monitored for previously mentioned reasons.)

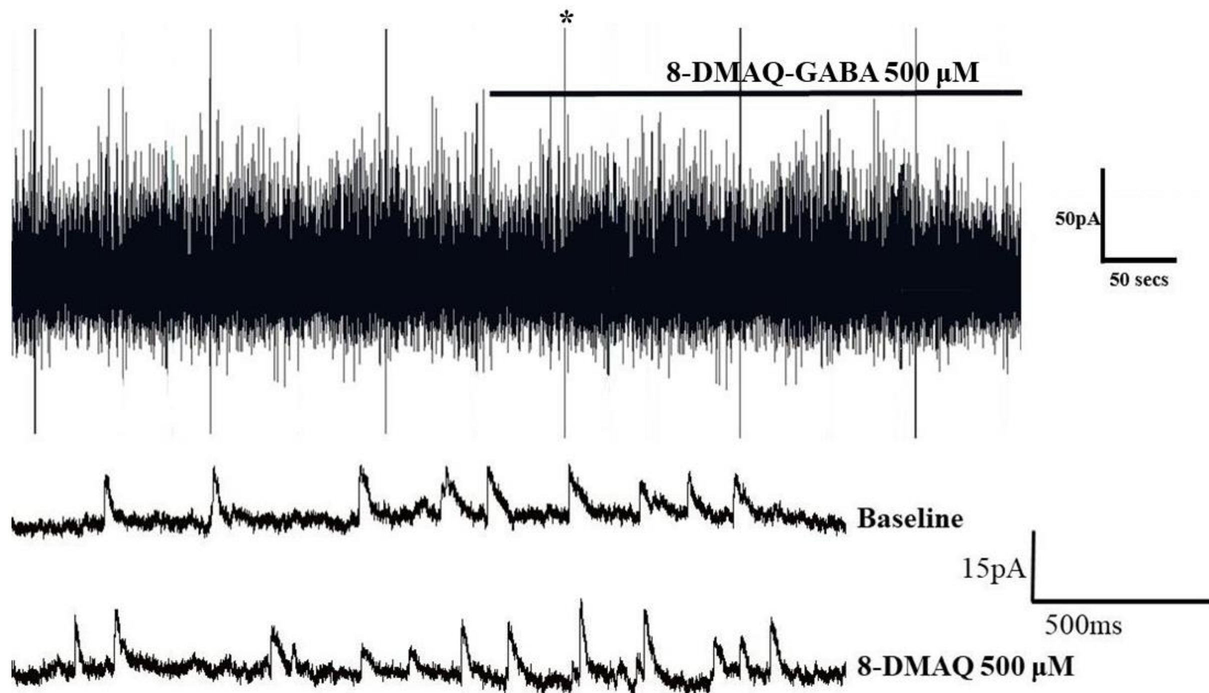

Figure 5.3.1: 8-DMAQ GABA does not display $GABA_AR$ antagonism even at 500 µM. Upper trace depicting baseline mIPSCs and their amplitudes after 8-DMAQ-GABA application scale. The transient full-amplitude deflections that are seen with a constant interval in the top trace are caused by access resistance monitoring. The inset lower traces depict baseline mIPSCs and mIPSCs post 8-DMAQ-GABA application in an expanded time scale. The absence of antagonism is evident in all the traces depicting undiminished mIPSC frequency and amplitudes. *Test pulse current transients elicited every two minutes to monitor access resistance were truncated.

Further analysis of parameters such as mIPSC frequency and amplitudes revealed a statistically significant trend where there was no $GABA_AR$ antagonism posed by 8-DMAQ. Paradoxically, this compound has a positive modulatory effect on both mIPSC frequencies and amplitudes (Figure 5.3.2). Paired and unpaired t-tests were used for both these parameters (amplitude and frequency) revealing statistically significant results.

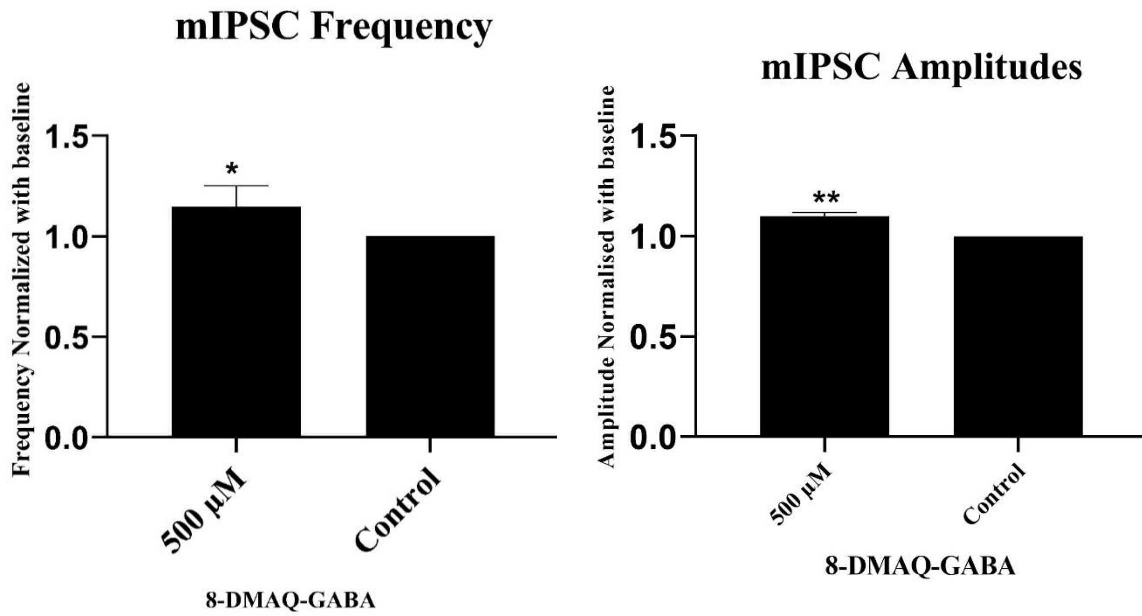

Figure 5.3.2: The effect of 8-DMAQ GABA at 500 µM on mIPSC frequencies and amplitudes. All numbers were normalized with the baseline values. n=6 for 8-DMAQ experiments with 6 controls. Statistical significance was observed in each case (paired and unpaired t-tests were the statistical measures employed here p values: for frequencies = 0.0325 * and amplitudes = 0.0042 **). This clearly indicates the positive modulation elicited by the caged form of 8-DMAQ GABA.

6. Discussion

One of the primary objectives of this study was to, using electrophysiological experiments, establish an antagonism profile for RuBi-GABA by observing its effects on mIPSCs in its caged form when applied extracellularly. No systematic assessment of the $GABA_A$ receptor antagonism profile of RuBi-GABA is provided in the existing literature. Rial Verde and colleagues (2008) reported that RuBi-GABA does not display $GABA_A$R antagonism in the low micromolar range: “at concentrations<20µM we have never detected any effect on either membrane resistance, or frequency or amplitudes of mEPSCs or mIPSCs (Rial Verde et al., 2008)”. This is especially problematic as this compound is currently one of the most widely used caged GABA compounds. Furthermore, papers describing the antagonistic actions of other caged GABAs appear to describe very different numbers, for example, micromolar concentrations typically employed for uncaging are able to antagonize $GABA_A$Rs (Molnár & Nadler, 2000).

The aim furthermore was to determine the lowest concentrations at which the compound poses $GABA_A$R antagonism. This preponderantly used caged GABA has been reported thus far to elicit significant $GABA_A$R antagonism only in the millimolar concentration range (Rial Verde et al., 2008). There are therefore no publications explicitly affirming the exact antagonism profile with realistic values. The major discovery of this study is that this caged compound poses severe $GABA_A$R antagonism already at low micromolar concentrations. At 100 µM, RuBi-GABA abolishes mIPSCs and at 10 µM causes a marked diminishment of mIPSC frequencies and amplitudes. Even at a very low concentration of 5 µM, $GABA_A$R antagonism elicited by RuBi-GABA persists and is distinguishable. These values from the results of this thesis are similar to values described for the antagonism elicited by other caged GABA forms, for example, cGABA (Molnár & Nadler, 2000).

As discussed in the introductory chapters, an ideal caged compound must not have any interactions with the biological system in its unphotolysed form, and neither should non-target by-products elicit any biochemical alterations. However, many caged compounds do have inadvertent biological activity in their unphotolysed form, and a multitude of caged neurotransmitters act as receptor antagonists in the caged form (Adams & Tsien, 1993; Amatrudo et al., 2015; G. C. R. Ellis-Davies, 2007; A. Gurney, 2008; Hess et al., 2014; Jerome & Heck, 2011; J H Kaplan & Somlyo, 1989; Mayer & Heckel, 2006; Richers et al., 2017).

Hence, the synthesis and design of caged compounds must be primarily propelled by the objective minimizing all off-target reactions and biochemical alterations. The antagonism profile must be optimized if not negated in the case of photo activated neurotransmitters. Furthermore, a sagacious approach would be to properly quantify the antagonism profile and its various interactions with the biological system if at all they are inevitable. Proper quantification of such effects such as antagonism would enable valid results and accurate experimental methodology. Moreover, apposite and exhaustive comprehension of the compounds properties would allow us to exploit all facets of their activity. For example, even the antagonistic properties of such compounds can be exploited in experimental procedures that necessitate such elements (Molnár & Nadler, 2000). All in all, this would yield valid empirical results. Thus, this study has increased our knowledge on the properties of the caged GABA compound RuBi-GABA, by revising its antagonism profile.

The study was also impelled by the objective of understanding the antagonism profile of the novel caged GABA compound 8-DMAQ-GABA in its unphotolysed form. The results of this study revealed that this compound does not pose significant antagonistic effects on $GABA_AR$ activity even at a remarkably high concentration as 500 µM. In fact, in the present experiments a small but statistically significant degree of positive modulation, where mIPSC frequencies and amplitudes were consistently increased. A parsimonious explanation to such an effect is that part of 8-DMAQ-GABA had spontaneously degraded into free GABA or been photolyzed by exposure to a light source. The latter option is effectively excluded as care was taken to maintain the both the dry stock and aliquots of the compound in darkness even during the experiments, which were conducted under a dim infrared light directed away from the patch setup. The exact mechanism of this positive modulation is beyond the ambit of this study and requires further scrutiny using mass spectrometry to assess whether spontaneous degradation releasing free GABA has occurred in the stock solution. Of note, in a pilot experiment conducted earlier this year, no positive modulation by 500 µM 8-DMAQ-GABA was observed (M. Puskarjov, unpublished observation). Furthermore, single channel activity of $GABA_ARs$ could be monitored with this compound to delve further into the kinetics and intricacies of this positive modulation.

Since 8-DMAQ does not antagonize $GABA_ARs$ as indicated by this study, this photolabile implement could serve to be particularly useful (once the underlying mechanism of positive modulation is illumined). 8-DMAQ may be used in very high concentrations to mimic synaptic

cleft neurotransmitter concentrations; which is otherwise unachievable with alternative caged GABA forms. This is because their high GABA receptor antagonism levies a ceiling concentration limit, which is significantly lower than cleft concentrations. Hence, 8-DMAQ GABA shows substantial promise as a novel candidate in this arena.

Reaction kinetics of evoked responses by both the caged GABAs were also examined in this study to understand their properties of photolysis. The study clearly revealed that RuBi-GABA is able to elicit evoked responses that mimic endogenous mIPSCs to a degree, displaying the characteristic "sharp" rise of endogenous mIPSCs. Though a bit slower than endogenous mIPSCs with respect to rise times (10-90% rise time), RuBi-GABA appears to be a great candidate for mimicking spontaneous events, as endorsed by previous literature (Amatrudo et al., 2015) (Rial Verde et al., 2008). Furthermore, these rise kinetics can be optimized by simple methodological improvements by altering the location of uncaging along the neurons, for example, to elicit fast responses closely mimicking mIPSCs (Rial Verde et al., 2008). RuBi-GABA is able to produce reliable evoked responses at low concentrations such as 5 μM, necessitating very brief laser flashes (0.1 ms for this concentration under the present experimental conditions) to produce events with the amplitudes of endogenous mIPSCs. Such attributes are ideal for minimizing any photo damage to the cell.

Currents evoked by 8-DMAQ-GABA uncaging on the other hand, display much slower rise time kinetics than RuBi-GABA, (as seen with the 10-90% rise time and slope), producing evoked responses that do not exhibit the characteristic sharp rise of mIPSCs. The rise time and slope parameters of the evoke responses by 8-DMAQ also differ from those of endogenous mIPSCs. Thus this compound is perhaps not applicable for eliciting evoked responses closely imitating spontaneous events. The compound also requires a laser flash duration longer than necessitated by RuBi-GABA (10 ms laser flash for an evoked response with an amplitude response that was close to that of the mIPSC at 5 μM concentration). This attribute of 8-DMAQ GABA is conceivably a corollary of unspecific absorption (Trigo et al., 2009), thus necessitating optimization. This compound is able to produce evoked responses at concentrations as low as 1 μM, and also reliably responds to laser flashes (consistent uncaging with respect to timing of flash), as does RuBi-GABA.

The slow rise times of 8-DMAQ are possibly indicative of the velocity of the dark reactions that occur after the photon has impacted the bond triggering photolysis; which in 8-DMAQ's case possibly proceed slowly.

In this study, the lowest concentration of RuBi-GABA employed was 5 μM for uncaging responses, which necessitated only a 0.1 ms flash (longer flashes elicited responses of huge amplitudes in comparison to mIPSCs). This indicates that the compound may be used at much lower micromolar concentrations (by calibrating laser duration appropriately) for uncaging experiments. 8-DMAQ was also able to produce evoked events at 1 μM concentration with a 10 ms flash, however mIPSC level amplitudes were only obtained with a 5 μM concentration. These results provide clues about lowest useful concentrations that may be implemented in the case of each compound for uncaging experiments. The fact that in the present experiments GABAAR currents could be evoked by uncaging GABA from cage concentrations as low as 1 μM strongly indicate that such currents are mediated by extrasynaptic high-affinity $GABA_A$Rs. This is an interesting conclusion, as typically, as done also presently, kinetics of uncaging evoked responses are compared to synaptic mIPSCs or sIPSC, which are mediated by synaptic $GABA_A$Rs. On the other hand both mIPSCs and currents evoked from 5 μM caged GABA were equally blocked by sub micromolar concentrations of Gabazine, which are known to preferentially target synaptic $GABA_A$Rs. This suggests that at the concentrations typically used for uncaging >5 μM (Puskarjov 2017 Anesthesiology; Chamma et al. 2013 JNS, Real Verde et al. 2008) the evoked events are mediated by a mixture of both tonic and phasic $GABA_A$Rs.

In conclusion, the revised antagonism profile for RuBi-GABA yielded by this study illumine the intrinsic properties, limitations and novel applications of this caged compound. This experimental thesis also scrutinized the photo-kinetic profile of this compound, emphasizing its robust attribute that may be used to mimic endogenous events. The visible-light absorption of RuBi- GABA is its endorsed asset; however, as is evident from the literature (cite here the review with the wavelengths table) and shown also in study shows that photolysis of the compound is possible with a UV- range light source.

The novel compound 8-DMAQ shows promise as a caged GABA candidate due to the absence of any $GABA_A$R antagonism. Hence, the compound may be used in very high concentrations to specifically target synaptic populations and also mimic cleft concentrations. However, it does

exhibit a surprising trait of positively modulating GABAAR activity, which requires further scrutiny. The study revealed its comparably slower photolysis and kinetics, an attribute which may levy certain limitations. If the minute intricacies of kinetics are not very important to an experimental methodology, 8-DMAQ is a good candidate to provide reliable uncaging responses. This caged compound is also not sensitive to visible light, thus reliable for uncaging without spontaneous or non-specific photolysis via any ambient light source or stray photons (whereas RuBi-GABA requires a dark setting for reliable uncaging).

Thus, this study elucidates certain intrinsic properties of two forms of caged GABA, augmenting the perspectives on their applications and future directions for these persuasive implements. Evidently both RuBi-GABA and 8-DMAQ possess their inherent merits and also some inadequacies. The choices made with respect to such apparatuses always depends on the requisites of the experimental system and the queries that are pursued. The results of this study clearly demarcate a few aspects concomitant to RuBi-GABA and the novel contender 8-DMAQ; however the remaining questions are certainly persuasive for imminent research.

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