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Unambiguous evaluation of the relative photolysis rates of nitro indolinyl protecting groups critical for brain network studies

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ABSTRACT

Nitrated indolinyl photoprotecting groups are crucial tools extensively used in the study of neuronal signal transduction. Mononitrated photolabile protecting groups have been used effectively, however, recent advances in the introduction of a second nitro group have shown improvement in the photo efficiency of neurotransmitter (agonist) release, albeit, to varying extents, depending on the assessment methods employed. An unambiguous method is discussed based on Nuclear Magnetic Resonance (NMR), which is shown to be an effective technique in the relative overall rate comparison amongst varying nitrated protecting groups. Mononitrated and dinitrated photolabile protecting groups such as CDNI-Glu and MNI-Glu are used as an example to assess the relative value of adding a second nitro group in photoactive cage designs. Using this technique, it was shown that the second nitro group in CDNI systems enhances the overall relative rate of photocleavage by a factor of 5.8. This reported method can also be used to unambiguously determine relative rate of agonist photorelease.

1. Introduction

The mammalian brain contains a complex circuitry of neurons with numerous synaptic connections integrated with one another. For a thorough understanding of brain function and subsequently brain disorders, it is crucial to decode the neural circuitry with the resolution of a single synapse. Electrostimulation techniques lack such spatial resolution [1]. Neuroscience research endeavours have made recent use of neurological chemical tools known as caged neurotransmitters [1]. A caged neurotransmitter is a neurotransmitter, such as glutumate (Glu), attached to an inactivating molecular entity known as the cage. Photocleavable cages have gained popularity due to their ability to release the active neurotransmitter upon demand with a focused beam of photons of specific wavelengths. Newer technologies make use of two photon laser spectroscopy to enable superior penetrative light properties and a lower phototoxicity relative to the corresponding energy of a single photon in the UV region [2]. Photocleavable caged neurotransmitters enable a high degree of resolution leading to an ultimate spatio temporal control in the stimulation of a single synapse.

There has been a great deal of interest into the design of efficient cages for use in photolysis experiments [1]. It is inherently necessary to understand which properties can be altered to enhance photolysis. For instance, adding one nitro group to a mononitrated caged molecule renders this dinitrated compound more efficient [3]. However, it is not necessarily always straightforward to assess the photorelease efficiencies of these new molecular designs using UV–vis. Current methods use UV–vis as a technique to evaluate the role of having an additional nitro group on the nitroindolinyl ring. It is naturally the preferred means due to its rapid results and directness of measurement. However, as highlighted in the report by Timothy Dore and Hunter Wilson, there are inevitable challenges encountered when trying to assess the efficiency of mono and di-nitro molecules [4]. An unambiguous evaluation of the role of the additional nitro using sequential NMR measurements is presented herein. There is a need to introduce additional alternative methods to assess newly designed and synthesized molecules when they are not amenable to UV–vis interpretations. A recent report using 2D NMR to study reaction kinetics *in situ* was also highlighted [5]. In this communication, direct 1D ^{1}H NMR is used to gain reliable relative photorelease information with reproducible results.

The most common commercially available cage for a neurotransmitter is the 4-methoxy-7-nitroindolinyl cage. Remarkable advances in caged neurotransmitter design have been made

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independently by both Ellis-Davies [7] and Corrie [8] leading to cages that carry two nitro groups instead of one. One such example is the photochemical protecting group 4-methoxy-5,7-dinitroindolinyl (MDNI) which they both reported to show an improvement in quantum efficiency over its mononitrated analogue, (MNI) [7,8].

A related caging group, 4-carboxymethoxy-5,7-dinitroindolinyl (CDNI), was reported by Ellis-Davies [3] in 2007. It showed an improved solubility over MDNI due to the presence of a carboyxlate side chain in place of the methoxy group in MDNI. The presence of the additional nitro group on the cage (in MDNI and CDNI) improved the quantum efficiency of Glu release, as shown by UV–vis kinetic measurements. Using UV–vis kinetic measurement in the case of the mononitrated cage, MNI-Glu, was a direct and rapid technique to determine the kinetics of photorelease, resulting in a clear isosbestic point reflecting the clear presence of two distinct species, the starting caged compound and the spent cage. However, the additional nitro group, results in a UV–vis spectrum, which is somewhat less resolved with a less well-defined isosbestic point, rendering a more challenging kinetic evaluation of these improved cage designs. This could be due to several competing factors.

Although, ^{1}H NMR has been used for several years to gain kinetic information, it has not been employed in this way for photocaged molecules, except in showing the mere presence of the products. This research uses this NMR method as an alternative to make comparisons between the well-established MNI and MDNI cages, as evidence of our methodology, and for the additional benefit of using such technique to evaluate the relative rates of photorelease of our future cage designs. This is especially useful to researchers who may not have access to LASER photolysis equipment or Ultra High Performance Liquid Chromatography (UHPLC) apparatus.

2. Materials and methods

2.1. UV–vis studies

The rates of disappearance of the photochemical protective group were initially investigated using UV–vis studies, which, as mentioned above, have many advantages including low sample concentrations, rapid analysis, and ease of quantitation of results [9–12].

In all UV–vis studies the sample was dissolved in the appropriate solvent and serially diluted to the appropriate concentration. An aliquot was transferred into a UV cuvette which was then irradiated in a Rayonet photochemical reactor at 350 nm. UV–vis spectra were collected on a JASCO V-650 UV–vis spectrophotometer from 250 to 600 nm, and the predominant absorbance signals were fitted to a first-order decay or rise using Sigmaplot 11.

Commercially obtained MNI-Glu was dissolved in a water solution (pH = 7.39 with $NaHCO_3$) and serially diluted to a concentration of 0.074 mM. An aliquot was transferred into a clear UV cuvette which was then irradiated in a Rayonet® photoreactor at 350 nm [7,8,13]. MDNI-Glu and CDNI-Glu were synthesized from commercially available starting materials and UV experiments were run as above.

2.2. ^{1}H NMR studies

The general experimental set-up follows. A sample about 2 mM was transferred to a transparent quartz NMR tube which was then seated in a 125 mL Erlenmeyer flask and irradiated in the Rayonet photochemical reactor at 350 nm (75Wx16 UV lamps). After each irradiation period, a ^{1}H NMR spectrum was collected using a Bruker 400 MHz NMR. The NMR spectra were then calibrated for the solvent peak, and then a sample peak was chosen as an internal calibration. The data was fitted to global (common rate constant) and individual first-order decay, rise and second-order rise using the statistical software Sigmaplot 11®.

Commercially obtained MNI-Glu (0.33 mg in 0.500 mL D_2O, $[\ ]_0 = 2.04$ mM) was transferred to a transparent quartz NMR tube which was then seated in a 125 mL Erlenmeyer flask and irradiated in the Rayonet® photochemical reactor at 350 nm (75Wx16 UV lamps) for initially 20 s and subsequently 40 s intervals. After each irradiation period, a ^{1}H NMR spectrum was collected. The NMR spectra were then calibrated for the D_2O peak at 4.79 ppm, and the j+j' peaks integrated to 2.00, providing an internal calibration as the total concentration of caged Glu (j) and free Glu (j′) should always be 2 protons. Upon complete photolysis, the sample turned dark yellow from light yellowish beige. The data was fitted to global common rate constant and individual 1st order decay and rise to a maximum using the statistical software Sigmaplot 11®. MDNI-Glu and CDNI-Glu were synthesized from commercially available starting materials and ^{1}H NMR experiments were run as above.

3. Results

For the identification of free glutamic acid in the studied cages, Fig. 1 shows the UV–vis spectrum of glutamic acid on the same graph as the caged and uncaged MNI-Glu. This shows that the increasing absorption at 285 nm in this experiment and all of the caged glutamate experiments is due to the uncaged glutamate and the other absorbances are from either the cage molecule or remnant cage after photolysis.

For the commercially available MNI-Glu, results were reproducible and matched the published data for UV [6]. UV spectra were collected from 250 to 550 nm, and the absorbance at easily identifiable inflection points were fitted to a 1st order decay and rise to a maximum (Fig. 2).

The UV rates were more accurate (stronger R^2 correlation) for the rise at 403 nm than the decay at 332 nm. The 1st order rise to a maximum showed the highest rate constant (k) of 26.2×10^{-6} ms^{-1}. Overall, UV kinetics was fast but somewhat limited when assessing other cages or in providing mechanistic details.

Furthermore, MDNI-Glu was synthesized and the resultant UV spectrum was similar to those of Corrie et al. (Fig. 3) [8]. The UV rates showed a stronger R^2 correlation for the rise at 398 nm than the rise at 348–287 nm. The 1st order rise to a maximum showed

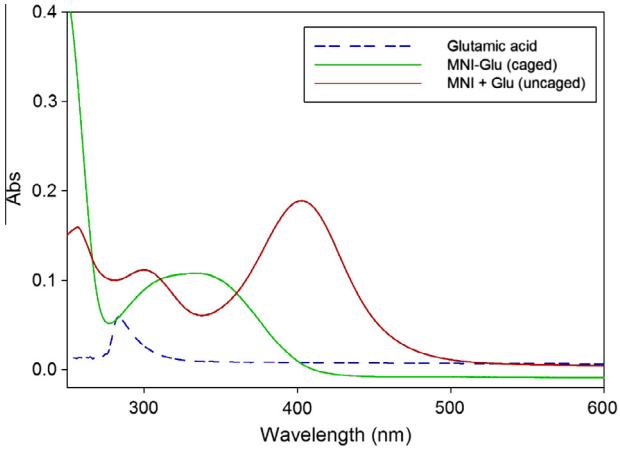

Fig. 1. Net UV–vis spectrum of glutamic acid, MNI-Glu (caged), and MNI + Glu (uncaged).