Photochemically Knocking Out Glutamate Receptors In Vivo

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Supporting Information

Docking

The crystal structure of the GluR2 ligand-binding core complexed with DNQX (PDB: 1ft1) was used for all molecular docking studies. To prepare the ligand binding core for docking, the Tripos *Sybyl* 6.6 Biopolymer package was first used to block the ends (remove the N-terminal NH_3^+ and C-terminal COO^- charges), add all hydrogens and electron lone pairs, and calculate AMBER All-Atom charges.[1] Next the ligand binding pocket was defined using *Sybyl* to identify heavy atom residues lying within a 20 Å radius and a molecular surface for the pocket was generated using the program *dms*.[2] Next, the electrostatic, steric, and chemical properties (property grids) of the pocket were calculated using the program *grid*.[3] Inhibitor structures were built with Gasteiger charges using *Sybyl*, and initial rotamer conformations generated and minimized using *Omega*.[4] Finally, the program *DOCK* was used to generate placements for the inhibitor molecules.[5]

Synthesis

All reagents were commercially available and used without further purification unless otherwise indicated. Melting points were determined using a Buchi B-540 apparatus and are uncorrected. ^{1}H NMR spectra were recorded using a 400 MHz Varian Inova NMR spectrometer. Chemical shifts (δ) are reported relative to the internal standard tetramethylsilane (TMS). Fast atom bombardment (FAB) mass spectra were obtained at the University of California at Berkeley Mass Spectrometry Facility. Thin-layer chromatography was performed using silica gel 60 F_{254}-coated plates (EM Science, Gibbstown, NJ). Flash column chromatography was carried out using silica gel 60 (EMD Chemical, Inc., Cincinnati, OH). All reactions were carried out under an inert atmosphere of argon unless otherwise indicated.

6-Nitro-1,4-dihydroquinoxaline-2,3-dione. The title compound was synthesized according to the literature with modifications.[6] 4-Nitrophenylenediamine (25.0 g, 163 mmol) was suspended in diethyl oxalate (100 mL) and heated to reflux. After 24 h, the mixture was allowed to cool to ambient temperature and the precipitate collected via filtration, washing repeatedly with EtOH to afford an orange solid that was used without further purification (28.5 g, 86%): mp >315 ºC dec (lit. mp >300 ºC dec); ^{1}H NMR (400 MHz, $(CD_3)_2SO$) δ 7.25 (d, 1 H, Ar*H*, *J* = 8.8 Hz); 7.95 (m, 2 H, Ar*H*), 12.15 (s, 1 H, N*H*), 12.35 (s, 1 H, N*H*).

6-Amino-1,4-dihydroquinoxaline-2,3-dione. The title compound was synthesized according to the literature with modifications.[7] 6-Nitro-1,4-dihydro-quinoxaline-2,3-dione (7.0 g, 33.8 mmol) was dissolved in EtOAc:EtOH (95:5, 100 mL) and added to a Parr flask containing Pd/C (10% dry on charcoal, 0.5 g) in EtOH (10 mL). The flask was pressurized to 30 psi with H_2 and shaken overnight. After ~12 h, the heterogenous solution was filtered through Celite, washing with EtOAc and EtOH, and the filtrate rotary evaporated to afford a light yellow solid that was used without further purification (5.4 g, 90%): mp >315 ºC (lit. mp >330 ºC dec.); ^{1}H NMR (400 MHz, $(CD_3)_2SO$) δ 5.07 (bs, 2 H, ArNH_2), 6.31 (dd, 1 H, Ar*H*, *J* = 8.4, 1.6 Hz), 6.33 (d, 1 H, Ar*H*, *J* = 1.6 Hz), 6.82 (d, 1 H, Ar*H*, *J* = 8.4 Hz), 11.68 (bs, 2 H, 2 N*H*).

6-Azido-1,4-dihydroquinoxaline-2,3-dione. To a 0 ºC solution of 6-amino-1,4-dihydro-quinoxaline-2,3-dione (0.62 g, 3.5 mmol) dissolved in 2 N HCl (33 mL) was added slowly a solution of

$NaNO_2$ (0.25 g, 3.6 mmol, 0.5 M) in H_2O (7.0 mL). After stirring for 15 min, the reaction vessel was covered with aluminum foil and a solution of NaN_3 (0.23 g, 3.6 mmol, 0.25 M) in H_2O (13.0 mL) was added dropwise and the reaction was allowed to warm to ambient temperature. After stirring for 10 h, the mixture was cooled to 0 ºC and the precipitate collected via filtration, washing well with EtOH and 2N HCl to afford a tan solid that was used without further purification (0.56 g, 79%): mp 197-198 ºC dec; ^{1}H NMR (400 MHz, $(CD_3)_2SO$) δ 6.76 (d, 1 H, Ar*H*, *J* = 1.6 Hz), 6.82 (dd, 1 H, Ar*H*, *J* = 8.4, 1.6 Hz), 7.28 (d, 1 H, Ar*H*, *J* = 8.4 Hz), 11.83 (bs, 1 H, N*H*), 11.90 (bs, 1 H, N*H*); MS-FAB (*m*/*z*): 204 (M + H)$^+$.

6-Azido-7-nitro-1,4-dihydroquinoxaline-2,3-dione (ANQX). To a solution of 6-azido-1,4-dihydro-quinoxaline-2,3-dione (0.7 g, 3.5 mmol) in AcOH (35 mL) was added dropwise a solution of fuming HNO_3 (5.6 mL, 132 mmol). After stirring for 16 h, the resulting precipitate was collected via filtration, washing extensively with AcOH, 2N HCl, H_2O, and EtOH to afford a tan solid that was used without further purification (0.625 g, 72%): mp 301-302 ºC dec.; ^{1}H NMR (400 MHz, $(CD_3)_2SO$) δ 7.06 (s, 1 H, Ar*H*), 7.76 (s, 1 H, Ar*H*), 12.04 (bs, 1 H, N*H*), 12.16 (bs, 1 H, N*H*); MS-FAB (*m*/*z*): 249 (M + H)$^+$.

Molecular Biology.

The rat wild-type GluR1 (flop) was a gift from Dr. Stephen F. Heinemann. The GluR1 gene (minus the 5'UTR) was subcloned into a modified bluescript vector containing a 5'AMV sequence and a 3' poly A tail.[8] Site-directed mutagenesis (QuikChange Mutagenesis Kit; Stratagene, La Jolla, CA) was used to introduce the GluR1-L497Y point mutation and the sequence of the amplified region confirmed by DNA sequencing (Biomolecular Resource Center, University of California, San Francisco). The resulting construct was linearized with *Not I* and capped mRNA transcripts were synthesized *in vitro* using T7 polymerase (mMessage Machine Kit; Ambion, Austin, TX).

Electrophysiology.

Stage V-VI oocytes were surgically obtained from adult *Xenopus* laevis frogs (Nasco Intl., Fort Atkinson, WI) as previously described and stored at 18 °C in ND96-storage solution (96 mM NaCl, 2.0 mM KCl, 1.0 mM MgCl, 1.8 mM $CaCl_2$, and 5.0 mM HEPES, 1% horse serum, 2.5 mM Na pyruvate, 0.5 mM theophylline, 50 mg/mL gentamycin, pH 7.5).[9] Oocytes were injected in the cytoplasm (50 nL/oocyte) with GluR1 mRNA (1.0 μg/μl) using a positive-displacement injection apparatus (Drummond Nanoject).

Glutamate-evoked currents from AMPARs were recorded 24-48 h after injection using a two-electrode voltage clamp recording configuration (GeneClamp 500B, Axon Instruments, Union City, CA) at a holding potential of -80 mV, using electrodes (0.5 to 2 MΩ resistance) filled with 3 M KCl. Ooctyes were continuously perfused with ND96-recording solution (96 mM NaCl, 2.0 mM KCl, 1.0 mM MgCl, 1.8 mM $CaCl_2$, and 5.0 mM HEPES, pH 7.5).

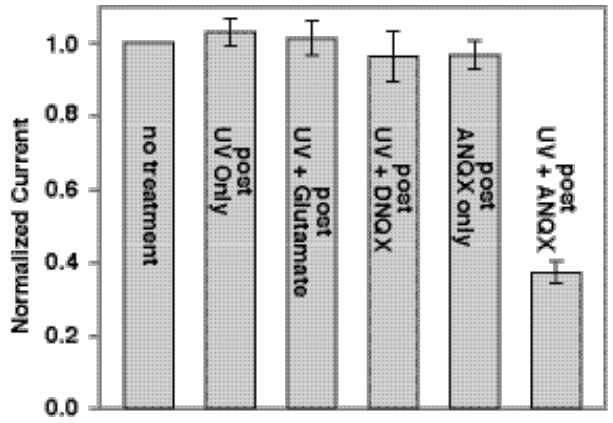
GluR1 peak dose response currents were recorded in response to 2 μM glutamate in the presence of varying concentrations of antagonist (0-150 μM). Antagonist solutions were prepared from fresh DMSO stocks of antagonist. Control experiments confirmed that the concentration of DMSO used during IC_{50} measurements (0.01-0.40% v/v DMSO) did not affect glutamate-evoked currents. Solution changes were controlled via an electronic valve control system with pinch-type valves (Valvelink 16, Automate Scientific, Inc., San Francisco, CA). Current responses were acquired using pClamp 9.0 software (Axon Instruments, Union City, CA) and a Digidata 1322A interface (Axon Instruments).

Data were analyzed and graphed using SigmaPlot 8.0 (SPSS, Inc., Chicago, IL). Responses were fit to the logistic equation $I = I_{max} \times (1/(1 + (IC_{50}/[ligand])nH))$, where I_{max} is the response at the specified concentration of ligand, IC_{50} is the concentration of ligand producing a half-maximal response, and nH is the Hill coefficient.

Photochemistry

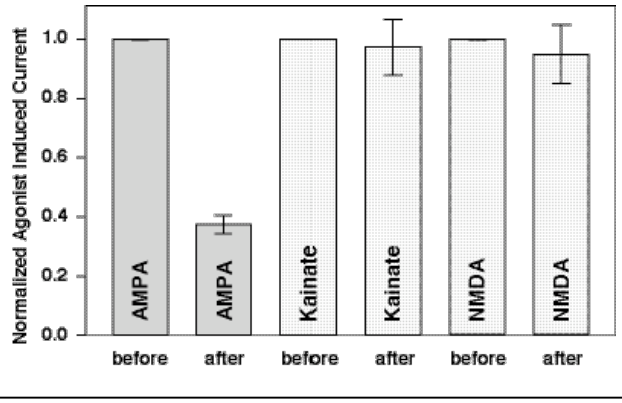
Oocytes expressing GluR1 were irradiated with UV light (100 W Hg or 1000 W Hg-Xe Oriel arc lamp outfitted with Schott UG1 350 nm band-pass and KG2 heat absorbing filters) in the presence of ANQX (100 μM). In the continuous recording/irradiation experiments (Figure 2 in the manuscript) on individual cells, each oocyte (expressing GluR1 L497Y) was irradiated (100 W) while clamped on the TEVC electrophysiology rig. Ultraviolet light piped in from the arc lamp was focused on the bottom half of the oocyte using a fiber optic cable fixed to the underside of the recording chamber outfitted with a quartz coverslip. Light passing by the oocyte was reflected and focused back onto the top half of the oocyte using a concave mirror sitting above the recording chamber. Following each experimental and control treatment (i.e. UV light only, UV light + 2 μM glutamate, UV light + 50 μM DNQX, 100 μM ANQX, UV light + 100 μM ANQX), peak AMPAR currents were recorded in response to 2 μM glutamate. The mean level of inhibition and standard error of the mean from seven ooctyes is shown in Figure 2 Supplement A.

Figure 2 Supplement A. Normalized glutamate-evoked AMPAR currents following treatment ± ultraviolet light (30 sec) in the presence and absence of agonist (2 μM glutamate) and antagonists (50 μM DNQX, 100 μM ANQX). Glutamate-evoked currents are irreversibly blocked following treatment with UV light + ANQX.



The specificity of ANQX for the AMPA subtype of glutamate receptors was investigated by irradiating oocytes heterologously expressing either kainate (GluR6) or NMDA (NR1 and NR2A) receptors in the presence of ANQX (100 μM) in the continuous recording/irradiation configuration. The kainate and NMDA receptors were irradiated in the presence of ANQX for ≥ 1 min. Only the AMPARs are irreversibly blocked by photoirradiation in the presence of ANQX. The data from these experiments (n = 7-10, error = SEM) is shown in Figure 2 Supplement B.

Figure 2 Supplement B. Peak agonist-induced currents recorded from AMPA, kainate, and NMDA receptors *before* and *after* irradiation with UV light in the presence of ANQX. AMPARs were irradiated for 30 s, kainate and NMDA receptors for ≥ 1 min.



In the batch irradiation experiments (Figure 3 in the manuscript), oocytes were placed in 4 dram glass vials containing 2 mL of ANQX (100 μM in ND96) and irradiated with UV light (1000 W) for 1-60 seconds with one second pulses of light and inhibitor solution changes in between each light pulse. To facilitate equal exposure of all sides of the 1 mm diameter oocytes to light, the vials were rotated (~ 1 rpm) while being irradiated. Inhibitor solution changes in between each pulse of light were required to remove the photoinactivated ANQX, which is also a GluR1 antagonist (IC_{50} = 11.1 μM). Levels of irreversible GluR1 inactivation were recorded at several time points (1, 2, 4, 8, 16, 32 and 60 s of irradiation) by measuring peak glutamate-evoked currents in response to 2 μM glutamate. Control oocytes were identically irradiated in the absence of ANQX. The 'percentage current remaining' after irradiation was determined by dividing the peak current from the irradiated oocytes (n = 5 oocytes per time point) by the peak current from non-irradiated oocytes (n = 5) and multiplying by 100. For

Abbreviations:
AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; GluR1, glutamate receptor 1 (GluR1); ionotropic glutamate receptors (iGluRs); ultraviolet (UV); 6,7-dinitroquinoxaline-2,3-dione (DNQX); 6-azido-7-nitro-1,4-dihydroquinoxaline-2,3-dione (ANQX); DMSO, dimethyl sulfoxide; WT, wild type; TEVC, two-electrode voltage clamp.

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