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Brief Article

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Controlling Ca^{2+} permeable α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) Receptors with Photochromic Ion Channel Blockers

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ABSTRACT: Ionotropic glutamate receptors (iGluRs) play a critical role in normal brain function and neurodegenerative diseases. Development of light-dependent compounds would enable studies of iGluRs within intact mammalian neural tissue, as light is non-invasive and can be applied with high spatiotemporal precision. Here we develop a potent photochromic antagonist that selectively targets the Ca^{2+} permeable AMPA-type of iGuRs, thus providing an important tool study the contribution of AMPA-type iGluRs on neuronal activity.

INTRODUCTION

Optical control of neuronal activity has been achieved through coupling of exogenous photosensitive receptors from bacteria or protozoa into neurons as well as through photosensitization of endogenous receptors using synthetic photoswitchable small molecules.[1] Of these endogenous receptors, the family of ionotropic glutamate receptors (iGluRs) is a promising target for integrating photosensitivity to neuronal activity, due to its importance in neuronal activity in the vertebrate brain, where it plays an essential role in mediation of the excitatory synaptic communication.[2] Furthermore, the iGluRs are considered an attractive drug target, as they are essential to normal brain function and dysfunction is believed to play an important role in several neurological and psychiatric disorders.[2-3] The iGluR family comprises the three subfamilies of cation selective ligand-gated ion channels, the *N*-methyl-D-aspartate receptors (NMDARs), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) and kainate receptors (KARs). The iGluRs assemble as hetero- and homo-tetrameric receptor subtypes that are Ca^{2+}-permeable or -impermeable.[2] In most glutamatergic synapses, the AMPARs and NMDARs are co-localized in the postsynaptic membrane, where they generate a dual-component response to glutamate, mediating a fast and slow response component, respectively.[4] The role of the KARs is more complex, as they modulate release of both inhibitory and excitatory neurotransmitters and forms post-synaptic ion channels.[5]

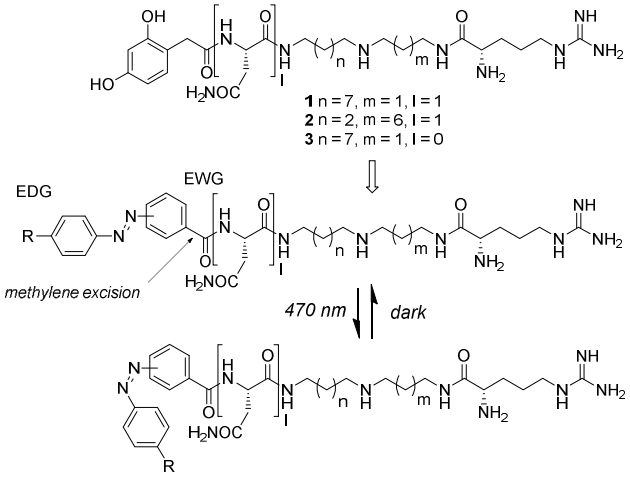
The iGluRs have been photosensitized using caged ligands,[6] photoswitchable tethered agonists[7], photochromic agonists[8] and recently a photoswitchable tethered antagonist[7a] as well as a photochromic antagonist[9]. Such antagonists are highly useful for studying the role of iGluRs in neuronal activity. In particular, selectively targeting Ca^{2+}-permeable AMPARs with photochromic antagonist is of great interest, as these receptors play key roles in brain development, synaptic plasticity as well as in excitotoxicity, which is believed to be associated with excessive receptor activity.[2, 10] Ideally, photochromic antagonists should enable receptor control, while preserving responsiveness to the native input. The existing tethered photoswitchable and photochromic antagonists inhibit the NMDAR or AMPARs and KARs, respectively, by binding to the orthosteric binding site in the ligand binding domain.[7a, 9] Thus, acting as competitive antagonists, that interferes with glutamate binding and activation of the receptor. Development of photochromic antagonists that target the receptor beyond the orthosteric binding site is therefore of great interest. To this end, selective targeting of Ca^{2+}-permeable iGluRs would further provide a tool to decipher the role of individual iGluR subtypes in neuronal excitability and excitotoxicity.

Polyamine toxins are a class of small molecules isolated from spiders and wasps that are potent use- and voltage dependent open-channel blockers of iGluRs.[11] They are important pharmacological tools for examining the iGluRs, owing to their high potency,[11-12] and importantly, polyamine toxins are capable of distinguishing between Ca^{2+}-permeable and -impermeable iGluR subtypes, with selectivity for the former subtype.[13]

Argiotoxin-636 (ArgTX-636) is a polyamine toxin isolated from the *Argiope lobata* spider, and is a potent, but

non-selective inhibitor of iGluRs. We have previously developed two analogues of ArgTX-636, denoted ArgTX-93 (**1**) and ArgTX-48 (**2**), which are highly potent and subfamily selective antagonists of the NMDARs and AMPARs, respectively.[14]

Furthermore, we have observed that these compounds tolerate substitution of the terminal resorcinol moiety with larger fluorophores with a clear correlation between size of the fluorophore and retention of potency.[15] Hence, smaller fluorophores such as coumarins and boron-dipyrromethene fluorophores could be accommodated in the iGluR ion channel vestibule, while larger flourophores rendered the compounds inactive. Based on this and the comparable size of the photoswitchable azobenzene scaffold to the smaller size fluorophores, we designed and developed polyamine toxins carrying an azobenzene moiety in place of the resorcinol group to incorporate photosensitivity (Scheme 1). One compound, **14b**, showed a significant difference in potency between the *cis* and *trans* isomers, and selectively targets the Ca^{2+}-permeable AMPARs.



Scheme 1. Design of photochromic argiotoxin analogues

RESULTS AND DISCUSSION

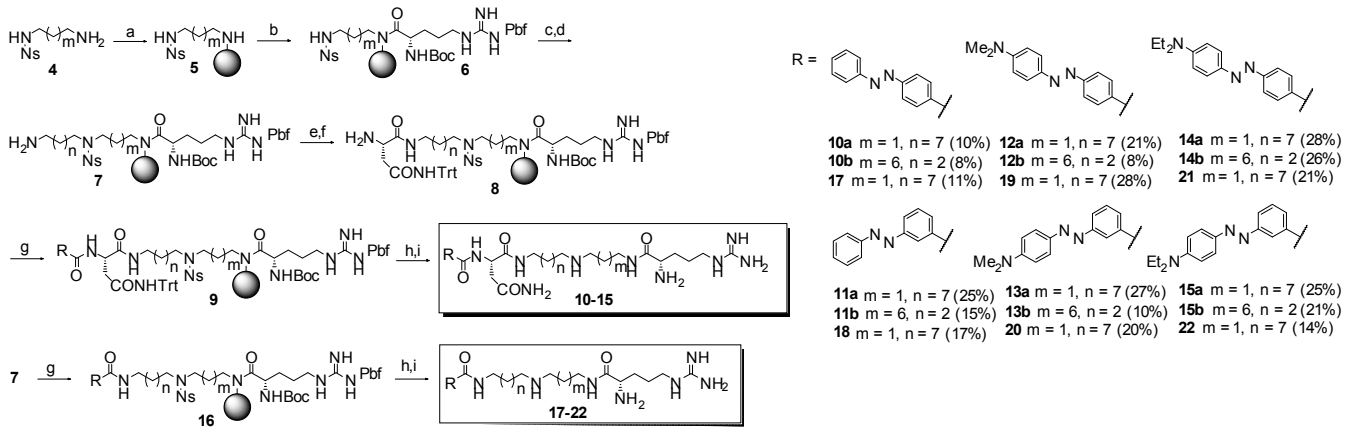
The class of azobenzenes was chosen as photoswitch due to its favorable geometric and photochemical properties.[1a, 16] Azobenzenes have well-defined cis and trans states with little overlap between the conformational space mapped by the two isomers, thus leading to significant geometric change upon isomerization.[16] Furthermore, azobenzenes are photostable, have high isomerization rates, molar extinction coefficients and quantum yields, and their spectroscopic properties are highly tunable through ring substitution.[16]

The azobenzenes were incorporated in the polyamine toxin templates of **1** and **2**, as well as an analogue of **1** without the asparagine moiety (**3**), as this excision has shown to improve potencies in an earlier study (Scheme 1).[15] For each of the three templates, six different azobenzene moieties were selected. For initial screening of the ability of the azobenzene scaffold to be accommodated in the ion channel vestibule, we selected the smallest possible, unsubstituted azobenzenes. To test differences between the orientations of the terminal phenyl ring, both the para and meta substituted azobenzenes were tested. To improve the spectroscopic properties, four azobenzenes were selected that carried electron-donating groups, dimethylamine or diethylamine, in the 4'-position. Furthermore, the methylene group connecting the azobenzene to the rest of the polyamine toxin was removed, thus attaching the azobenzene directly to the electron withdrawing amide moiety, which ensured an asymmetric push-pull electron distribution leading to redshifted absorption.[16] Redshifted absorption was desirable, as lower energy light has deeper tissue penetration and lower risk of tissue damage when applied in living systems.[17] Substitution at the 4' position had the additional effect of increasing the size of the photoswitchable moiety, thus potentially increasing the difference between the conformational space mapped by the cis and trans isomers.

The photoswitchable compounds were synthesized using a solid-phase organic procedure inspired by a previously reported argiotoxin synthesis (Scheme 2).[15] The synthesis began with loading of mono nosyl protected diamines (**4**) on a polystyrene resin by reductive amination to a backbone amide linker (BAL).[18] An arginine amino acid was subsequently coupled to the resin-bound secondary amine (**5**) providing intermediate **6**, which was extended with N-Teoc protected amino alcohols via a Fukuyama-Mitsunobu alkylation reaction.[19] The Teoc protection group could subsequently be removed with TBAF, resulting in intermediate **7**, an intermediate for all synthesized compounds. For synthesis of analogues of **1** and **2**, the intermediate **7** was coupled to an asparagine amino acid, followed by deprotection of the Fmoc group, to give resin-bound **8**. Six carboxyl azobenzenes were then coupled to **8**, and subsequent deprotection of the Ns group, cleavage from the resin and simultaneous deprotection of acid labile protection groups provided the desired photochromic analogues **10a–b** and **15a–b** in good yields (8–25%, 9 steps, 76–87% average yields per step). For the synthesis of analogues of template **3**, six carboxy azobenzenes were coupled directly to **7**, followed by deprotection of the Ns group, cleavage from the resin and simultaneous deprotection of acid labile protection groups (**17–22**, 11–28%, 7 steps, 73–83% average yields per step).

The substituted azobenzenes had been designed to tune their spectroscopic properties towards redshifted absorption. To evaluate this, representative absorption spectra were recorded for the six compounds carrying azobenzenes coupling to template **2** (Figure S1 in Supporting Information), showing redshift of absorption maxi-

mum from 323–329 nm (**10b–11b**) to 460–487 nm (**12b–15b**).



Scheme 2. Solid-phase synthesis of photochromic compounds 10–15 (a–b) and 17–22. Reagents and conditions: a) BAL PS resin, $NaBH(OAc)_3$, DMF/AcOH (9:1); b) Boc-Arg(Pbf)-OH, HATU, DIPEA, DCM/DMF (9:1); c) N-Teoc 9-amininonan-1-ol or N-Teoc 4-aminobutan-1-ol, Bu_3P, ADDP, DCM/THF (1:1); d) TBAF, THF, 55°C; e) Fmoc-Asn(Trt)-OH, HATU, DIPEA, DCM/DMF (9:1); f) 20% piperidine, DMF; g) carboxy azobenzene, HATU, DIPEA, DCM/DMF (9:1); h) DBU, ß-mercaptoethanol, DMF; i) TFA/DCM/TIPS/H_2O (75:20:2.5:2.5). ADDP = 1,1'- azodicarbonyldipiperidine, Boc = tert-butyl carbamate, DBU = 1,8-diazabicyclo[5.4.0]undecane, DIPEA = N,N-Diisopropylethylamine, DMF = N,N-dimethylformamide, Fmoc = fluorenylmethyloxycarbonyl, HATU = N-((dimethylamino)-1H-1,2,3-triazolo[4,5,b]pyridin-1-ylmethylene)-N-methylmethanaminium hexafluorophosphate N-oxide, Pbf = 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl, Teoc = 2-(trimethylsilyl)ethyloxycarbonyl, TFA = trifluoroacetic acid, THF = tetrahydrofuran, TIPS = triisopropylsilane, Trt = trityl.

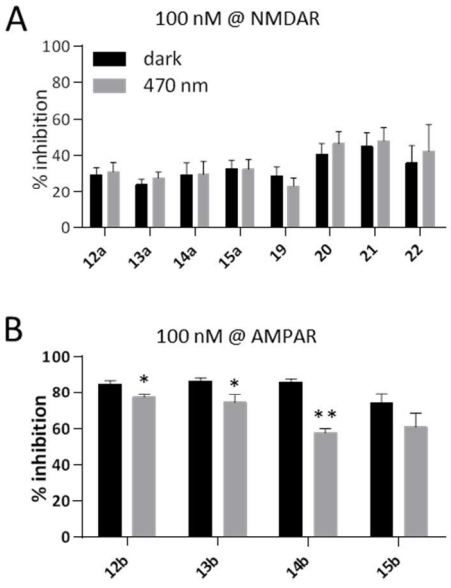


Figure 1. Screening of compounds. Percentage inhibition by 100 nM photochromic ligand at (A) NMDAR subtype GluN1/2A and (B) AMPAR subtype GluA1, in the dark (black bars) and under illumination with blue light (470 nm, grey bars). Bar graphs are showing mean ± SEM (standard error of the mean) for n = 3-16 oocytes at a membrane potential of –80 mV. *Statistical difference between inhibition by dark adapted trans state and illumination adapted cis state of the photochromic antagonists (P<0.05, students t-test). **Statistical difference between inhibition by dark adapted trans state and illumination adapted cis state of the photochromic antagonists (P<0.001, students t-test)

Compounds carrying an unsubstituted azobenzene moiety (**10a–11a, 10b–11b** and **17–18**) were initially screened in the dark to investigate whether this azobenzene scaffold could be accommodated in the ion channel vestibule (Figure S2, Supporting Information). This showed to be the case for the AMPAR targeting compounds **10b–11b** and the NMDAR targeting compound **18**, which showed inhibitions around 70–80% in the dark-adapted trans form. The substituted analogues were screened both in the dark (black bars) and under illumination with blue light (470 nm, grey bars, Figure 1). Encouragingly, compounds **12b–15b** all showed more than 70% inhibition of the AMPAR in the dark-adapted trans form, while in comparison the NMDAR targeting compounds (**12a–15a** and **19–22**) showed significantly lower inhibition in the 20–50% range.

Interestingly, illumination of compounds **12b–15b** with blue light (470 nm) led to a reduction in inhibition, especially for compound **14b**, which showed a remarkable 30% drop in inhibition upon illumination (Figure 1, grey bars). This difference was even more pronounced when inhibition was measured at a 10 nM concentration of the photochromic compound, where **14b** had approximately three times higher inhibition in the dark than when illuminated (dark state inhibition: 60% ± 5%, n=3; Illuminated state

inhibition: 20% ± 5%, n=3) (Figure 2B). At the same concentration of **14b** less than 5% inhibition was observed at the NMDAR, both in the dark and under illumination, showing that **14b** had retained selectivity toward the AMPAR (Figure 2A-B).

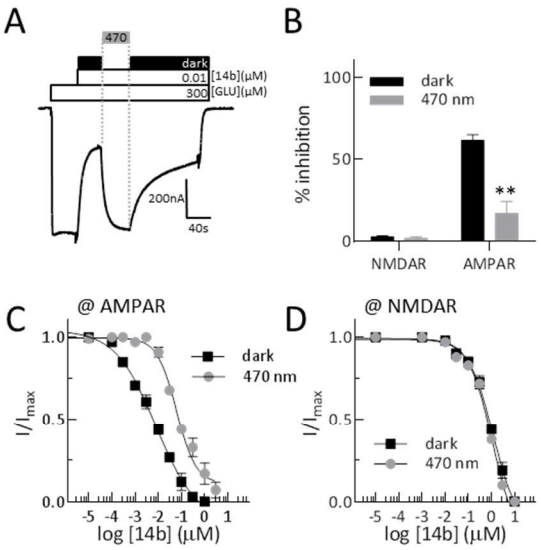


Figure 2. Pharmacological characterization of photochromic antagonist 14b. A: Representative electrophysiological trace showing shift in inhibition upon illumination with blue light (470 nm, highlighted in grey). B: Screening of percentage inhibition by 10 nM photochromic ligand at the NMDAR subtype GluN1/2A and the AMPAR subtype GluA1 in the dark (black bars) and under illumination with blue light (470 nm, grey bars). Bargraphs are showing mean ± SEM (standard error of the mean) for n = 3-10 oocytes held at a membrane potential of –80 mV. C and D: Determination of IC_{50} values at NMDAR subtype GluN1/2A and AMPAR subtype GluA1. Data points represent the mean ± SEM (standard error of the mean) for n = 3-10 oocytes held at a membrane potential of -80 mV. **Statistical difference between inhibition by dark adapted trans state and illumination adapted cis state of **14b** (P<0.001, students t-test).

Full dose-response curves were generated for **14b** at the AMPAR and NMDAR subtypes to determine the IC_{50} values, both in the dark and under illumination (Figure 2C-D). The dark-adapted trans isomer of **14b** showed to be a highly potent inhibitor of the AMPAR, having an IC_{50} value as low as 8 nM [4.3–15], thus being more potent than the parent polyamine toxin, **2** (Table 1).[14] Upon illumination, **14b** showed significantly lower potency, inhibiting with an IC_{50} value of 61 nM [49–77]. Thus, **14b** acts as a trans antagonist, where the inhibition can be reduced by 8-fold upon stimulation with blue light. Moreover, the difference in potency between the cis and trans isomers is most likely even bigger, as illumination cannot convert **14b** fully into the cis configuration, thus a part of the observed inhibition is caused by the 10–20% still found as the trans isomer. IC_{50} values were determined on the NMDAR as well to investigate subfamily selectivity, and **14b** showed significantly lower potencies (IC_{50} > 1 μM), thus displaying high selectivity toward the AMPAR subfamily. This study includes GluA1 and GluN1/2A as representative subtypes of the AMPAR and NMDAR subfamilies, respectively. On that note, polyamines, such as spermine, can act as potentiators by binding to a low affinity binding site on the amino terminal domain (ATD) of the GluN1/2B NMDAR subtype.[20] Thus, the polyamine tail of **14b** could potentially interact with the ATD of this particular subtype, consequently leading to an overall loss of inhibition and increased antagonistic selectivity towards the AMPAR subfamily. This selectivity is further substantiated by the inherent specificity for the Ca^{2+}-permeable subtype of AMPARs that polyamine toxins demonstrate.

Table 1. Potency of **14b** at NMDAR and AMPARs subtypes.

Receptor	Illumination	IC_{50} (μM)[a]	n_H[d]
NMDAR[b]	Dark	1.1 [0.7–1.9]	-0.8
	470 nm	1.2 [0.8–1.9]	-0.9
AMPAR[c]	Dark	0.008 [0.0043–0.015]	-0.5
	470 nm	0.061 [0.049–0.077]	-1.0

[a] IC_{50} values at NMDAR subtype GluN1/2A and AMPAR subtype GluA1 recombinantly expressed in oocytes and a membrane potential of –80 mV. Numbers in brackets denote the 95% confidence interval for IC_{50}. [b] Inhibition of the current elicited by 100 μM L-Glu and 100 μM Gly by simultaneous co-application of the antagonist to oocytes injected with a 1:10 ratio of cRNA coding for GluN1 and GluN2A subunits, respectively. [c] Inhibition of the current elicited by 300 μM L-Glu by simultaneous co-application of the antagonist to oocytes injected with cRNA coding for GluA1. [d] n_H indicates the Hill slope of the curves.

CONCLUSION

In summary, we have designed and synthesized a series of photochromic analogues of ArgTX-93 (**1**) and ArgTX-48 (**2**) by incorporating photoswitchable azobenzene moieties in place of their headgroups. Through a combination of different azobenzene moeitites and modification of the polyamine toxin scaffold, we have tuned the spectroscopic properties toward red-shifted absorption, while increasing the difference between the conformational space mapped by the cis and trans isomers. Pharmacological characterization of the developed compounds have resulted in the discovery of a photochromic antagonist, **14b**, that acts as a highly potent and selective dark-adapted trans antagonist of Ca^{2+}-permeable AMPARs. This photochromic antagonist, **14b**, binds outside the orthosteric binding site, thus we expect that **14b** can be used to add an additional input signal to Ca^{2+}-permeable AMPA receptors, that does not perturb with binding of the endogenous agonist. To this end, given the importance of AMPARs in neuronal activity, we envision that **14b** could be a highly useful tool for studying the role of this receptor in excitatory neuronal communication.

EXPERIMENTAL SECTION

Experimental Chemistry.

General procedures.

Unless otherwise stated, starting materials were obtained from commercial suppliers and were used without further purification. The BAL resin used was 2-(3,5-dimethoxy-4-formylphenoxy)ethyl (DFPE) polystyrene with a loading of 0.87 mmol/g, which was purchased from Novabiochem. N-(3-Aminopropyl)-2-nitrobenzenesulfonamide,[21] N-(8-aminooctyl)-2-nitrobenzenesulfonamide,[15] 2-(trimethylsilyl)ethyl (4-hydroxybutyl)carbamate,[22] (E)-4-(phenyldiazenyl)benzoic acid[23] and (E)-3- (phenyldiazenyl)benzoic acid[23] were prepared according to literature procedures. Tetrahydrofuran (THF), N,N-dimethylformamide (DMF) and dichloromethane (DCM) were dried, degassed and scrubbed using a Glass Contour Solvent Purification System (SPS) immediately before use.

Preparative HPLC was performed on a Agilent 1100 system using a C18 reverse phase column (Zorbax 300 SB-C18, 21.2 × 250 mm) with a linear gradient of the binary solvent system of water/acetonitrile/formic acid (A: 95/5/0.1% and B: 5/95/0.1%) with a flow rate of 20 mL/min and UV detection at 215 nm and 254 nm. ^{1}H and ^{13}C NMR spectra were recorded on an Avance 400 spectrometer (at 400 or 100 MHz, respectively), using CD_3OD, $CDCl_3$ or DMSO-d_6 solvents. Chemical shifts are reported in ppm (δ) using residual solvent as an internal standard; $CDCl_3$: 7.26 (^{1}H), 77.16 (^{13}C) ppm; CD_3OD: 3.31, 49.00 ppm; DMSO-d_6: 2.50, 39.52 ppm. Coupling constants (*J*) are given in Hz. The purity of the compounds was determined by LC-MS and all compounds are >95% pure. LC-MS analysis was performed on an Agilent 6410 Triple Quadrupole Mass Spectrometer instrument using electron spray coupled to an Agilent 1200 HPLC system (ESI-LC/MS) with autosampler and diode-array detector using a gradient (0–100% B over 3 min, then 100% B for 3 min) of the binary solvent system of water/acetonitrile/TFA (A: 95/5/0.1% and B: 5/95/0.1%) with a flow rate of 1 mL/min. During ESI-LC/MS analysis evaporative light scattering (ELS) traces were obtained with a Sedere Sedex 85 Light Scattering Detector, which was used for estimation of the purity of the final products. Analyses at intermediate stages of the synthesis were performed by cleaving samples from the resin as follows: a 1 mL screwcap vial was charged with a 1 mg sample of the resin and 0.4 mL TFA/DCM/EDT (75:20:5). The vial was agitated periodically over 1 h and filtered using a syringe filter and disposable syringe. The crude product was analyzed by LC-MS. High resolution mass spectra (HRMS) were obtained using a Micromass Q-Tof II instrument. Absorption spectra were recorded on a Thermo Scientific UV-Vis spectrophotometer. (See supporting information)

Experimental biology

Expression of glutamate receptors in Xenopus oocytes. The cDNA encoding rat GluA1 (flip isoform), rat GluN1-1a and rat GluN2A subunits inserted in the vectors pGEM-HE and pCIneo were used for preparation of capped cRNA transcripts. cDNA was linearized using the NheI or NotI restriction enzymes (New England Biolabs, Ipswich, MA) and cRNA transcription was performed using the Amplicap-max T7 high Yield Message Maker mRNA-capping kit (Ambion, Austin, TX, USA) The quality and quantity of the synthesized cRNA was assessed by gel electrophoresis and spectroscopy. Stage V and VI oocytes were surgically removed from the ovaries of Xenopus laevis frogs and prepared as described in Poulsen et al.[24] under license 2014-15-0201-00031 from the Danish Veterinary and Food Administration. Oocytes were injected with 13-46 nL of diluted cRNA (1 ng/nL of GluA1; 0.5 ng/nL of GluN1 and GluN2A in a 1:10 mixture) and incubated in 1-3 days in Barth's medium (in mM: 88 NaCl, 1 KCl, 0.33 $Ca(NO_3)_2$, 0.41 $CaCl_2$, 0.82 $MgSO_4$, 2.4 $NaHCO_3$, 10 HEPES; pH 7.4) with gentamicin (0.10 mg/mL).and used for recordings 1 to 3 days post-injection.

Voltage-clamp recordings from *Xenopus oocytes*. Two-electrode voltage-clamp (TEVC) recordings were made 1–3 days post-injection. Oocytes were placed in a 500 µL recording chamber with a single perfusion line delivering 5 mL/min perfusion. Voltage and current electrodes were filled with 3 M KCl. Recordings were made using a Warner OC725B two-electrode voltage clamp (Warner Instruments, Hamden, CT) configured as recommended by the manufacturer. Oocytes were perfused with a solution comprised of (mM): 115 NaCl, 2 KCl, 5 HEPES and 1.8 $BaCl_2$, pH 7.6. All experiments were performed at room temperature (23 °C). Data acquisition and voltage control was accomplished using a CED 1401plus analog-digital converter (Cambridge Electronic Design, Cambridge, UK) interfaced with a PC running WinWCP software (available from Strathclyde Electrophysiology Software, University of Strathclyde, Glasgow, UK).

Screen of photochromic antagonists and determination of IC_{50}. A screen of the photochromic antagonists was carried out by measuring the maximal current resulting from bath application of a saturating concentration of agonist to the oocyte expressing either the NMDAR subtype GluN1/2A or AMPAR subtype GluA1, held at a membrane potential of -80 mV (300 µM L-glutamate for GluA1; 100 µM L-glutamate and 100 µM glycine for GluN1/2A). Subsequently varying concentrations of antagonist was applied in the presence of agonist. The resulting current was normalized to the response produced by the agonist alone. Measurements in the dark were carried out in a dark room. Measurements at 470 nm were conducted by addition of photochromic antagonist in the dark, followed by illumination using a handheld flashlight emitting light with intensity of 470 nm.

Dose-response measurements of polyamine inhibition of the receptors were performed by measuring agonist-evoked currents in step-wise increasing concentrations of polyamine toxin. For determination of IC_{50}, data were pooled among individual experiments at 3 to 10 individual oocytes and the composite dose-response data were fitted using GraphPad Prism software (GraphPad Inc, San Diego, CA, USA) by the equation:

$$Response = \frac{1}{1+10^{((\mathrm{Log}IC_{50}-X)*n_H)}}$$

Response is the agonist-evoked current response measured at a given inhibitor concentration normalized to the agonist-evoked current response in absence of inhibitor, IC_{50} is the concentration of inhibitor that produces a half-maximal inhibition, X is the logarithm of the concentration of the inhibitor concentration, and n_H is the Hill slope.

ASSOCIATED CONTENT

Supporting Information. Preparation of building blocks and ^{1}H and ^{13}C NMR spectra of final compounds, as well as Figures S1 and S2. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

ADDP, 1,1-(azodicarbonyl)dipiperidine; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ArgTX, argiotoxin; BAL, backbone amide linker; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DCM, dichloromethane; DIPEA, diisopropylamine; DMF, N,N-dimethylformamide; HATU, 2-(1*H*-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; NMDA, *N*-methyl-D-aspartate; Ns, 2-nitrobenzenesulfonamide; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; TBAF, tetra-n-butylammonium fluoride; TFA, trifluoroacetic acid; Teoc, 2-(trimethylsilyl)ethyloxycarbonyl; TIPS, triisopropylsilane.

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