Optochemical Genetics

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A Photochromic Agonist of AMPA Receptors**

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Optochemical genetics uses small photoswitchable molecules to control neuronal activity. These can be attached covalently or noncovalently to their target proteins, which can be ligand or voltage-gated ion channels as well as G-protein coupled receptors. The integration of the resulting hybrid photoreceptors into excitable cells allows for the control of neural networks with the temporal and spatial precision that only light provides.[1]

Among different targets for optochemical genetics, ionotropic glutamate receptors (iGluRs) stand out owing to their central role in synaptic transmission. These ion channels are gated by glutamate (**1**) and fall into three major classes, each of which is marked by a distinct pharmacology (Figure 1 b).[2] The so-called AMPA receptors (GluAs), named after the selective agonist 2-amino-3-(5-methyl-3-hydroxyisoxazol-4-yl)propanoic acid (**2**), can be considered the workhorses of glutamatergic synapses. They are responsible for the major part of the excitatory neurotransmission in the mammalian central nervous system and are mostly positioned at the center of the postsynaptic density. Kainate receptors (GluKs), named after kainic acid (**3**), in contrast, are located primarily on the periphery of the synaptic cleft and play more of a supporting and modulatory role. NMDA-receptors, named after their selective response to the agonist *N*-methyl-D-aspartate (**4**), are both voltage- and ligand-sensitive ion channels and function as coincidence detectors of postsynaptic depolarization and synaptic glutamate release.[3]

Our group has a longstanding interest in converting iGluRs into photoreceptors for optochemical applications. So far, our efforts have been focused on kainate receptors owing to the well-defined architecture of their clamshell-like ligand-binding domain (LBD) and their readily interpretable pharmacology (Figure 1 a). Our efforts have yielded LiGluR, a light-gated ionotropic glutamate receptor, wherein a derivative of glutamate was covalently tethered to the surface of the LBD through a photoswitchable linker ("photoswitched tethered ligand", PTL).[4] Shortly thereafter, we introduced a photochromic ligand (PCL) for kainate receptors that functions as a "reversibly caged glutamate" (Figure 1 c,d).[5] This molecule, termed 4-GluAzo (**5**), is an azobenzene derivative of glutamate that changes its affinity and efficacy to GluK1 and GluK2 upon photoisomerization. As such, it can be used to reversibly control neuronal activity with different wavelengths of light.

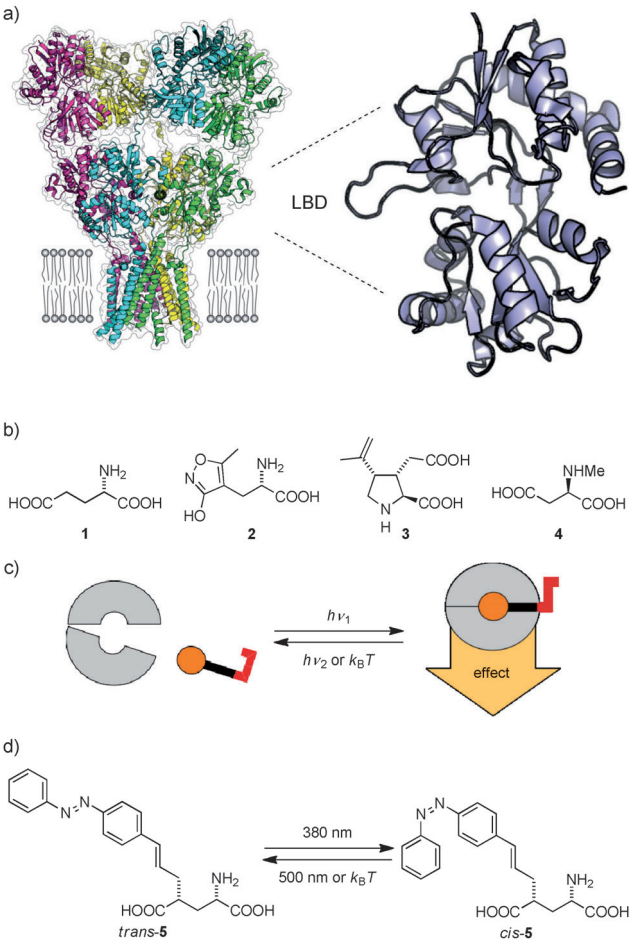

Figure 1. a) General structure of a glutamate receptor (derived from protein data bank (PDB): 3KG2) with a close-up of an LBD (PDB: 2P2A). b) The universal agonist and three subtype-selective agonists of iGluRs. c) Schematic mode of action of a photochromic agonist. d) 4-GluAzo (**5**), a PCL acting on kainate receptors.

The rational design of suitable photoswitches for AMPA, kainate and NMDA receptors has been greatly facilitated by the availability of numerous X-ray crystal structures.[6] The clamshell-like ligand-binding domains of these receptors have been crystallized in conjunction with a variety of agonists, such as AMPA (**2**) and domoic acid, antagonists, such as DNQX, as well as modulators, such as cyclothiazide. Very recently, the crystal structure of a full tetrameric GluA2 receptor assembly has been disclosed.[7] This seminal work provided insights into the overall architecture and symmetry

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of AMPA receptors in particular and ionotropic glutamate receptors in general.

Although the clamshell-like LBDs of AMPA and kainate receptors share a similar overall architecture, there are subtle differences that apparently prevent 4*R*-substituted glutamate derivatives, such as 4-GluAzo (**5**), from acting as agonists. One of the reasons for this inhibition may be that the LBD of AMPA receptors is closed more tightly around the ligand in the activated form. Thus, side chains attached to a glutamate molecule cannot be as easily accommodated as in the case of GluKs.

Accordingly, glutamate derivatives with a photoswitchable side chain, such as 4-GluAzo (**5**) were deemed unsuitable for the light-dependent stimulation of AMPA receptors and we decided to radically change our molecular design. We now report a new class of molecules termed ATAs (azobenzene tetrazolyl AMPAs, **7a–d**, Figure 2). These compounds are photochromic derivatives of AMPA itself, which selectively target GluA receptors expressed in HEK cells and neurons. One of them (**7c**) effectively triggers neuronal firing in the dark and quickly inactivates when irradiated with blue–green light. As such, it could be a useful tool for the study of neural circuitry controlled by AMPA receptors and a potential therapeutic tool for the restoration of vision with artificial photoswitches.

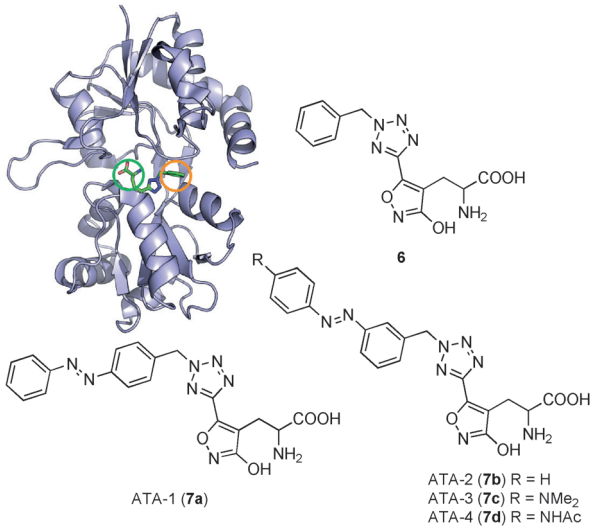
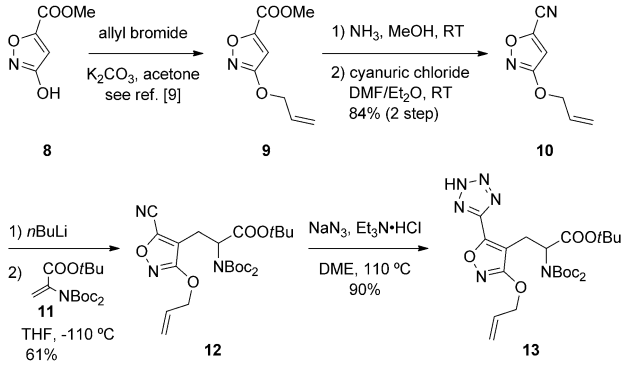


Figure 2. The structure of BnTetAMPA (**6**) and its complex with the GluA2 LBD (PDB: 2P2A) and the structures of ATA-1 (**7a**) and ATA-2–4 (**7b–d**) shown in their respective *trans*-form. The green circle indicates the previous "exit tunnel", whereas the orange circle shows the approximate location of the newly identified "exit tunnel".

The design of **7a–d** is based on a recently published X-ray structure of the GluA2 LBD in conjunction with the potent and highly selective agonist BnTetAMPA (**6**).[8] This molecule is a derivative of AMPA, wherein the methyl substituent on the isoxazole ring is replaced with a tetrazole benzylated in the N2-position. As seen in the crystal structure, the benzyl substituent occupies a cleft in the receptor that is different from the "exit tunnel" that we previously exploited in the kainate receptors (Figure 2). This structure suggested to us that extending the benzene to an azobenzene would allow the molecule to reach the solvent-exposed surface while still permitting the ligand binding domain (LBD) to close sufficiently for receptor activation. It could also be deduced that this elongation would require a *meta*-substitution with respect to the first benzene ring of the azobenzene unit.

To test our hypothesis that ATAs would function as photochromic agonists, we synthesized several variants (**7a–d**; Figure 2). These molecules have different substitution patterns and photophysical properties owing to the presence or absence of substituents in the 4′-position of the azobenzene. The dimethylamino substituent in **7c**, for instance, shifts the absorption spectrum of the *trans*-isomer toward the red ($\lambda_{max} = 456$ nm), which allows the use of longer wavelengths that are better tolerated by cells during prolonged exposure.

The synthesis of ATA-3 (**7c**) was modeled after the published route to BnTetAMPA (**6**),[8] but relied on an improved protecting-group strategy (Schemes 1 and 2). It

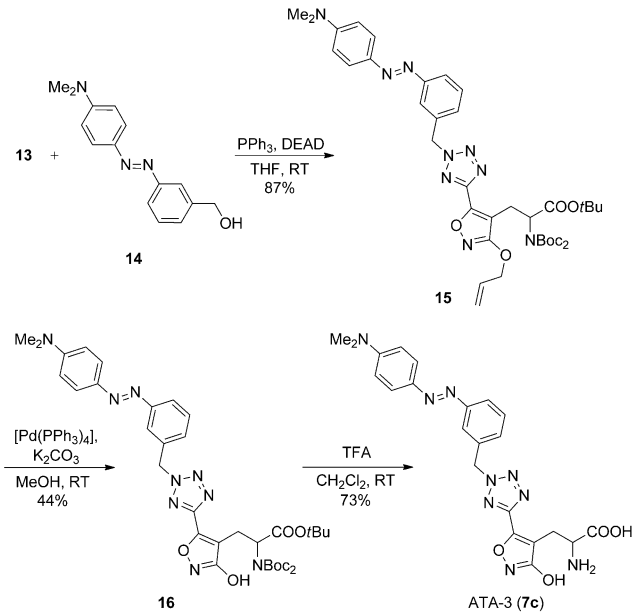


Scheme 1. Synthesis of racemic key intermediate **13**.

commenced with the known hydroxy isoxazole **8**,[9] which was protected as an allyl ether to afford methyl ester **9**.[10] This intermediate was then converted into nitrile **10** in a two-step procedure.[11] Lithiation of **10**, followed by conjugate addition of the obtained organolithium compound to dehydroalanine **11**, yielded racemic AMPA derivative **12**. This step required careful optimization but could be carried out reliably and on large scale at very low temperatures. A 1,3-dipolar cycloaddition of hydrazoic acid to nitrile **12** then furnished tetrazole **13**, which served as a common intermediate in the synthesis of all the ATAs (**7a–d**).

Mitsunobu coupling of tetrazole **13** with azobenzene **14**[13] gave the N2-alkylated tetrazole **15** as the major isomer (Scheme 2). The undesired, N1-alkylated regioisomer could be separated by HPLC after removal of the allyl group under mild conditions,[12] which yielded hydroxy isoxazole **16**. Subsequent global deprotection using trifluoroacetic acid (TFA) gave the free amino acid **7c** after reversed phase chromatography. The synthesis of **7b** and **7d** as well as the *para*-substituted control compound **7a** was carried out analogously.[13]

As anticipated, ATA-3 (**7c**) and ATA-4 (**7d**) showed significant red-shifted absorption spectra compared to ATA-



Scheme 2. Synthesis of the PCL ATA-3 (**7c**). DEAD = diethyl azodicarboxylate.

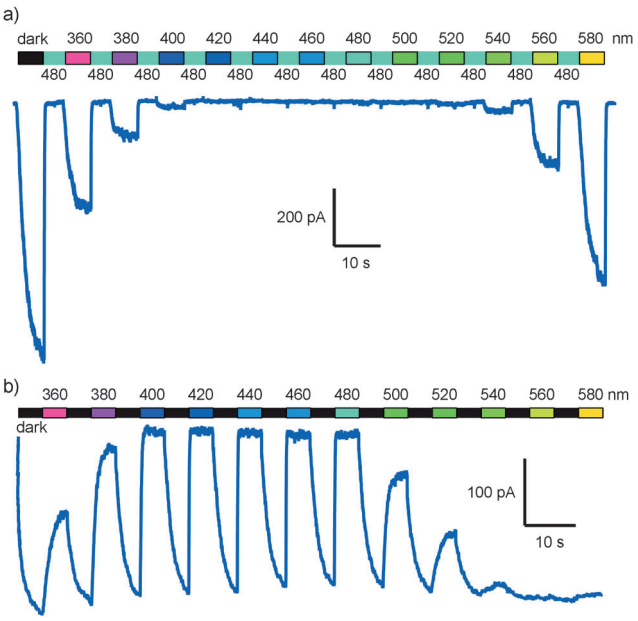


Figure 3. Light-induced current recorded from GluA2-expressing cells a) Action spectrum of ATA-3 (**7c**), b) action spectrum recorded with intermittent darkness from a different cell ($c = 50\ \mu M$).

1 (**7a**) and ATA-2 (**7b**).[13] In contrast to its congeners, ATA-3 (**7c**) could not be actively switched from its *cis*- to its *trans*-state in physiological buffer solution because of the complete overlap of the π–π* and n–π* absorption bands. However, thermal relaxation was found to be sufficiently fast under these conditions.

With **7a–d** in hand, we assessed their biological activity in mouse cortical slices and HEK293T cells using whole-cell patch-clamp electrophysiology (Figures 3 and 4). Whereas **7a** was found to be completely inactive in both preparations and **7b** gave inconclusive results, compounds **7c** and **7d** proved to be effective *trans*-agonists of GluA2 transiently expressed in HEK293T cells. This result confirmed that the *meta*-substitution at the azobenzene moiety is a necessary structural requirement.

Since **7c** showed the highest activity and best kinetics we decided to focus our further investigations on this PCL. Its action spectrum at GluA2 recorded in HEK293T cells is shown in Figure 3. As designed, **7c** elicited the strongest inward current in its dark-adapted *trans*-state. Irradiation with varying wavelengths of light then produced photostationary states that gave less or no current at all (Figure 3a). In addition, the kinetics of ATA-3 (**7c**) currents in the dark and at different wavelengths of light were determined (Figure 3b). We found that the fastest abrogation of current was achieved by switching from darkness to $\lambda = 480$ nm light [$\tau_{off} = (47.2 \pm 7.7)$ ms].[13] When **7c** was tested against GluK2, no response (neither agonistic nor antagonistic) was observed.[13] This result demonstrates the selectivity of the ATA chemotype for AMPA over kainate receptors.

As a final characterization step in non-excitable cells, we determined the efficacy of **7c** at GluA2 expressed in HEK293T cells. The compound acts as a partial agonist with an EC_{50} of 24 μM in the dark and is virtually inactive under 480 nm illumination.[13] However, enantiopure material will likely show considerably higher activity, as has been observed for other AMPA-derivatives.[14]

We evaluated the ability of **7c** to control action potentials in excitable cells. Action potentials were recorded from layer 2/3 neurons in mouse cortical slices. When **7c** was applied at 50 μM concentration, trains of action potentials could be generated reliably upon switching from 480 nm light to darkness (Figure 4). Owing to the moderate intensity of the

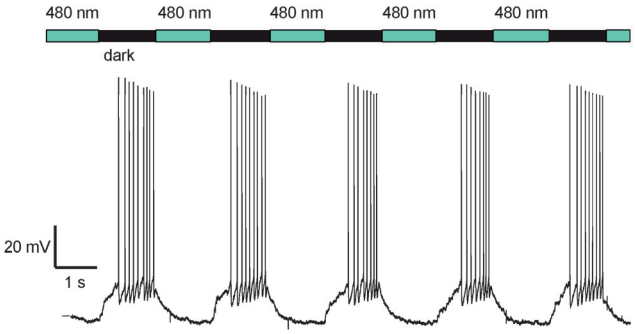


Figure 4. Reversible generation of action potentials by **7c** in mouse cortical neurons (resting potential −65 mV).

light used (20 $mW\,m^{-1}\,m^2$)[13] recordings could be performed over prolonged periods without any apparent photodamage.

The activity of **7c** in neural networks was further probed using known glutamate receptor pharmacology. GYKI-52466,[15] an AMPA-selective antagonist, blocked the effect of the PTL as did the AMPA/kainate antagonist CNQX. Additional experiments with the selective NMDA antagonist AP5[16] failed to prevent action potential firing.[13] This result

demonstrates that **7c** can discriminate between all three subtypes of iGluRs and selectively acts on AMPA receptors.

In summary, we have developed a photochromic agonist of AMPA receptors that stimulates neurons in the dark but can be rapidly turned off when irradiated with blue–green light at moderate intensities. ATA-3 (**7c**) uses a novel "exit tunnel" that should allow for the design of new, highly AMPA-selective ligands, which are not necessarily photochromic. Given the prominence of AMPA receptors in synaptic transmission, this molecule could be used as an effective tool to optically control neuronal activity in a wide variety of networks. Its response to light matches the logic of OFF-bipolar cells, and by extension OFF-retinal ganglion cells, which increase firing in the darkness and show decreased activity when light reaches their receptive field. As such, **7c** could be a useful component of our ongoing efforts to restore vision with small photochromic molecules.

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