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Restoring Light Sensitivity in Blind Retinae Using a Photochromic AMPA Receptor Agonist

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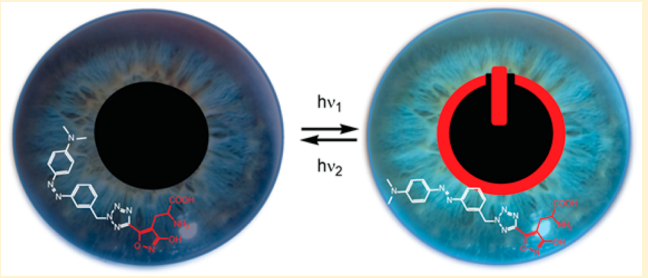
Supporting Information

ABSTRACT: Retinal degenerative diseases can have many possible causes and are currently difficult to treat. As an alternative to therapies that require genetic manipulation or the implantation of electronic devices, photopharmacology has emerged as a viable approach to restore visual responses. Here, we present a new photopharmacological strategy that relies on a photoswitchable excitatory amino acid, ATA. This freely diffusible molecule selectively activates AMPA receptors in a light-dependent fashion. It primarily acts on amacrine and retinal ganglion cells, although a minor effect on bipolar cells has been observed. As such, it complements previous pharmacological approaches based on photochromic channel blockers and increases the potential of photopharmacology in vision restoration.



KEYWORDS: *Photopharmacology, retinal degeneration, AMPA receptors, photochromic ligand, ATA*

Vision is probably the most important sensual perception in humans, and its loss can be devastating. To date, diverse approaches for restoration of vision are under investigation. The most advanced in terms of clinical application are retinal implants, such as Argus II or the alpha-IMS subretinal microchip[1−4] Other, more experimental approaches use viral gene transfer to introduce channelrhodopsin-2 and related photosensitive proteins in either bipolar or retinal ganglion cells (RGCs).[5−8] Although these strategies have improved significantly in the past decade, each comes with its own caveats and trade-offs. For instance, the lifetime of retinal implants is limited due to mechanical degradation or loss of electrical contacts.[9] Furthermore, the resolution of these devices is restricted by electrode density and their implantation is invasive and expensive. In the case of gene therapy, it remains to be determined whether introduction of nonhuman genes into the human retina has undesirable side effects, such as immunogenicity.

Recently, we described a novel approach toward vision restoration that relies on photochromic ligands (PCLs). PCLs typically contain an azobenzene moiety, which is chemically modified to function as a selective photoswitchable ligand for various receptors.[10−14] Illumination with specific wavelengths induces a change in the conformation of the PCL, thereby reversibly toggling the molecule between its active and inactive state.[15,16]

PCLs could provide an attractive approach to restoring vision to the blind. Pathways to their clinical approval should resemble those of regular drugs. If proven successful they could be more affordable and more widely applicable than alternative approaches.

So far, photochromic potassium channel blockers, such as AAQ and DENAQ, were shown to elicit light-dependent spiking in RGCs and were able to confer visually entrained memories to blind mice.[17,18]

Here, we extend the photopharmacological toolbox for vision restoration approaches, introducing a new class of PCLs based on the excitatory amino acid AMPA. We have modified this well-known ligand to feature a photoswitchable side-chain, yielding ATA (former ATA3[12]) (Figure 1A). ATA is active in its thermodynamically more stable *trans*-form, that is, in the dark, and becomes inefficacious when irradiated with blue light. Furthermore, ATA was shown to be a specific photochromic agonist for AMPA receptors[12,19] and could be used to control neuronal activity in acute cortical brain slices. We now report the activity of ATA in light-insensitive mouse retinae providing a conceptually new way for the restoration of vision.

RESULTS AND DISCUSSION

ATA Conveys Light-Sensitivity to Blind Retinae. To investigate the activity of ATA in a blind retinae we generated a new triple knockout mouse line. The retina of this mouse line is

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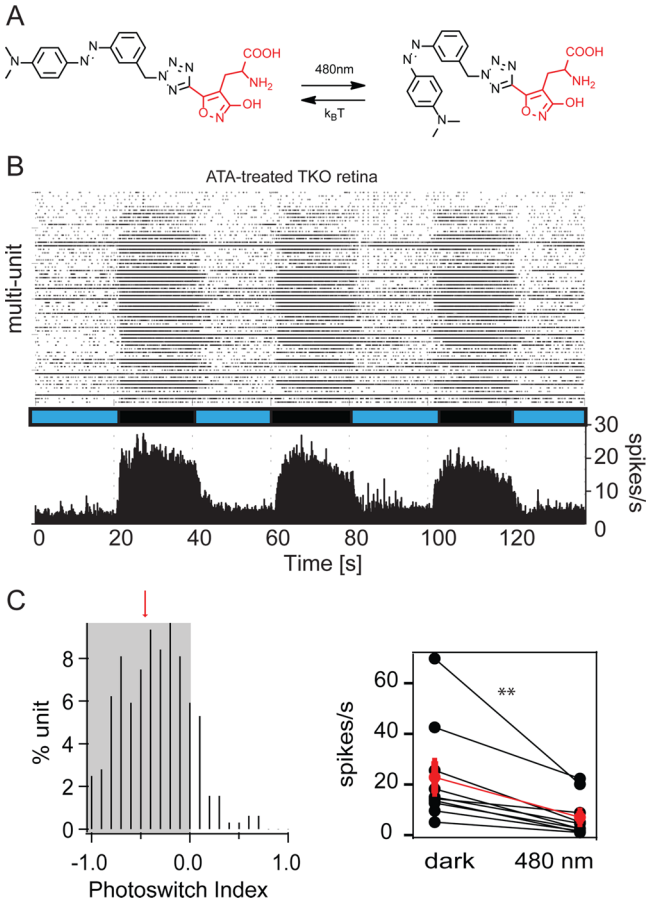

Figure 1. ATA induces robust light-responses in blind retinae. (A) ATA is a photoswitchable amino acid that bears structural resemblance to AMPA (depicted in red). Switching from the active *trans*-configuration to the inactive *cis*-configuration is achieved using blue light. *cis*-ATA thermally relaxes to the *trans*-state within milliseconds.[12] (B) Raster plot and histogram of MEA recording of ATA-treated TKO mouse retina. (C) Statistics of light responses in ATA-treated TKO retinae. (Left) Distribution of photoswitch index for RGC populations (n = 962 cells). The red arrow indicates the mean photoswitch index for all recorded cells (photoswitch index = −0.50 ± 0.05). (Right) Average spiking rate in darkness and with blue light; mean indicated in red (mean ± SEM) (n = 10 retinae). Significance level p = 0.011, Wilcoxon rank sum test.

completely light-insensitive since it lacks rhodopsin in rod photoreceptor cells (*rho*), cyclic-nucleotide gated channel A3 in cone photoreceptor cells (*cngA3*), and melanopsin in intrinsically photosensitive RGCs (*opn4*). Using multielectrode-array recordings (MEA), we monitored response to light in the presence of our photoswitchable compound. After a 3 min incubation with ATA (25 μM) (Figure 1A), switching between blue light (480 nm) and darkness induced robust light responses, without a significant rundown in light-dependent spiking frequency over several hours (dark, 22.9 ± 6.36 spikes/s; and 460 nm, 7.01 ± 2.49 spikes/s; Figure 1B). This is quantified using the photoswitch index (PI) as previously defined by Polosukhina et al.[17] A positive PI indicates an increased spiking activity upon switching light on and vice versa. ATA-mediated light responses exhibit an overall negative PI. This is due to the fact that ATA is active in its thermodynamically stable dark-adapted *trans* isoform and can be quickly inactivated by switching to the *cis*-isoform upon illumination with blue light (400−480 nm) (PI = −0.50 ± 0.05, n = 10) (Figure 1C left, red arrow).[12]

On a single cell level a population of RGCs respond to light on, whereas others respond to light off (Supporting Information (SI) Figure 1). This response pattern results from ATA-mediated activation of AMPA receptors expressed on different cell types, that is, RGCs, bipolar cells and amacrine cells. The level of activation depends on the profile of AMPA receptor expression. Therefore, PIs are distributed over a wide range. Without ATA treatment, no light-dependent spiking behavior could be detected (data not shown).

In terms of sensitivity, ATA can be inactivated by irradiation with relative low light intensities (10^{14} photons/cm^2·s). Thus, it is at least as sensitive as previously published photochromic blockers.[17,18,20] Like these, it can be switched to the off-state using blue light (440−480 nm), but it also works with broad-spectrum white light (SI Figure 4).[12,18]

ATA application to the retinal tissue requires a short preincubation time of 3−5 min at low concentrations (25 μM). Following the preincubation, strong light responses can be induced over several hours in ex vivo experiments. This might be due to integration of the amphiphilic molecule into the membrane during the initial incubation period, and constant release of the compound afterward. Our observation of a yellow staining of the retinal tissue after incubation supports this hypothesis.

RGCs and Amacrine Cells Primarily Shape the ATA-Mediated Light Response. To verify AMPA receptors as the main targets of ATA in the retina, we tested the compound on different glutamate receptor types, that is, kainate, NMDA and mGluR6 (see the Supporting Information).

Application of D-AP5, a selective NMDA receptor antagonist, had no effect on ATA-mediated currents (SI Figure 5). CPPG, an mGluR6 selective antagonist, slightly increased the baseline firing, but had no effect on the overall light response (SI Figure 6). This may be due to activation of on-bipolar cells, which express high levels of mGluR6.[21] Because kainate receptors cannot be easily distinguished from AMPA receptors pharmacologically, we heterologously expressed GluK1 and GluK2 in HEK293T cells. In these cells, ATA did not induce light-mediated membrane currents (SI Figure 7).

In contrast, application of the selective AMPA receptor antagonist NBQX (25 μM) completely abolished light-dependent firing. The spiking levels in darkness and under 480 nm light were comparable (3.56 ± 0.41 spikes/s and 2.74 ± 0.44 spikes/s, respectively) (Figure 2A) and the overall PI was −0.06 ± 0.004 (n = 8) (Figure 2B, left, red arrow).

Therefore, we conclude that ATA selectively activates AMPA receptors and does not affect NMDA, kainate receptors or metabotropic glutamate receptors. This confirms our previous results in cortical neurons.[12] In retinal tissue, AMPA receptors are widely distributed over several cell types.[22−26] To determine the target cell type of ATA in the retina, we pharmacologically isolated RGCs via application of $CdCl_2$ (500 μM), a blocker of synaptic transmission. While light-dependent spiking activity can still be observed under these conditions, the shape of the response is significantly altered. Whereas the basal activity has not changed, there is a pronounced and short off-response (Figure 3B bottom). The spiking frequency was slightly reduced during the sustained response (Figure 3B bottom), and due to the short but strong off-response, the PI value is even lower with $CdCl_2$ present (PI (w/$CdCl_2$) = −0.60 ± 0.09, n = 6 Figure 3B and D). A few experiments in the presence of

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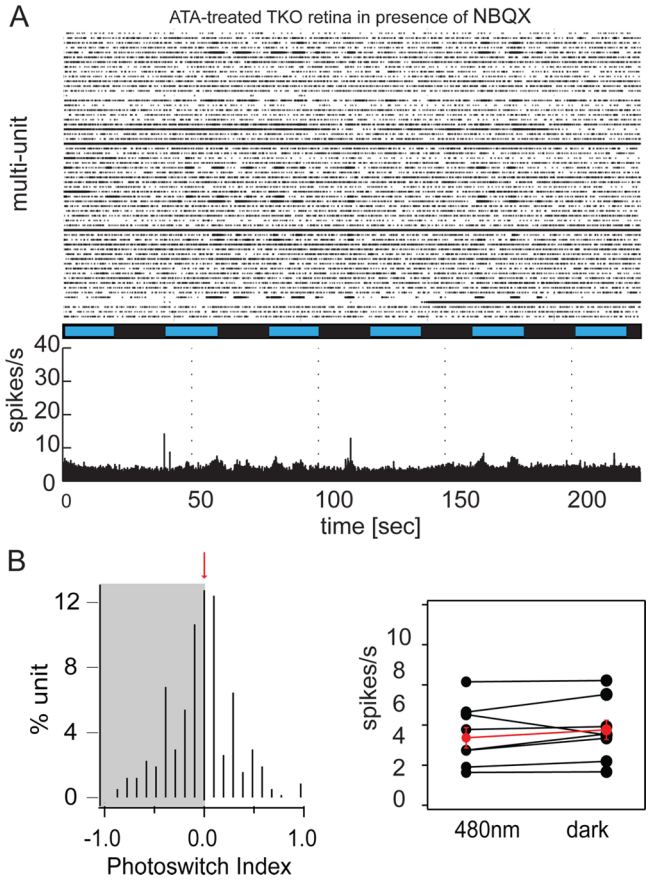

Figure 2. ATA exclusively sensitizes AMPA receptors in blind retinae. (A) Raster plot and histogram of MEA recording of ATA-treated TKO retina in the presence of 25 μM NBQX. (B) Statistics of light responses in ATA-treated TKO retinae. (Left) Distribution of the photoswitch index for RGC populations (n = 286 cells). The red arrow indicates the mean photoswitch index for all recorded cells (photoswitch index = −0.06 ± 0.04). (Right) Average spiking rate in darkness and with 480 nm light (n = 8 retinae).

$CdCl_2$ showed not only brief transient light-off but also transient light-on responses (SI Figure 2).

From these experiments, we conclude that ATA is directly activating RGCs in a light-dependent fashion. However, the change in the overall shape of the light response upon synaptic isolation indicates that RGCs are not the only target cells.

Because of the difficulties with addressing bipolar cells with a specific pharmacology, we tested the effect of amacrine cell input onto ATA-mediated light responses. We eliminated this inhibitory input to RGCs using a combination of strychnine (1 μM), picrotoxine (5 μM) and TPMPA (10 μM).[17,18] Under these conditions we observed an inversion of the light response (PI = 0.36 ± 0.03, n = 10, Figure 3C and E and SI Figure 3). Furthermore, the onset of spiking increase is slightly delayed compared to the light response without blockers (Figure 3A and C).

These results indicate that ATA primarily targets both amacrine cells and RGCs. However, bipolar cells are directly affected as well, which is compatible with ubiquitous expression of AMPA receptors throughout the retina.

Taken together, we were able to validate that ATA exclusively activates AMPA receptors, ruling out other glutamate receptors as molecular targets. Furthermore, we demonstrate that ATA primarily affects amacrine cell and RGC activity and our experiments with $CdCl_2$ show that ATA can act on RGCs alone. The pronounced off-response under these conditions is likely due to directly activating AMPA receptors on RGCs followed by desensitization. In the absence of blockers, the transient off-response is probably masked by the inhibitory input of amacrine cells.

When the inhibitory input is blocked, the light response is inverted. This surprising result is difficult to interpret, as it is not clear how the excitatory input by bipolar cells can invert the ATA-mediated RGC response. Our NBQX and CPPG experiments, however, rule out the possibility that ATA activates mGluR6, which in an intact retina are specifically expressed by on-bipolar cells.

Over the past decade, different approaches for restoring light sensitivity in blind retinae have been investigated. Retinal implants demonstrated that electrical stimulation of RGCs or bipolar cells is able to produce low-resolution images.[1,2] Additional studies in mice have shown that expression of halorhodopsin, channelrhodopsin or LiGluR confer light sensitivity to the remaining retinal network after photoreceptor loss.[6,27,28] In collaboration with the Kramer group, we recently introduced a simple photopharmacological approach that relies on photoswitchable molecules targeting voltage-gated potassium channels in the retina.[17,18] These molecules, such as AAQ and DENAQ, are able to confer light-sensitivity to blind mice in vivo after bolus injection into the intravitreal cavity.[17,18] We now extend this approach to photoswitchable agonists for AMPA receptors. Our lead molecule, ATA, bears several new features.

First, it exhibits a higher solubility in aqueous solution than our previous photopharmaceuticals. No additional excipients are therefore needed. Second, it operates on ligand-gated ion channels as opposed to voltage-gated ion channels. And third, it functions as a photoswitchable agonist and not as a photochromic blocker. As such, ATA-mediated stimulation provides a more natural stimulus mimicking excitatory synaptic transmission.

However, the analysis of our pharmacological results is difficult to interpret and is further complicated by recent studies demonstrating that retinal remodeling during degeneration involves massive changes in glutamate receptor expression levels. This can even result in conversion of retinal on- to retinal off-bipolar cells.[29−32] In addition, Lin et al. showed that not only the expression pattern but also the AMPA receptor composition changes after light-induced retinal degeneration.[33] This remodeling, however, not only affects our approach, but all attempts to restore vision based on the manipulation of the extant retina. Whether it also applies to human patients suffering from retinal degeneration remains to be determined.

Regardless, our results indicate that photoswitchable AMPA receptor agonists are powerful tools to control neural network activity with light. Despite the fact that AMPA receptors are ubiquitously expressed in the retina, output at the RGC level is reproducible and reliable. This raises expectations that compounds like ATA will find a place in human vision restoration. Here, we show for the first time that a freely diffusible photochromic receptor *agonist* is able to confer light sensitivity to blind retinae without the need for continuous application. Previously, photochromic channel blockers had been used. Future work aims at the development of second-generation photochromic glutamates that show higher subtype selectivity and are inactive in the dark.

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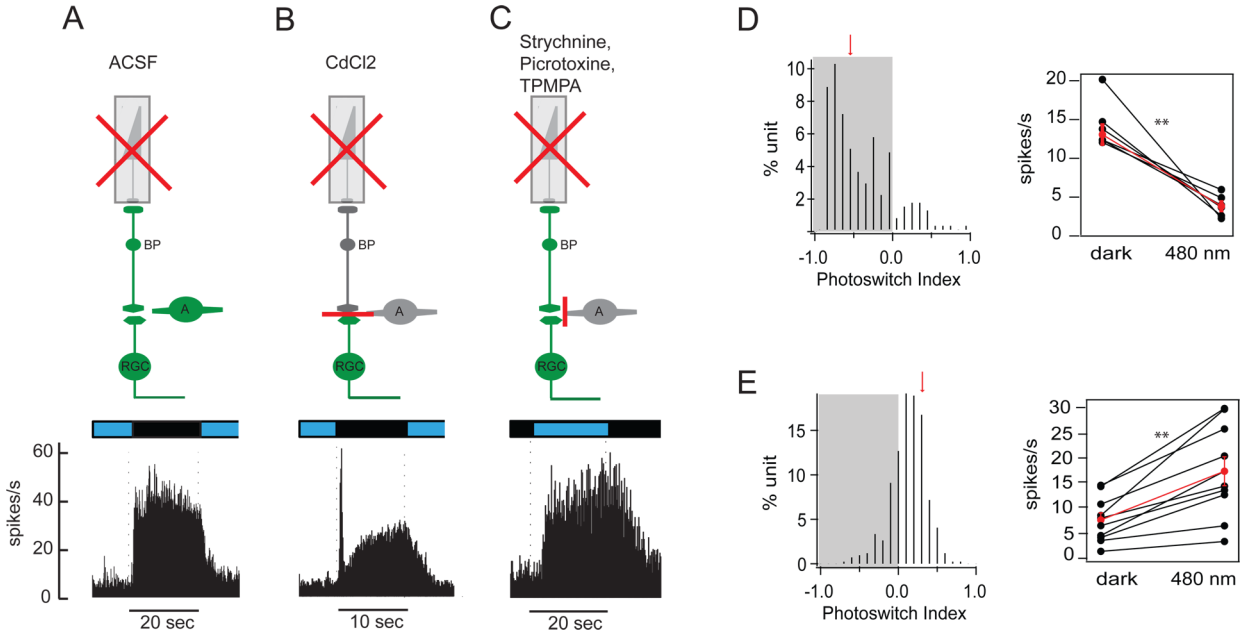

Figure 3. Pharmacology reveals that ATA primarily acts on RGCs and amacrine cells. Top: Schematic drawing of retinal cell types. Cells depicted in green may contribute to the light-dependent effect of ATA on RGC-output. Bottom: Histogram of ATA-induced light-response. (A) No blockers are applied; therefore, all retinal cell types may contribute to RGC output. (B) Synaptic inputs from amacrine and bipolar cells are blocked using 500 μM $CdCl_2$ to synaptically isolate RGCs from the remaining retinal cells. (C) Inhibitory input on RGCs is specifically blocked by strychnine, picrotoxine and TPMPA. RGC signal output consists of bipolar cell and RGC-mediated components. (D). Statistical analysis of light responses in ATA-treated TKO retinae in the presence of 500 μM $CdCl_2$. (Left) Distribution of photoswitch index for RGC populations (n = 797 cells). The red arrow indicates the mean photoswitch index for all recorded cells (photoswitch index = 0.6 ± 0.09). (Right) Average spiking rate in darkness and with 480 nm light (n = 6 retinae). Significance level p = 0.002, Wilcoxon rank sum test. (E) Statistical analysis of light responses in ATA-treated TKO retinae in the presence of strychnine, picrotoxine and TPMPA. (Left) Distribution of photoswitch index for RGC populations (n = 418 cells). The red arrow indicates the mean photoswitch index for all recorded cells (photoswitch index = 0.36 ± 0.03). (Right) Average spiking rate in darkness and with 480 nm light (n = 10 retinae). Significance level p = 0.007, Wilcoxon rank sum test.

METHODS

Chemicals. ATA was synthesized as an HCl salt in accordance with the synthetic route described by Stawski et al. 2012. All other chemicals were purchased from Abcam or Tocris Bioscience.

Heterologous Expression of GluK1 and GluK2 in HEK Cells. HEK293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Biochrom, Merk Millipore, Germany) in a 10% CO_2 atmosphere at 37 °C. Transfections were performed using JetPrime (Polyplus-transfections, France) according to the manufacturer's instructions 48 h before experiments.

GluK1 and GluK2 were transfected using 350 ng of DNA per coverslip together with 50 ng per coverslip of yellow fluorescent protein (YFP) plasmid DNA.

Animals and Tissue Preparation. Completely light-insensitive homozygous triple knockout $cnga3^{-/-}$ $rho^{-/-}$ $opn4^{-/-}$ (TKO) mice, were generated crossing the $cnga3^{-/-}$ $rho^{-/-}$ double knockout mouse with the $opn4^{-/-}$ provided by Dr. Samen Hattar.[34−36] TKO mice were used for all multielectrode experiments. Mice were sacrificed by cervical dislocation. After dissection of the retinae, they were kept in artificial cerebrospinal fluid (ACSF) at room temperature containing (in mM): 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 1 $MgCl_2$, 2 $CaCl_2$, 26 $NaHCO_3$, and 20 D-glucose, aerated with 95% O_2/5% CO_2.

Acute hippocampal brain slice were prepared from C57Bl6JRj mice (postnatal day 10−13, both male and female animals were used without known experimenter bias). Mice were decapitated and the brain was rapidly removed and transferred to ice-cold saline solution composed of (in mM): 87 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 0.5 $CaCl_2$, 7 $MgCl_2$, 25 glucose, 75 sucrose saturated with carbogen (95% O2/5% CO_2). Using a Campden vibratome 7000 smz-2 (NPI Electronic), 250 μm thick hippocampal brain slices were prepared and incubated at 34 °C for 30 min in ACSF. After incubation, slices were stored at room temperature from 1 to 5 h before recordings were started. All experiments were performed at room temperature and in the presence of 1 μM TTX. The 25 μM ATA solution in ACSF was prepared from a 25 mM stock (ddH_2O).

Patch-Clamp Recordings of Hippocampal Neurons and HEK Cells Heterologously Expressing GluK1 or GluK2. Cells were patched using glass electrodes with a resistance of 7−8 MΩ and an intracellular solution containing (in mM): 140 K-gluconate, 10 HEPES, 12 KCl, 4 NaCl, 4 Mg-ATP, 0.4 Na2-GTP. Recordings were made with an EPC 10 USB amplifier, which was controlled by the Patchmaster software (HEKA). Data was filtered at 2.9−10 kHz and digitized at 50 kHz. Cells were rejected if leak currents were >200 pA for hippocampal neurons or >300 pA for HEK cells, or with a series resistance >15 MΩ. Data was analyzed using the Patcher's Power Tools (MPI Göttingen) and routines written in IgorPro (Wavemetrics).

Multielectrode Array Electrophysiology. For extracellular recordings, a flat-mounted retina was placed ganglion cell layer down onto a multielectrode-array (MEA 1060-inv-BC, Multi-Channel Systems). We used 8 × 8 rectangular arranged MEA electrodes with a diameter of 30 μm and a spacing of 200 μm. Retinae were incubated with 25 μM ATA, while applying alternating 10 s 480 nm/dark pulses for 3 min at room temperature. After mounting on the MEA, retinae were washed for at least 20 min and recordings were made at 34 °C, while constantly perfusing ACSF without ATA. Extracellular spikes were high-pass filtered at 300 Hz and digitized at 20 kHz. A spike threshold of 3.7 SD (standard deviation) was set for each channel. In general electrodes with a width of 30 μm record from one to three RGCs.[18] To distinguish single RGC light responses, spike waveforms were manually sorted using the MC_Rack software (Multi-Channel Systems). Due to possible false negatives and false positive sorting events, we used the term "multiunits".

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Light Stimulation. MEA recordings were performed with a MCS- and Patchmaster-controlled 480 nm LED-lamp with a light-intensity of 3.1×10^{16} photons cm^{-2} s^{-1}, if not otherwise indicated. For white light stimulation we use a xenon lamp with an intensity of 10.5 mW/cm^2. A typical MEA stimulation protocol consisted of 10 cycles of alternating 10 s, 15 s or 20 s light/dark intervals.

Pharmacology (MEA). In order to pharmacologically isolate RGCs, 500 μM $CdCl_2$ was perfused. Whereas inhibitory currents were selectively blocked using 1 μM strychnine (glycine receptor antagonist), 5 μM picrotoxine ($GABA_A$ receptor blocker), and 10 μM 1,2,5,6-Tetrahydropyridin-4-yl)methylphosphinic acid (TPMPA) ($GABA_C$ receptor antagonist).[17,18] For verification that AMPA receptors are the molecular target of ATA, 25 μM NBQX was perfused.

Data Analysis and Statistics. We calculated the RGC firing rate in 100 ms bins for individual retinae in light and in darkness. In order to normalize light-elicited changes in firing rate of individual RGCs in retinae, we calculated the photoswitch index (PI) = (mean firing rate in the light−mean firing rate in darkness)/(mean firing rate in the light + mean firing rate in darkness) as previously described.[17,18] RGC firing was determined between the last second of dark and the first second of light intervals to calculate the PI. Statistical significance (p values) was calculated using the Wilcoxon rank sum test. Results were considered significant with $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. Error bars are presented as mean ± standard error of the mean (SEM). Data analysis was performed using custom routines within the IgorPro software (Wavemetrics) and Matlab.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschemneuro.5b00234.

Additional figures and data sets of experiments regarding the pharmacology of ATA (PDF)

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Author Contributions

L.L. performed and analyzed patch-clamp and MEA experiments. K.H. and P.S. carried out the synthesis of ATA. S.M., C.S., and M.B. designed and generated the provided Cnga3/Rho/Opn4 TKO mouse line. L.L., D.T., and M.P.S. designed the project and wrote the paper. All authors contributed to the writing of the paper.

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Notes

The authors declare no competing financial interest.

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