Kainate Receptors Mediate Signaling in Both Transient and Sustained OFF Bipolar Cell Pathways in Mouse Retina

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A fundamental question in sensory neuroscience is how parallel processing is implemented at the level of molecular and circuit mechanisms. In the retina, it has been proposed that distinct OFF cone bipolar cell types generate fast/transient and slow/sustained pathways by the differential expression of AMPA- and kainate-type glutamate receptors, respectively. However, the functional significance of these receptors in the intact circuit during light stimulation remains unclear. Here, we measured glutamate release from mouse bipolar cells by two-photon imaging of a glutamate sensor (iGluSnFR) expressed on postsynaptic amacrine and ganglion cell dendrites. In both transient and sustained OFF layers, cone-driven glutamate release from bipolar cells was blocked by antagonists to kainate receptors but not AMPA receptors. Electrophysiological recordings from bipolar and ganglion cells confirmed the essential role of kainate receptors for signaling in both transient and sustained OFF pathways. Kainate receptors mediated responses to contrast modulation up to 20 Hz. Light-evoked responses in all mouse OFF bipolar pathways depend on kainate, not AMPA, receptors.

Key words: glutamate sensor; kainate receptor; mouse; OFF bipolar cell; retinal circuitry; two-photon imaging

Introduction

A fundamental property of all sensory systems is the simultaneous encoding of the environment by multiple cellular pathways. In the visual system, parallel processing starts at the first synapse in the retina (Wässle, 2004; Masland, 2012; Sterling, 2013). Parallel processing is especially evident at the output of cone photoreceptors, which mediate daylight vision (Fig. 1A). Each cone releases glutamate onto approximately a dozen types of cone bipolar cell that divide into ON and OFF classes (Haverkamp and Wässle, 2000; Wässle et al., 2009). Cones hyperpolarize to light increments, and depolarize to light decrements. OFF bipolar cells express non-NMDA-type ionotropic glutamate receptors (iGluRs: AMPA/kainate receptors) and thereby follow the sign of the cone’s response (i.e., depolarize to light decrements; DeVries, 2000), whereas ON bipolar cells express metabotropic glutamate receptor type 6 (mGluR6) and thereby invert the sign of the cone’s response (i.e., depolarize to light increments; Slaughter and Miller, 1981; Nakajima et al., 1993; Morgans et al., 2009; Shen et al., 2009). ON and OFF classes each comprise 5–6 distinct types that mediate additional levels of parallel processing along spatial, temporal, and chromatic dimensions (Cohen and Sterling, 1990; Kouyama and Marshak, 1992; Li and DeVries, 2006; Wässle et al., 2009; Breuninger et al., 2011; Baden et al., 2013).

The mechanism for distinct temporal responses in parallel pathways, fast/transient versus slow/sustained, is unresolved. All ON bipolar cell types express mGluR6 receptors (Vardi et al., 2000), so distinct temporal responses must depend on cell type-specific differences in intrinsic (Awatramani and Slaughter, 2001; Ivanova et al., 2006; Cui et al., 2012; Saszik and DeVries, 2012) or circuit properties (e.g., inhibitory feedback; Eggers and Lukasiewicz, 2011). For OFF bipolar cells, it has been hypothesized that transient and sustained pathways may be generated instead by cell-type-dependent expression of specific glutamate receptors. Transient cells would express AMPA-type receptors (AMPARs), with relatively fast recovery from desensitization, and sustained cells would express kainate-type receptors (KARs) either exclusively or predominantly, with relatively slow recovery from desensitization (DeVries, 2000; Lindstrom et al., 2014). The evidence for this hypothesis derives primarily from paired cone—OFF bipolar cell recordings in slice preparations of ground squirrel retina (DeVries, 2000; DeVries et al., 2006; Li et al., 2010; Lindstrom et al., 2014). Physiological experiments in other species do not clearly support this model: iGluR agonist application in mouse retinal slices, and light-evoked stimulation in rabbit retinal slices suggested that some OFF bipolar cell types express a mixture of AMPARs and KARs (Buldyrev et al., 2012; Puller et al., 2013). Neither study indicates a major role for AMPARs in encoding light-evoked synaptic release in OFF bipolar cells.
A crossover inhibition pathway is shown: ON bipolar cell excites (+) amacrine cell, which inhibits (−) OFF bipolar cell terminals. Orange lines illustrate the stratification depths of ON- and OFF-type starburst amacrine cells, a commonly used anatomical marker in the retina. Dashed line indicates the ON/OFF boundary (see B).

**Materials and Methods**

The procedures for retinal preparation, patch-clamp recording and two-photon imaging of iGluSnFR fluorescence were identical to those described previously (Borghuis et al., 2013). All procedures were conducted in accordance with National Institutes of Health guidelines under protocols approved by the Yale University or University of Louisville Animal Care and Use Committees. The eyes of C57/B6 mice of either sex (2–6 months, n = 6 locations, three retinas) and DNQX + 1 μM strychnine (right; n = 4 locations, two retinas); *p < 0.01.

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**Figure 1.** ON-pathway crossover inhibition drives glutamate release in all OFF-levels of the IPL. A, UV light stimuli were projected onto the photoreceptors (cones, c; rods were saturated and are omitted for schematic clarity). Light-evoked glutamate release from ON-type (light gray) and OFF-type (dark gray) bipolar cells (b) was measured with two-photon fluorescence imaging of iGluSnFR (green), expressed on the dendritic arbors of ganglion (g) and amacrine cells (a). A crossover inhibition pathway is shown: ON bipolar cell excites (+) amacrine cell, which inhibits (−) OFF bipolar cell terminals. Orange lines illustrate the stratification depths of ON- and OFF-type starburst amacrine cells, a commonly used anatomical marker in the retina. Dashed line indicates the ON/OFF boundary (see B). **B**, Left, Fluorescence image of labeled ON (open symbols) and OFF (closed symbols) response at each level were defined as the amplitude of the fluorescence signal in 200 ms windows (indicated with magenta and green lines) relative to baseline. **C**, Relative Z (μm) amacrine cell, which inhibits (−) OFF bipolar cell terminals. Orange lines illustrate the stratification depths of ON- and OFF-type starburst amacrine cells, a commonly used anatomical marker in the retina. Dashed line indicates the ON/OFF boundary (see B). **B**, Left, Fluorescence image of labeled ON (open symbols) and OFF (closed symbols) response at each level were defined as the amplitude of the fluorescence signal in 200 ms windows (indicated with magenta and green lines) relative to baseline. **D**, Average fluorescence response at 12 levels of the IPL, centered on the OFF layers (control; left). All OFF-type responses persisted in the presence of the iGluR antagonist DNQX (100 μM; center). Additional block of the ON-pathway (L-AP4, 20 μM) eliminated all responses (data from a single retinal location). **E**, Average ON (open symbols) and OFF (closed symbols) response amplitudes in control, DNQX and DNQX + L-AP4 (n = 6 locations, three retinas). Response after wash is shown with control (top). The ON/OFF junction was located ~25–30 μm distal to the ganglion cell layer (see Materials and Methods). In this panel and in subsequent figures z-positions are expressed relative to the ON/OFF junction (relative Z). **F**, Schematic of the drug perturbations. L-AP4 blocks synaptic input to OFF bipolar cells (light gray); DNQX blocks synaptic input to OFF bipolar cells (dark gray). **G**, Bar graph of response amplitudes averaged across the principal OFF levels (14 μm Z-range, indicated by shaded region in B) in the presence of DNQX + L-AP4 (left; n = 6 locations, three retinas) and DNQX + 1 μM strychnine (right; n = 4 locations, two retinas); *p < 0.01.

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**Here, we tested the roles of AMPARs and KARs in OFF bipolar pathways using two-photon fluorescence imaging of a glutamate biosensor and whole-cell recording in the intact mouse retina in vitro. Our results show that light-evoked responses of all OFF bipolar cell types, both transient and sustained, critically depend on KARs but not AMPARs, and that KARs are sufficiently fast to signal light-evoked release modulated up to 20 Hz.**
months of age) were removed immediately following death. Each retina was dissected in carboxengated (95% O2–5% CO2) Ames Medium (Sigma-Aldrich) using a microscope equipped with infrared viewers, as described previously (Borghuis et al., 2013). The retina was then transferred to a chamber on the stage of an Olympus microscope and perfused with heated, carboxengated Ames medium (~6 ml/min; 34–36°C). The receptor agonists, antagonists and transporter blockers (purchased from Tocris Bioscience), described below, were added to Ames medium from drug reservoirs. The KAR selective antagonists ACET (1 μM) and UPB310 (50 μM) showed similar effects in our experiments. They were used interchangeably, and data obtained with either drug are combined in Figure 3 B, D.

Light stimuli were designed to stimulate cones. The majority of mouse cones coexpress two opsins with a gradient of coexpression along the dorsal-ventral axis (Rölich et al., 1994; Applebury et al., 2000). Cones in the dorsal retina express primarily a middle-wavelength (M)-sensitive opsin with peak sensitivity to green light (510 nm), whereas cones in the ventral retina express almost exclusively a short-wavelength (S)-sensitive opsin with peak sensitivity to UV light (Nikonov et al., 2006; Wang et al., 2011; Baden et al., 2013; 365 nm). All measurements were made in the ventral retina, except for the data presented in Figure 3D. Light stimuli delivered from a customized projector (peak output, 395 nm) through the condenser activated ~106 photoisomerizations (R* cone “sec”−1); rods are largely suppressed at this light level and in the presence of the laser used for two-photon imaging, as described previously (Borghuis et al., 2013). The standard stimulus was a disk (0.4-mm diameter) centered on a background at mean luminance (4 X 3 mm on the retina). Luminance of the disk was modulated with a 1 Hz temporal square-wave at 100% Michelson contrast. For the electrophysiological measurements presented in Figure 3E; two stimulus conditions were used in addition to the standard stimulus: in the first, the mean light level was reduced by 2 log-units using a neutral density filter; in the second the disk was presented on a dark background, to maximize contrast (effective contrast ratio of 1000:1). For the temporal tuning measurements presented in Figure 6, A and B, the stimulus was a 0.3 × 0.3-mm square generated with a software-controlled light-emitting diode (peak output, 405 nm) at 100% contrast (same mean R* rate as the projector).

Two-photon fluorescence measurements were obtained with a custom-built microscope controlled with ScanImage software (www.scanimage.org; Pologruto et al., 2003), using a Olympus 60X, 1.0 NA, LUMPLANFl/IR objective and an ultrafast pulsed laser (Chameleon Ultra II, Coherent) tuned to 910 nm, as described previously (Borghuis et al., 2013). Whole-cell recordings were made from ganglion and bipolar cells in the whole-mount retina using the following intracellular solution (in mM): 100 Cs-methanesulfonate, 5 TEA-Cl, 10 HEPES, 10 BAPTA, 3 NaCl, 2 QX-314-Cl, 4 ATP-Mg, 0.4 GTP-Na2, and 10 phosphocreatine-Tris, pH 7.3 (280 mOsm). Excitatory currents were recorded with a holding potential near EcL (−67 mV) after correcting for the liquid junction potential (~9 mV).

Stimulus-evoked fluorescence responses (32 frames/s) were acquired in z-stacks that encompassed the entire OFF half of the inner plexiform layer (IPL). The z = 0 μm level was defined as the focal plane that hemisected the ganglion cell somas. Stacks were acquired from the vitreal side of the ON/OFF boundary (typical z = 20 μm) to the inner nuclear layer (i.e., focal plane that intersected the first layer of cell bodies; typical z = 48 μm). The light stimulus consisted of 1.5 s of a gray screen followed by 3.5 s of contrast modulation of a 0.4-mm-diameter disk (1 Hz square wave). The stimulus was repeated three times with a 3 s interstimulus interval; after the third repeat, the focus was automatically advanced 2 μm toward the inner nuclear layer, and the stimulus sequence was repeated. Obtaining a complete z-stack between the middle of the IPL and the inner nuclear layer required ~7 min.

Images (20 X 20 μm) were down-sampled in software to 32 X 32 pixels (0.63 X 0.63 μm per pixel), using boxcar averaging. Pixels whose fluorescence intensity during the respective stimulus on- and off-phase (300 ms window) exceeded one SD above the average intensity just before stimulus onset (500 ms window) were defined as significantly modulating ON- and OFF-responding pixels, respectively (Fig. 1, B, C). These pixels were grouped into separate ON and OFF ROIs. Where ON and OFF responses are not presented separately, the average of all significantly modulating pixels is shown (Fig. 1C). Population data shown in Figure 6E were based on a total of 30 line scans for each condition. Using a computer algorithm, each line scan (25 μm length) was divided into 10 equal-sized ROIs. ROIs were sorted based on the modulation amplitude of their response to 1 Hz stimulation. The two most strongly modulating ROIs from each line scan were selected and the modulation amplitude of their response to 7.5 Hz was combined, culminating in data for 60 ROIs in each condition in Figure 6E.

All data are reported as mean ± SEM. The effects of drug applications were evaluated by two-tailed repeated measures t tests.

Results
Glutamate imaging in the intact mouse retina

We monitored light-evoked synaptic release of OFF-type bipolar cells during selective perturbations of AMPARs and KARs. Synaptic release from bipolar cells was measured using two-photon fluorescence imaging of the glutamate biosensor intensity-based glutamate-sensing fluorescent reporter (iGlurSnFR; Borghuis et al., 2013; Marvin et al., 2013) expressed on the dendrites of their postsynaptic targets, the ganglion and amacrine cells (Fig. 1A).

iGlurSnFR was delivered by in vivo intraocular injection with adeno-associated virus under control of the human synapsin-1 promoter (AAV2/1-hSynapsin-iGlurSnFR), as described previously (Borghuis et al., 2013). Retinas were recorded 2–3 weeks after injection. Cones were stimulated with patterned UV light (λmax = 395 nm) projected through the microscope’s condenser (Borghuis et al., 2013) while fluorescence responses were recorded at multiple levels of the IPL, including all levels where OFF bipolar terminals stratify (Fig. 1A, B). At each level, ON- and OFF-responding pixels were detected, based on a threshold and the response sign, and grouped into ON- and OFF-responding regions accordingly (see Materials and Methods; Fig. 1B, C).

The iGlurSnFR signals reflected the basic organization of the IPL. ON responses localized to the proximal half of the IPL, nearest to the ganglion cell layer and OFF responses localized to the distal half, nearest to the inner nuclear layer (INL; Fig. 1B, D). In an intermediate layer, both ON and OFF signals occurred simultaneously in spatially nonoverlapping regions (Fig. 1B, C; Borghuis et al., 2013). OFF layers included both transient and sustained responses (Fig. 1C, D), with transient signals nearest the center of the IPL, and sustained signals nearest the INL. Some stacks showed additional transient responses at the INL border (Fig. 1D).

Kainate receptors mediate both transient and sustained OFF bipolar pathways

Two distinct pathways mediate glutamate release from OFF bipolar terminals following light decrements. First, cones release glutamate onto iGlurRs (AMPArs or KARs) expressed on the bipolar cell dendrites, causing depolarization. Second, crossover ON amacrine cells relieve their tonic inhibition of the OFF bipolar terminal, also causing depolarization. Hence the cones and crossover amacrine pathways form a push-pull circuit with the same influence on OFF bipolar terminals (Fig. 1A, F): light decrement evokes glutamate release (Werblin, 2010). To study the specific contribution of the cone → OFF bipolar cell synapse, we first designed experiments to distinguish this contribution from the crossover amacrine cell pathways.

Cone input to OFF bipolar dendrites was blocked by bath-application of the AMPAR/KAR antagonist DNQX (100 μM). In the presence of DNQX, iGlurSnFR responses persisted in all OFF layers of the IPL and were significantly increased (n = 4; 0.10 ± 0.01 vs. 0.17 ± 0.01, 63.9 ± 12.3% increase, t = 4.12, p = 0.0062;
OFF layers remained in the presence of DNQX, including layers that were predominantly transient or sustained (Fig. 1D). We hypothesized that release from OFF bipolar terminals was mediated by the crossover inhibition from the ON pathway described above (Welblin, 2010). Although multiple circuits are possible, including circuits composed of serial inhibitory synapses, the simplest circuit is ON bipolar → ON amacrine → OFF bipolar (Fig. 1F).

Blocking the ON pathway with the group III mGluR agonist L-AP4 (20 μM), which hyperpolarizes ON bipolar cells (Slaughter and Miller, 1981), blocked all DNQX-resistant activity (Fig. 1D–G). DNQX and L-AP4 have no direct effect on iGluSnFR (Marvin et al., 2013). Therefore, the lack of OFF responses in the presence of L-AP4 and DNQX supports a model where DNQX blocks all cone-to-OFF bipolar cell transmission, and persisting release depends on ON-pathway crossover inhibition (Fig. 1F). Averaging across layers, the fluorescence response in DNQX (0.25 ± 0.02; n = 4) was reduced by 98.0 ± 0.4% after adding L-AP4 (0.005 ± 0.002; t = 12.2, p = 0.0012). Responses returned after washing out all drugs (Fig. 1E). To further test the mechanism underlying the crossover inhibition we selectively blocked glycine receptors. In the presence of DNQX, strychnine (1 μM) suppressed, but did not eliminate, the response across OFF layers (0.38 ± 0.04 vs 0.11 ± 0.02; 70.1 ± 18% reduction, t = 6.24, p = 0.0083). This shows that glycinergic amacrine cell pathways mediate the majority of crossover inhibition, but that other (presumed GABAergic) pathways contribute as well (Arman and Sampath, 2012). We conclude that relief from crossover inhibition from the ON pathway can drive synaptic release from OFF bipolar terminals at each OFF level of the IPL. Consequently, to isolate OFF bipolar responses mediated by direct cone inputs, all subsequent experiments were performed in the presence of L-AP4 (Fig. 1F).

In the presence of L-AP4, iGluSnFR responses were absent in the ON layers, as expected, but responses remained in both transient and sustained OFF layers (Fig. 2A). In the presence of L-AP4, we blocked either AMPARs or KARs. Based on the prevailing model (DeVries, 2000), our hypothesis was that transient responses would be sensitive to AMPAR block, whereas sustained responses would be sensitive to KAR block. Instead, we found that OFF responses in all layers remained (and increased) in the presence of the AMPAR preferential antagonists GYKI 52466 (100 μM) and GYKI 53655 (100 μM); respectively, n = 4, 0.43 ± 0.05 vs 0.55 ± 0.10, t = 0.81, p = 0.48, ns; and n = 4, 0.28 ± 0.03 vs 0.49 ± 0.04, t = 7.91, p =
The increased release in GYKI 53655 is likely explained by the loss of inhibitory feedback from horizontal cells and amacrine cells that use AMPARs (i.e., similar to the effect of DNQX above). OFF responses in all layers were eliminated by the KAR antagonists UBP310 (50 μM; 0.08 vs 0.00, t = 4.57, p = 0.020) and ACET (1 μM; 0.26 ± 0.02 vs 0.01 ± 0.00, t = 14.1, p < 0.001; Pinheiro et al., 2013; Fig. 2A–C). These results strongly suggest that all mouse OFF bipolar pathways, both transient and sustained, depend on KARs at the cone–bipolar cell synapse.

Figure 3. Kainate receptor block eliminates excitatory currents in OFF-type ganglion cells. A. Excitatory currents in an ON-α cell (black) persisted in the presence of NMDA and ACh antagonists (D-AP5, 100 μM + hexamethonium, 100 μM, blue) and after additional block of KARs (top; ACET 1 μM, red) but were blocked by antagonists of AMPARs (bottom; GYKI 53655, 50 μM, red; GYKI 53655, 100 μM, cyan dashed). Gray curve shows the response after 15 min wash to control condition. B. Effect of KAR and AMPAR block in three ON-α cells (gray, individual cells, black mean ± SEM; amplitude was calculated over 200 ms window, 100 ms following onset of the preferred stimulus phase; *p < 0.01. C. Excitatory currents in OFF-α- and OFF-β-type ganglion cells under control conditions (black) and in the presence of L-AP4 (blue). Responses in the presence of L-AP4 were eliminated by additional block of KARs (red; UBP310, 50 μM or ACET, 1 μM). Gray curves as in A, B. D, Effect of KAR block in OFF-α- and -β cells (individual cells: dashed/green = dorsal retina; solid/magenta = ventral retina; black, mean ± SEM). Amplitudes were calculated as the mean leak-subtracted current 100–300 ms after onset of the preferred stimulus phase; *p < 0.01. E. Effect of ACET and D-AP5 on excitatory currents in an OFF-α and an OFF-β cell. Green indicates response after wash to control condition after additional block with L-AP4. Gray, blue, and magenta show responses under three different stimulus conditions, standard: typical conditions used throughout; dim light: 2 log-unit lower mean light level; dark: photopic intensity flash against dark background (see Materials and Methods). Inset (bottom, right) shows magnification of the trace above. F, Response amplitudes under control conditions and in the presence of ACET and D-AP5 (as in E) for all recorded cells (OFF-α: n = 4; OFF-β: n = 3).
from an ON α ganglion cell. The bipolar→ganglion cell synapse uses primarily AMPARs (Lukasiewicz et al., 1997; Cohen, 2000; Jacoby and Wu, 2001; Beaudoin et al., 2008), whereas the cone→ON bipolar synapse uses mGluR6 receptors. We isolated AMPAR- and KAR-mediated currents by eliminating other known sources of excitation. To this end, NMDA and nAch receptors were blocked with D-AP5 (100 μM) and hexamethonium (100 μM), respectively; these drugs in fact had little effect (Fig. 3A). Under these conditions, 97.7 ± 9.0% of light-evoked responses in ON-α cells were blocked by GYKI 53655 (n = 4; p = 0.0008; remaining current 18.6 ± 2.64 pA; Fig. 3A,B), with similar remaining currents at 50 and 100 μM, demonstrating that the concentration used above (100 μM) blocked AMPARs adequately. The small GYKI-resistant current (2.3% of control) was insensitive to KAR block (ACET, 1 μM; n = 1; data not shown).

Responses measured in the presence of D-AP5 and Hexamethonium were insensitive to subsequently applying KAR antagonists UBP310 (50 μM) and ACET (1 μM) (n = 4; mean difference: 0.87 ± 18.2 pA, t = 0.05, p = 0.97, ns; Fig. 3A,B). Thus, KARS do not mediate light-evoked responses at the ON bipolar→ON α cell synapse, as expected, and UBP310 and ACET have no apparent effect on AMPARs at the concentrations applied in this study.

If KAR block in the presence of L-AP4 eliminates light-evoked synaptic release from both transient and sustained OFF bipolar cell types, then this should be apparent in the excitatory currents of their postsynaptic ganglion cell targets. To test this, we recorded from OFF-α cells, whose dendrites costratify with transient OFF bipolar cells, and OFF-δ ganglion cells, whose dendrites costratify with sustained OFF bipolar cells (Fig. 1A; Tagawa et al., 1999; Margolis and Detwiler, 2007; van Wyk et al., 2009). Indeed, in the presence of L-AP4 (to block crossover inhibition), blocking KARS with either ACET or UBP310 eliminated all excitatory responses in both OFF-α and OFF-δ ganglion cell (OFF α ventral retina, n = 4, 265 ± 46.0 pA vs 1.74 ± 0.87 pA, t = 5.77, p = 0.010; dorsal retina, n = 5, 344 ± 52.8 pA vs 9.35 ± 2.71 pA, t = 6.46, p = 0.003; OFF-δ ventral retina, n = 3, 354 ± 32.7 pA vs 25.3 ± 13.2 pA, t = 9.05, p = 0.012; dorsal retina, n = 7, 329 ± 32.0 pA vs 8.58 ± 1.49 pA, t = 10.3, p < 0.0001; Fig. 3C,D). Results were consistent between recordings in ventral and dorsal retina, where cones express primarily either S (UV-sensitive) or M (green-sensitive) opsin (see Materials and Methods). Combining data from dorsal and ventral retina, KAR antagonists blocked, respectively, 98.2 and 96.0% of the light-evoked excitatory current in OFF-α cells and OFF-δ cells. These data show that light-evoked glutamate release from cones activates OFF bipolar cells through kainate receptors.

Next we verified that the absence of excitatory currents in OFF-α and OFF-δ cells in the presence of L-AP4 and UBP310 or ACET was not caused by direct block of KARS expressed on the ganglion cells themselves. To test this, we isolated the putative
AMPAR-mediated response in the ganglion cells by pharmacological block of KARs and NMDARs (ACET, 1.0 μM; D-AP5, 100 μM). Under this condition, excitatory currents in OFF-α and OFF-β cells persisted (mean difference for OFF-α: 2.96 ± 19.8 pA, t = 0.15, p = 0.89, n.s.; OFF-β: −30.8 ± 18.9 pA, t = 1.63, p = 0.24, ns; Fig. 3 E, F). These currents presumably reflected ON-pathway-mediated OFF bipolar release onto the ganglion cells’ AMPARs. Indeed, these currents were suppressed by subsequently blocking ON-pathway crossover inhibition with L-AP4 (Fig. 3E). In the latter condition, not only was the response to our standard stimulus (high contrast on a photopic background) blocked, additional stimuli, including contrast modulation at a standard stimulus (high contrast on a photopic background), did not block stimulus-evoked glutamate release from photoreceptors measured at Muller cell processes in the IPL, but did not block stimulus-evoked glutamate release from photoreceptors measured at Muller cell processes in the OPL. These data confirm the imaging results and suggest that both transient and sustained OFF bipolar pathways rely on KARs at the cone—OFF bipolar synapse. Strong evoked release at the level of the photoreceptors does not cause AMPAR-mediated activation of either transient or sustained OFF bipolar cells sufficient to evoke synaptic release.

Cone photoreceptor release persists with kainate receptors blocked
We next tested the possibility that the combination of L-AP4 and KAR block indirectly suppressed glutamate release at the level of the cone synapse. We visualized glutamate signaling in both the outer plexiform layer (OPL) and IPL by selectively targeting iGluSnFR expression to Muller glia, using the GFAP (glial fibrillary acidic protein) promoter (Borghuis et al., 2013). Muller glia extend vertically across the entire retina with dense local branching in the OPL and IPL (Fig. 4A). Under control conditions, Muller glia processes were exposed to glutamate in the IPL (strong fluorescence responses) but not in the OPL (no fluorescence responses; Fig. 4 B, C). In the OPL, glutamate uptake apparently occurs through a glia-independent, neural mechanism and is efficient enough to prevent glutamate exposure to the Muller glia processes (Hasegawa et al., 2006). However, stimulus-evoked glutamate release in the OPL could be visualized after bath applying the nonspecific glutamate transporter blocker TBOA (20 μM; Fig. 4G).

By blocking glutamate uptake, TBOA increased glutamate concentrations near photoreceptor synaptic release sites enough to expose iGluSnFR on nearby Muller cell processes (Fig. 4B). As expected, TBOA also increased the amplitude and duration of ON and OFF responses in the IPL (Fig. 4C; Marvin et al., 2013). In the presence of TBOA, application of L-AP4 and UBP310 eliminated all stimulus-evoked glutamate release from ON and OFF bipolar cells measured at Muller cell processes in the IPL, but did not block stimulus-evoked glutamate release from photoreceptors measured at Muller cell processes in the OPL. These data show that cone glutamate release persists in the presence of L-AP4 and KAR block. Furthermore, they also demonstrate that UBP310, like other glutamate receptor antagonists
(Marvin et al., 2013), does not affect iGlurSNFR function. Even with transporters blocked, and glutamate spillover in the OPL increased, we did not detect AMPAR-mediated glutamate release from OFF bipolar cells, whereas KARs were required for the function of all OFF-type bipolar cells. Therefore, glutamate spillover in the OPL is not sufficient for evoking AMPAR-mediated responses in OFF bipolar cells.

Cone to type-4 OFF bipolar cell synapses rely on kainate receptors
To further substantiate the role of KARs in OFF bipolar cell responses, we made direct recordings of light-evoked excitatory currents in OFF bipolar cells in the whole-mount retina. Type-4 OFF bipolar cells reportedly express both AMPARs and KARs (Puller et al., 2013), based on agonist application, and these cells
can be selectively targeted for recording in the 5HT2Ra-GFP line (Lu et al., 2009). A GFP+ cell was recorded with a patch pipette filled with red dye; following recording, the green cell showed red fluorescence, and the filled axon terminal fitted an apparent mosaic of neighboring GFP+ terminals in the IPL (Fig. 5A). Type-4 cells showed characteristic changes in their light-evoked currents at varying holding potentials (Fig. 5B), including an excitatory conductance at light offset, and an inhibitory conductance at both light offset and onset (Fig. 5C). The inhibition at light onset is direct evidence for crossover inhibition from the ON pathway. In the presence of L-AP4, the KAR antagonist UBP310 (50 μM) blocked the excitatory response (n = 4; 30.9 ± 9.3 pA vs 3.3 ± 0.76 pA; mean difference 27.6 ± 9.3 pA, t = 2.97, p = 0.029; Fig. 5D,F).

Next we recorded from type-4 bipolar cells and applied L-AP4 and the AMPAR antagonist GYKI 53655 (100 μM). Although there was some variability in the response in GYKI, as expected during prolonged whole-cell recording from bipolar cells in the intact retina, on average the response was not changed from the control condition (n = 3; mean difference 2.09 ± 14.3 pA, t = 0.146, p = 0.90, ns; Fig. 5E,G). These measurements support the imaging results and demonstrate that the cone–OFF bipolar synapse of the type-4 bipolar cell is directly dependent on KARs, with no apparent contribution from AMPARs.

Kainate receptors support rapid temporal processing in OFF bipolar cell pathways

KARs are known for having slow kinetics at many CNS synapses (Castillo et al., 1997; Vignes and Collingridge, 1997; Erreger et al., 2004; Copits and Swanson, 2012; Tomita and Castillo, 2012). Accordingly, paired synaptic recordings of cones and individual OFF bipolar cells in the ground squirrel retina suggested a slow time constant for recovery from desensitization, ~0.5–2 s (DeVries and Schwartz, 1999; DeVries, 2000; Straub et al., 2011). This slow recovery suggested that KARs might be inadequate for encoding of high temporal frequencies, but in fact, high-frequencies are commonly encoded by sustained visual pathways. For example, sustained ganglion cells in cat (βX-type) and monkey (midget/parvocellular-type) encode temporal frequencies >30 Hz (Frishman et al., 1987; Lee et al., 1990). Our results in Figure 1 show that ON-pathway crossover inhibition can modulate release from OFF bipolar terminals. Conceivably, this crossover pathway, rather than direct cone input, mediates rapid modulation of KAR-expressing OFF bipolar cells. To test whether OFF bipolar cells inherit their fast temporal kinetics from the ON-pathway, we measured OFF bipolar cell output in response to high temporal frequencies in the absence of crossover inhibition by the ON pathway.

Under control conditions, excitatory currents in ganglion cells responded to sine-wave modulation at frequencies up to 20 Hz, with a peak near 8 Hz (Fig. 6A,B). ON-α (n = 8) and OFF-α (n = 7) cells showed similar tuning; whereas OFF-β (n = 4) cells showed low-pass tuning, and lower response amplitudes compared with the α cells (Fig. 6B). Blocking ON bipolar cell-mediated crossover inhibition, with L-AP4 (20 μM), altered the tuning curve of OFF-α cells, decreasing the response at low-frequencies and increasing the response at high frequencies. L-AP4 increased the response in OFF-β cells at all frequencies. The different effects of L-AP4 on OFF-α and -β cells implies that distinct presynaptic circuits, each with a diverse influence from the ON-pathway, converge onto the presynaptic bipolar terminals; this would include more complicated circuits than that depicted in Figure 1F. Importantly, in both OFF-α and OFF-β cells, the response at the highest temporal frequency tested (20 Hz) persisted in the presence of L-AP4 (Fig. 6B) and was significantly greater than zero (OFF-α: 100 ± 7.8 pA, t = 13.0, p < 0.0001; OFF-β: 44.2 ± 5.2 pA, t = 8.6, p < 0.0001). Hence, the fast temporal kinetics of OFF bipolar cell release onto ganglion cells does not rely on the ON bipolar cell pathway through crossover inhibition. Instead, KARs on OFF bipolar cells mediate fast visual processing.

We verified with iGlurSNFR measurements that KARs mediated OFF bipolar responses to 7.5 Hz stimulation. This frequency approximates the peak of the tuning curves of α cells and falls within the resolution limit of iGlurSNFR (temporal cutoff ~15 Hz). Line scans across bipolar cell axon terminals showed response modulation at 7.5 Hz in the ON and OFF layers of the IPL (Fig. 6C,D). Responses in OFF layers persisted after blocking ON-pathway crossover inhibition (L-AP4, 20 μM) and AMPARs (GYK153655, 100 μM; Fig. 6C,D,E); 0.13 ± 0.01 ΔF/F. These results demonstrate that transmission of this temporal frequency from cones to OFF bipolar cells does not require AMPARs. Furthermore, because KAR block eliminates all synaptic activity in OFF bipolar cells, we conclude that KAR signaling is the principal mechanism underlying cone-driven bipolar cell activation, including responses at high temporal frequencies.

Discussion

A prevailing theory proposes that differential expression of AMPARs and KARs at OFF bipolar cell dendrites generates parallel fast/transient and slow/sustained pathways for vision in the mammalian retina (DeVries, 2000; DeVries et al., 2006; Masland, 2012; Sterling, 2013). Here, we tested this hypothesis in the mouse retina, using light-evoked synaptic stimulation, two-photon imaging of iGlurSNFR, patch-clamp recording of ganglion cells and bipolar cells, and pharmacological perturbations. Our results show that the transient and sustained OFF pathways in the mouse retina are not mediated by differential expression of AMPARs and KARs. Instead, all OFF pathways (evaluated with crossover inhibition blocked; Fig. 1) depend critically on KARs (Figs. 2–5). Results were consistent across multiple conditions, including: different mean light and contrast levels (Fig. 3E); different halves of the retina, where cones express primarily either M or S opsin (Fig. 3D); and in the presence of glutamate transporter block, which increases spillover (Fig. 4). If there are any conditions where AMPARs contribute substantially to OFF bipolar pathways in mouse, they must be outside of the range we tested and possibly require long-term changes in receptor expression associated with prolonged exposure to specific stimulus conditions (Jones et al., 2012).

Our results show that OFF bipolar cell KARs support responses to high-frequency light-evoked synaptic stimulation in the physiological range (up to 20 Hz). Notably, both the transient and sustained OFF pathways, which converge onto OFF-α and OFF-β ganglion cell types, followed 20 Hz modulation (Fig. 6). Hence, KAR signaling in OFF bipolar cells is not limited to slow synaptic integration, as has been demonstrated elsewhere in the nervous system (Castillo et al., 1997; Vignes and Collingridge, 1997; Copits and Swanson, 2012; Tomita and Castillo, 2012).

Using a fluorescent biosensor for glutamate, we show for the first time that crossover inhibition from the ON pathway controls glutamate release from OFF bipolar terminals at all levels of the IPL (Fig. 1). This suggests a complementary role for cone → OFF bipolar cell and ON amacrine → OFF bipolar cell synapses in modulating glutamate release from OFF bipolar cells. Crossover inhibition has been observed directly in some rabbit and mouse...
bipolar cells (Molnar and Werblin, 2007; Pang et al., 2012) and was implied by pharmacological experiments in rabbit OFF brisk-sustained ganglion cells (Buldyrev et al., 2012). Crossover inhibition likely plays a prominent role in generating distinct responses across OFF bipolar cell types.

A model that emphasizes the importance of AMPARs for fast/ transient OFF pathways was supported at the functional level primarily from data by the ground squirrel retina (DeVries, 2000). In response to S-cone, M-cone, or rod depolarization, a type-b2 OFF bipolar cell’s response was blocked by GYKI (Li and DeVries, 2006; Li et al., 2010). To our knowledge, there is limited additional functional evidence for OFF bipolar cells whose light response depends predominantly on AMPARs. In rabbit, OFF bipolar cells of unknown type were mostly blocked (77–86%) by UBP310 (10 μM), with a minor component blocked by GYKI (10–20%; Buldyrev et al., 2012). Some mouse bipolar cell types showed a GYKI-sensitive response to direct AMPA application (Puller et al., 2013). However, experiments with puff-applied agonists should be interpreted with caution. For example, GFP + type-4 bipolar cells in the 5-HTR2a mouse retina showed GYKI-insensitive, KAR-mediated light responses in our study (Fig. 5) but mixed AMPAR/KAR-mediated responses to puff-applied agonists in a previous study (Puller et al., 2013). Direct application of glutamate receptor agonists might activate nonsynaptic receptors (including AMPARs) on OFF bipolar cells that are not stimulated during synaptic glutamate release from cones, or, alternatively, act through an indirect pathway involving horizontal cells, which express AMPARs and feedback onto cones. Some salamander OFF bipolar cell light-evoked responses were completely blocked by GYKI (Cadetti et al., 2005), emphasizing that expression patterns may differ across species.

The second line of evidence for AMPARs on mammalian OFF bipolar cells comes from anatomical studies, performed across several species (Morigiwa and Vardi, 1999; Haverkamp and Wässle, 2000; Li et al., 2004; Puller et al., 2007). It is difficult to fully evaluate this evidence for two reasons. First, in most studies the specificity of the antibodies was untested (Rhodes and Trimmer, 2006). Second, even if the antibodies were specific, confocal fluorescence imaging does not permit differentiating AMPARs expressed on horizontal cells, which terminate near the ribbon release sites in invaginations, from AMPARs expressed by OFF bipolar terminals, whose dendrites in squirrel are semi-invaginating (DeVries et al., 2006). Previous studies in mouse showed apparent AMPAR expression in basal (noninvaginating) contacts on both rod and cone photoreceptors (Hack et al., 1999, 2001), but our functional measurements offer no support for a role of AMPARs at these conventional sites of OFF bipolar cell dendrites. AMPARs and KARs appear to form layers beneath the cone pedicle (Haverkamp et al., 2000), but some of these layers may include nonsynaptic receptors, as described above. Importantly, several studies also demonstrated AMPAR expression on ON bipolar cell dendrites (including rod bipolar cell dendrites), but there are no light-evoked AMPAR-mediated currents in ON bipolar cells (Hughes et al., 1992; Morigiwa and Vardi, 1999; Hack et al., 2001).

We propose an alternative model for parallel OFF pathways in the retina, where both transient and sustained bipolar cells use kainate, but not AMPA, receptors. How can KARs, with their slow recovery from desensitization, function in the presence of tonic synaptic release from the photoreceptors, and how can they mediate transient responses and fast temporal processing? Cones typically rest near −35 mV and have ongoing release of ~20 – 40 vesicles synapse−1 second−1 (Ashmore and Copenhagen, 1983; DeVries et al., 2006; Jackman et al., 2009). Thus, the time constant of the KARs’ recovery from desensitization (~0.5–2 s; DeVries and Schwartz, 1999; DeVries, 2000; Straub et al., 2011) is approximately an order of magnitude longer than the average interval between quantal events (~25–50 ms). KARs would therefore operate primarily in a desensitized state, with only a fraction of channels capable of mediating current for a given quantal event at each synapse. Indeed, this explains the relatively small, tonic currents measured in KAR-expressing OFF bipolar cells when a presynaptic cone is clamped at –35 mV (DeVries and Schwartz, 1999; DeVries et al., 2006). However, the combined current from all 5–10 presynaptic cones (Wässle et al., 2009) (order of 10–100 pA) is sufficient to modulate the OFF bipolar cell’s membrane voltage throughout the physiological range (DeVries and Schwartz, 1999). Thus, under physiological levels of cone depolarization, an OFF bipolar cell’s KARs would faithfully report the presynaptic glutamate release rate, with relatively stable quantal response amplitudes at each synapse.

How do OFF bipolar cell KARs encode high temporal frequencies in the presence of persistent desensitization? The limitation on temporal resolution depends not on the time constant for recovery of the desensitized receptors, but rather on the time constant of receptor deactivation for the nondesensitized receptors, estimated to be 15–20 ms (DeVries and Schwartz, 1999; Straub et al., 2011). This allows encoding most of the cone-mediated operating range, in which mouse extends to ~30 Hz (i.e., 33 ms cycle−1; Wang et al., 2011). A glutamate receptor’s recovery from desensitization would play a substantial role in signaling only for stimuli strong enough, and for frequencies low enough, to suppress glutamate substantially for a prolonged period of time. As an example, AMPARs on the ground squirrel’s b2 bipolar cell at bright light levels would apparently recover from desensitization during stimulation at a temporal frequency that suppresses glutamate release for >100 ms (DeVries, 2000).

For mouse and other mammals that lack AMPARs at the cone—OFF bipolar cell synapse, the division between transient and sustained signaling must depend on alternative mechanisms, and our data support this: block of iGluRs apparently removed inhibitory feedback, and the lack of amacrine signaling caused sustained OFF release to become transient (Figs. 1D, 2A). Additional shaping of release kinetics could be implemented at the bipolar cell’s ribbon synapse; diversity between bipolar pathways would then follow from differences in resting potential and accompanying differences in vesicle depletion (Jarsky et al., 2011; Ke et al., 2014). It remains to be determined whether responses of the fastest OFF bipolar cell types in mouse are aided by selective expression of KARs with fast kinetics through specific receptor subunit combinations, splice isoforms, and the inclusion or exclusion of auxiliary proteins (Contractor et al., 2003; Straub et al., 2011; Tang et al., 2011; Copits and Swanson, 2012; Tomita and Castillo, 2012).

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