Figures and figure supplements

Endocannabinoid signaling enhances visual responses through modulation of intracellular chloride levels in retinal ganglion cells

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**Figure 1.** Immunolocalization of CB1R in the Xenopus laevis tadpole eye. (a) Cartoon of tadpole retinotectal system. (b) DAPI (gray) and CB1R-IR (red) co-labeling of a retinal cryosection. Dashed lines highlight ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL) and outer nuclear layer (ONL) regions. (c) DAPI (blue), GFP-expressing retinal ganglion cells (green, Isl2b:EGFP transgenic) and CB1R-IR (red) in histological section of the retina. (d) Cell culture of dissociated cells from retina of isl2b:GFP animals. DAPI (blue), GFP (green) and CB1R-IR (red). Scale bar = 100 μm in b and 25 μm in c,d.

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Figure 1—figure supplement 1. Cells of the Xenopus retina. (a) Schematic representation of the retinal circuitry with different layers (ONL: outer nuclear layer, OPL: outer plexiform layer, INL: inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer) and cell types (p: photoreceptor, h: horizontal cell, b: bipolar cell, a: amacrine cell, rgc: retinal ganglion cell), (b) Panels showing GFP-expressing horizontal, bipolar, amacrine and retinal ganglion cells (EGFP sparse electroporation) together with CB1R-IR (red), and DAPI (grey) in confocal histological sections of the retina.

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Figure 2. WIN 55,212-2 enhances visually evoked firing of RGCs without impacting activity in the outer retina. (a) Isolated eye preparation for extracellular retinal recordings. (b) Representative extracellular multi-unit RGC spike recordings in the isolated eye preparation in response (red) to 500 ms full-field light-ON (top) and light-OFF (bottom) stimuli, before and after application of the CB1R agonist WIN 55,212-2. (c) WIN 55,212-2 application increases RGC spiking rates to stimuli (n = 10 animals). (d) A different CB1R agonist ACEA also increases evoked firing to light-OFF stimuli (n = 10 animals, p=0.42 for light-ON). (e) Elevating endogenous AEA by application of URB597 also enhances RGC responsiveness (n = 10 animals). (f) ERGs were used to measure outer retina responses to 500 ms light-ON (top) and light-OFF (bottom) flashes before (control) and after WIN 55,212-2 application. No change was observed. (g) Peak amplitudes for each component of the ERG in response to light-ON (n = 6 animals) and light-OFF (n = 6 animals) stimulation were not significantly affected by application of WIN 55,212-2. *p<0.05, **p<0.01, two-way RM ANOVA with Holm-Sidak posttest.

ACEA, Arachidonyl-2’-chloroethylamide; AEA, anandamide; ERG, Electroretinogram; RGC, Retinal ganglion cell.

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**Figure 2—figure supplement 1.** Individual experiments for Figure 2c–e, and ERG components. (a–f) Individual raw spike rates from RGC recordings in response to (a–c) light-ON and (d–f) light-OFF stimulation (500 ms) during application of WIN 55,212-2 (1 μM), ACEA (1 μM) or URB597 (2 μM). For each experimental condition n = 10 animals, *p<0.01, **p<0.05, two-way RM ANOVA with Holm-Sidak post-test. (g) Blockade of metabotropic glutamate receptors with AP4 during ERG recording eliminates ON-center bipolar activation (b-wave). Representative ERG traces to 500 ms light-ON flashes before (top) and after (bottom) AP4 application. ACEA, Arachidonyl-2’-chloroethylamide; ERG, Electroretinogram; RGC, Retinal ganglion cell.

*Figure 2—figure supplement 1 continued on next page*
Figure 3. WIN 55,212-2 reduces some synaptic input strength onto RGCs. (a–i) In vivo imaging of GCaMP6s expressed in the retina was used to measure Ca$^{2+}$ influx at synaptic terminals of bipolar and amacrine cells. (a) Schematic representation of the imaging configuration and retinal circuitry showing bipolar and amacrine cell type locations. (b) GCaMP6s expressed in bipolar cells, with ROIs exemplifying ON- and OFF-specific bipolar axon terminals. (c) Average calcium responses of individual bipolar axon terminal to 10 light-ON (top) or 10 light-OFF (bottom) flashes before (black) and after (red) WIN 55,212-2 perfusion. (d) $\Delta F/F$ peak amplitudes in bipolar axon terminals ($n = 10$ terminals from four animals) to light-ON before and after WIN 55,212-2 perfusion ($p=0.17$). (e) A significant change in $\Delta F/F$ peak amplitude of bipolar axon terminals ($n = 10$ terminals from four animals) was observed to light-OFF following WIN 55,212-2 perfusion. (f) GCaMP6s expressed in amacrine cells, with ROI exemplifying ON- and OFF-specific terminals. (g) Average calcium responses of amacrine cell terminals to 10 light-ON (top) or 10 light-OFF (bottom) flashes before (black) and after (red) WIN 55,212-2 perfusion. (h) No change ($p=0.36$) in $\Delta F/F$ peak amplitude was observed in amacrine cell terminals ($n = 10$) to light-ON before and after WIN 55,212-2 infusion. (i) $\Delta F/F$ peak amplitude in amacrine cell terminals ($n = 10$) to light-OFF stimulation was also unchanged ($p=0.48$) by WIN 55,212-2 perfusion. (j–p) Voltage-clamp recordings of RGCs in vivo ($n = 7$) during light stimulation. (j) Schematic representation of whole cell RGCs recording configuration with light stimulation driven by red LED flashes conveyed to the eye through an optic fiber. (k) Left: Trace of IPSCs from RGC held at $-65$ mV, evoked in response to a 3 s light flash. Right: inset of the raw trace showing fast inward currents in response to light. (l) Left: Trace of EPSCs from RGC held at 0 mV, evoked in response to a 3 s light flash. Right: inset of the raw trace showing fast outward currents in response to light. (m–n) Average total integrated inward current responses to light-ON (m) or light-OFF (n) flashes before (black) and after (red) WIN 55,212-2 perfusion with RGCs held at $-65$ mV. $N = 10$, $p=0.025$ for simple effect of WIN application by two-way ANOVA. (o–p) Average total integrated outward current responses to light-ON (o) or light-OFF (p) flashes before (black) and after (red) WIN 55,212-2 addition with RGCs held at 0 mV. $N = 10$, $p<0.05$. **$p<0.01$, two-way RM ANOVA with Holm-Sidak post-test. EPSC, Excitatory postsynaptic current; IPSC, Inhibitory postsynaptic current; RGC, Retinal ganglion cell; ROI, Regions of interest. DOI: 10.7554/eLife.15932.006
CB1R-mediated increase in RGC responsiveness requires GlyRs and NKCC1 inhibition by AMPK. (a–h) Extracellular multi-unit spike rates in RGCs were evoked with a 500 ms light-OFF stimulus. After establishing baseline response rates (Before), various pharmacological agents were washed on sequentially. (a–c) The CB1R inverse agonist AM-251 prevented (a) WIN 55,212-2-, (b) ACEA- and (c) URB597-mediated increases in spiking rates (n = 10 animals for each group), confirming the requirement for CB1R activation. (d) The GABA-A receptor blocker gabazine did not prevent the enhancement of RGC excitability, but the selective GlyR antagonist strychnine completely abolished the enhancement when applied either (e) before or (f) after WIN 55,212-2. (g) The NKCC1 blocker bumetanide mimicked and occluded the effects of WIN 55,212-2, and (h) no additional effect was observed if bumetanide was applied after WIN 55,212-2 (n = 10 animals for d–g, n = 9 animals for h). (i) RT-PCR confirmed expression of the cation-chloride cotransporters NKCC1 and KCC2 in the Xenopus retina at stage 45. (j) Western blots show a 60 kD band staining for phospho-SPAK/phospho-OSR1, and β-tubulin, in controls or following treatment with WIN-55,212-2 (1 µM) in the Xenopus retina at stage 45. (k) Bar graph showing fold-change in phosphoprotein levels following treatment with WIN-55,212-2 (1 µM) of NKCC1-phospho Thr212+Thr217, and phospho-SPAK/phospho-OSR1 normalized to control levels (n = 4). (l) In the presence of dorsomorphin (10 µM) to block AMPK, WIN-55,212-2 (1 µM) treatment no longer enhances, but instead reduces RGC firing rates (n = 10 animals). (a–h) *p<0.05 one-tailed RM ANOVA with Holm-Sidak posttest, (l) **p<0.01, paired t-test. RGC, Retinal ganglion cell.

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Figure 4—figure supplement 1. Similar trend of pharmacological agents on light-ON responses to that observed for light-OFF stimuli. Normalized spike rates of RGCs in response to light-ON stimuli before and after application of the CB1R inverse agonist AM-251, followed by the application of (a) WIN 55,212-2, or (b) URB597. Normalized spike rates of RGCs in response to light-ON stimuli, before and after the application of (c) the GABA-A receptor antagonist gabazine. (d) the GlyR antagonist strychnine, followed by the application of WIN 55,212. (e) WIN 55,212-2, followed by the application of strychnine, (f) the NKCC1 blocker bumetanide, followed by the application of WIN 55,212-2 or (g) WIN 55,212-2, followed by the application of bumetanide. (h) Summary of fold differences in extracellular RGC spike rates in response to light-ON and light-OFF stimuli before and after WIN 55,212-2 application for the various inhibitors used in a–g. (n = 10 animals for each condition). (i) Western blots of stage 45 retina showing a 130 kD band stained for phospho-NKCC1 (left) and total NKCC1 (right), in control condition (top) or following treatment with WIN-55,212-2 (1 µM) (bottom). *p<0.05, one-tailed, two-way RM ANOVA. Holm-Sidak tests were used to correct for multiple comparisons. RGC, Retinal ganglion cell. DOI: 10.7554/eLife.15932.008
Figure 5. WIN 55,212-2 decreases intracellular Cl\(^{-}\) in RGCs. (a) Cerulean(CFP)-Topaz(YFP) modified version of the FRET-based Cl\(^{-}\) indicator clomeleon provides a ratiometric readout of changes in intracellular Cl\(^{-}\) concentration due to the quenching of YFP fluorescence by Cl\(^{-}\). (b) Example time series of in vivo clomeleon-expressing retinal cells before (top) and after (bottom) WIN 55,212-2 application. Only cells with a stable baseline CFP/YFP ratio (<10% drift) were analyzed. (c) Somatic measurements of CFP/YFP from the cells in panel b, reveal an RGC-specific change in the CFP/YFP fluorescence...
Figure 5 continued

ratio in response to WIN 55,212-2. (d) Measurements of WIN 55,212-2-induced effects on CFP/YFP fluorescence ratios in all 15 RGCs imaged in seven tadpoles. (e) Mean CFP/YFP ratios in RGCs (n = 15), amacrine cells (n = 16), bipolar cells (n = 12) and Muller glia (n = 8) comparing the 10 min before (black) and the 10 min after (white) WIN 55,212-2 application. Only RGCs exhibit a change in CFP/YFP ratio. (f) CFP/YFP ratios of RGCs before and after WIN 55,212-2 application using a control Cl⁻-insensitive M69Q mutant of clomeleon (n = 5). Pre-application of the NKCC1 blocker bumetanide (n = 8), or AM-251 (n = 9) prevents the changes in RGC Cl⁻ levels. (g) With synaptic activity blocked using a cocktail containing DNQX, AP-4, picrotoxin, strychnine, and TTX, the ratio of CFP/YFP fluorescence in RGCs (n = 14), but not amacrine cells (n = 15), still is decreased in response to WIN 55,212-2 application ***p<0.005, two-way RM ANOVA with Holm-Sidak posttest. RGC, Retinal ganglion cell.
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Figure 6. CB1R activation hyperpolarizes RGCs by shifting the Cl⁻ equilibrium potential. (a) Schematic of the in vivo whole cell recording set-up to target RGCs for measurement of $E_{Gly}$. (b) Visualized patch clamping of RGC in the eye and (c) confirmatory loading with Alexa Fluor 488 hydrazide. (d) The axon terminal in the tectum of successfully loaded RGC provides confirmation of RGC identity. (e) Following WIN 55,212-2 application, a hyperpolarizing shift in the reversal potential of glycine receptor currents ($E_{Gly}$) is revealed as a leftward shift in I-V curves of peak currents evoked by puffing glycine ($AV = -3.23 \pm 0.84 \text{ mV}, n = 7, p < 0.01, \text{two-tailed paired t-test}$). (f) Bumetanide mimics the shift of $E_{Gly}$ caused by CB1R activation ($AV = -1.60 \pm 0.46 \text{ mV}, n = 5, p < 0.05, \text{two-tailed paired t-test}$). (g) GFP-expressing RGC in dissociated retinal cultures from Isl2b:GFP transgenic tadpoles. (h) Resting membrane potential of RGCs, recorded with gramicidin-perforated patch in dissociated retinal cultures shifted to more hyperpolarized after...
Figure 6 continued

WIN 55,212-2 application (n = 6 cells). (i) AM-251 prevented the shift (n = 6 cells; p=0.14, one-way RM-ANOVA). Membrane voltages were not corrected for the liquid junction potential, estimated to be 11.9 mV. (j) RGC spiking was evoked by injecting a 200 pA test pulse in current clamp mode 25 ms following pre-pulses ranging from −100 to 80 pA. (k) Plot of spike number evoked by the test pulse, as a function of pre-pulse current injected (n = 8 cells) confirms that initial hyperpolarization can result in a greater number of spikes generated in response to identical current injections. Insets are examples of spiking following hyperpolarizing (left) and depolarizing (right) pre-pulses. **p < 0.01, paired t-test. RGC, Retinal ganglion cell.

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Figure 7. Conditioning visual stimulation in peripheral subfields increases RGC spike rates and decreases [Cl\(^-\)], by a CB1R-dependent mechanism. (a) Isolated eye preparation for extracellular retinal recordings, and light stimulation with a changing surround luminance. (b) Conditioning visual stimulation protocol displayed on screen. During the initial test period (100 s), 10 light-OFF stimuli (1 s every 10 s) were presented in the central field with a constant dark periphery (Before). This continued during the conditioning period except that periphery visual fields were illuminated continuously for 100 s (conditioning stimulation). After conditioning another set of 10 test stimuli identical to the first set was presented (After). Representative extracellular multiunit RGC spike recordings are shown, in vehicle (Control) and AM-251 (5 μM) containing external solutions. (c, d) Binned PSTHs of recorded spikes from the examples illustrated in (b) show that the average response was strongly enhanced after conditioning stimulation in vehicle (c) but not with AM-251 treatment (d). (e) Group data showing the increase in spiking rates of RGCs induced by conditioning stimulation (n = 10 animals). (f) This effect is prevented when CB1Rs are blocked with AM-251. (n = 10 animals). (g–k) A similar conditioning protocol reduces intracellular Cl\(^-\) levels selectively in RGCs. Clomeleon signal was imaged in vivo under the two-photon microscope, and the objective was used to project an eyepiece-mounted OLED video display directly onto the imaged region of the retina. (h) Example of in vivo clomeleon-expressing retinal cells, and schematic of the visual conditioning protocol (CP). (i, j) The decrease in RGC (n = 12 cells) somatic ratios of CFP/YFP after conditioning stimulation (the first time point of the second imaging period compared to the mean of the 5 ratio values during the first imaging period) was blocked in AM-251 (n = 17). (k) Only RGCs (n = 22), but not amacrine cells (n = 19) or bipolar cells (n = 10) exhibited this decrease in intracellular Cl\(^-\) after conditioning stimulation. (e, f) *p<0.05, paired t-test, (k) **p<0.01, two-way RM ANOVA with Holm-Sidak posttest. RGC, Retinal ganglion cell.

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Figure 8. CB1R activation increases retinal inputs onto tectal neurons, expanding their input RFs, and enhances performance in a visual detection task.

(a) Schematic of in vivo amphotericin-perforated patch recordings of tectal neurons with extracellular electrical stimulation in the retina. (b) Peak  

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retinotectal EPSC amplitudes show an increase induced during perfusion of WIN 55,212-2 onto the tadpole (red line) in a representative experiment. Inset illustrates recording traces before (black) and after (red) drug. (c) The increase in retinotectal EPSC amplitude by WIN 55,212-2 (n = 11) was blocked by AM-251 (n = 5). Elevating endogenous AEA using URB597 (n = 7) similarly enhanced EPSC amplitudes. (d) An isolated brain preparation (n = 7) in which the eyes are not present and retinotectal axons are stimulated directly at the chiasm did not show EPSC enhancement following WIN 55,212-2 application, indicating a retinal site of action. (e) Tectal RF mapping was performed by projecting visual stimuli from a small OLED video monitor onto the retina through the microscope objective while recording from tectal neurons. (f) Example color coded RF maps of integrated postsynaptic charge evoked by OFF stimuli presented in a 7 × 7 grid of visual fields before and after WIN 55,212-2 application reveal an expansion of the RF. (g) Sample traces of the compound synaptic currents used to generate f. (h) Total postsynaptic charge evoked by stimulating at each field of the grid, and the mean sizes of tectal cell RFs both increased in response to WIN 55,212-2 application (n = 5 animals). (i–n) Visually guided escape behavior analysis (l) Avoidance response of a free swimming tadpole to a dark moving dot. (l) False-positive rate was measured during a 3 min pre-TEST where dots possess the same luminance as the background (‘invisible dots’); this was followed by a 5 min TEST period with dots darker than the background. (k) WIN 55,212-2-treated tadpoles swam at the same speed as control tadpoles prior to encountering dots, but swam away faster following an encounter. (l) The probabilities of eliciting avoidance reactions to dots presented at a range of contrasts on a bright background (luminance: 190 cd m⁻²) did not differ between control (n = 24) and WIN 55,212-2-treated (n = 24) tadpoles. (m) Dark dots moving on a dim background (luminance: 0.65 cd m⁻²) led to avoidance reactions in WIN 55,212-2-treated (n = 24), but not control (n = 24), tadpoles. (n) URB597-treated tadpoles (n = 24) also reacted with higher rates than matched control animals (n = 24). This was prevented by blocking CB1Rs with AM-251 (n = 24). Reaction rates represent the average fraction of encounters with dots per tadpole that resulted in a change in swimming velocity (>24 mm s⁻¹) in l, m and n horizontal dashed lines represent mean false-positive reaction rates measured pre-TEST using ‘invisible dots’. (c,d,k) *p<0.05, **p<0.01, two-way RM ANOVA with Holm-Sidak postest. (h) *p<0.05, one-sample t-test vs. pre-drug baseline. (l–n) †††p<0.001 two-way ANOVA with Holm-Sidak post-hoc tests comparing control and drug-treated groups, ***p<0.001; **p<0.01, *p<0.05 compared to pre-TEST false-positive rates. AEA, anandamide; EPSC, Excitatory postsynaptic current; RF, Receptive field.

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Figure 8—figure supplement 1. Effects of CB1R activation on tectal neurons. (a) EPSC amplitudes did not increase after application of either N-arachidonyl maleimide (NAM) (150 nM, n = 7) or JZL184 (100 nM, n = 7), two selective inhibitors of monoacylglycerol lipase (MAGL), the 2-AG degrading enzyme, suggesting AEA rather than 2-AG is the endogenous ligand for this effect. (b) EPSC amplitude increase induced by the CB1R agonist WIN 55,212-2 (n = 11) is blocked by the inverse agonist AM-251 (n = 7). It is not blocked by the GABAAR antagonist gabazine (n = 10), but is blocked by the GlyR antagonist strychnine (n = 6), Application of the NKCC1 inhibitor bumetanide (n = 5) also prevents the increase. (c,d) CB1R-IR is present in cell somata and retinorecipient neuropil of the optic tectum. DAPI (left) and CB1R-IR (right). Dashed lines delineate cell body layer and neuropil regions. (e) Holding current and (f) input resistance were not significantly different before (black) and after (grey) WIN 55,212-2 application. (g) WIN 55,212-2 (n = 6) or URB597 (n = 7) application did not significantly change paired-pulse ratios but (h) did induce an increase in CV/Co (n = 14, WIN 55,212-2, p<0.01, one-sample t-test). A similar trend was observed in response to URB597 (n = 7) application. Because CV/Co reflects the product of the number of release sites and the probability of release, a change in CV/Co in the absence of any change in PPR is more consistent with an increase in the number of evoked release sites or recruitment of additional inputs than with a change in P, at individual synapses (Shirke and Malinow, 1997).

*p<0.05; **p<0.001 two-way RM ANOVA with Holm-Sidak posttest. AEA, anandamide; EPSC, Excitatory postsynaptic current.

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