Imaging an optogenetic pH sensor reveals that protons mediate lateral inhibition in the retina

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The reciprocal synapse between photoreceptors and horizontal cells underlies lateral inhibition and establishes the antagonistic center-surround receptive fields of retinal neurons to enhance visual contrast. Despite decades of study, the signal mediating the negative feedback from horizontal cells to cones has remained under debate because the small, invaginated synaptic cleft has precluded measurement. Using zebrafish retinas, we show that light elicits a change in synaptic proton concentration with the correct magnitude, kinetics and spatial dependence to account for lateral inhibition. Light, which hyperpolarizes horizontal cells, causes synaptic alkalinization, whereas activating an exogenously expressed ligand-gated Na+ channel, which depolarizes horizontal cells, causes synaptic acidification. Whereas acidification was prevented by blocking a proton pump, re-alkalinization was prevented by blocking proton-permeant ion channels, suggesting that distinct mechanisms underlie proton efflux and influx. These findings reveal that protons mediate lateral inhibition in the retina, raising the possibility that protons are unrecognized retrograde messengers elsewhere in the nervous system.

Lateral inhibition is a key neural network phenomenon that enhances contrast sensitivity in nearly every sensory system. As the first laterally projecting neuron in the retina, horizontal cells initiate lateral inhibition in the visual system, but the synaptic mechanism involved in this process is still unclear. We know that photoreceptors continuously release the neurotransmitter glutamate in darkness to depolarize horizontal cells. Horizontal cells, in turn, transmit a negative feedback signal that inhibits activation of voltage-gated Ca2+ channels in the photoreceptor terminals, thereby reducing Ca2+-dependent glutamate release. However, the identity of the negative feedback signal has remained uncertain.

For many years, the inhibitory neurotransmitter GABA was thought to mediate negative feedback. Early studies showed that horizontal cells release GABA after depolarization and that cones possess GABA receptors. However, later studies showed that a wide variety of GABA receptor antagonists fail to alter negative feedback or lateral inhibition. Moreover, there is no evidence that the concentration of GABA in the synaptic cleft changes during illumination to account for lateral inhibition.

As an alternative, an ephaptic mechanism of negative feedback was proposed. In this scenario, depolarization of horizontal cells causes current to flow through open channels located in the tips of horizontal cell dendrites, which extend into the invaginated cone terminal. This current leads to an increase of extracellular potential, which has the same effect as intracellular hyperpolarization and is sensed by voltage-gated Ca2+ channels in the cone terminal, altering Ca2+ influx and Ca2+-dependent glutamate release. Hemichannels have been found to be concentrated in the dendritic tips of horizontal cells in fish, making them a candidate for transmitting the putative ephaptic signal from horizontal cells to cones. However, although modeling studies support the possibility of ephaptic signaling, there has been no direct experimental evidence that a local change in extracellular potential actually occurs during lateral inhibition.

Protons have also been proposed as the negative feedback transmitter. In this scenario, illumination hyperpolarizes horizontal cells, which decreases proton efflux. The resulting change in extracellular pH modulates the voltage-dependent gating of cone Ca2+ channels, with alkalization allowing the channels to open at a more negative membrane potential. The alkalization is a negative feedback effect because it increases cone release, as opposed to the direct effect of light on cones, which decreases release. Negative feedback and lateral inhibition can be blocked by adding a high concentration of exogenous pH buffer, which is consistent with the proton hypothesis. However, some of these buffers may acidify the intracellular pH and affect hemichannels, raising concerns about the mechanism of their blockade. And as is the case for the other putative signals, there has been no direct evidence demonstrating a light-dependent change in proton concentration in the synaptic cleft.

Extracellular pH can be measured with pH-sensitive microelectrodes, but they are too blunt and invasive for accurate measurements in the synaptic cleft. Measurements can be made with pH indicator dyes, but their spatial resolution is inadequate for accurate synaptic localization of the signal. Hence, to provide a reliable and accurate measure of pH in the synaptic cleft precisely, we engineered a genetically encoded pH indicator that is expressed on the plasma membrane of the cone terminal. We fused a pH-sensitive form of GFP (pHluorin) onto the extracellular side of a subunit of the cone Ca2+ channel. Hence, the pH indicator is on the same channel that normally serves as the effector of negative feedback. Using this optogenetic...
pH indicator, we observed a light-elicited change in fluorescence intensity that indicated a change in synaptic pH. Moreover, the signal had the appropriate magnitude, direction and spatial dependence to account for at least most of the negative feedback signal underlying lateral inhibition.

RESULTS
CalipHluorin: a genetically encoded reporter of synaptic pH
To detect changes in pH at the invaginating synaptic terminal of cones, we fused a supercretic pHluorin onto the N terminus of the α2δ4 subunit of the Ca2+ channel, and we named the resulting protein CalipHluorin (Fig. 1a,b). To determine whether CalipHluorin can report changes in pH in vivo, we first expressed the protein in cultured rat hippocampal neurons and measured fluorescence while superfus- ing solutions that were buffered to different pH values. Fluorescence dropped rapidly with an acidifying extracellular pH (Fig. 1c). Hence, the pH response must be attributed to CalipHluorin on the cell surface.

To confirm that the response to pH was mediated by CalipHluorin on the cell surface rather than in internal organelles, we inserted a thrombin proteolytic cleavage site between the extracellular pHluorin region and the membrane-spanning α2δ4 region. Thrombin treatment of cells expressing this construct resulted in removal of cell surface fluorescence and elimination of the response to acidification (Supplementary Fig. 1). Hence, the pH response must be attributed to CalipHluorin on the cell surface.

We then generated a transgenic zebrafish line expressing CalipHluorin in cones driven by the cone transducin-α promoter. We found that some CalipHluorin was expressed at the soma of cones (Fig. 1c–e). However, its fluorescence was concentrated particularly in puncta in cone terminals (Fig. 1c,f,g), which are known to possess clusters of voltage-gated Ca2+ channels adjacent to vesicular release sites near synaptic ribbons. We observed that the line of zebrafish that we generated in this study had CalipHluorin expression that was highest in the ultraviolet- and blue-sensitive cones (Fig. 1c,f), which can be identified by their characteristic sizes and repeating pattern in the retina.

Light induces alkalinization of the cone synaptic cleft
To optically measure changes in synaptic pH in response to light, we imaged CalipHluorin in flat-mounted retina. We obtained images with a series of 910-nm laser scans that we repeated at a constant rate (−8 Hz) throughout the experiment. Repeated two-photon scanning with infrared light results in some degree of light adaptation, but the retina can still generate robust responses to bright stimulus light. Hence, in the midst of the scan series, we stimulated photoreceptors with a flash of bright light (Fig. 2a). Comparison of the images revealed that CalipHluorin fluorescence at the cone terminals was brighter immediately after the flash and gradually returned to baseline within 0.5 s after a 0.5-s flash (Fig. 2b,c). On average, terminals exhibited a 5% increase in fluorescence intensity immediately after light stimulation (1.051 ± 0.008 (mean ± s.e.m.), n = 23; Fig. 2c). This fluorescence increase indicates that the synaptic cleft was alkalinized during the light flash, which should allow Ca2+ channels to open at more negative membrane potentials. We observed the change in fluorescence in synaptic terminals but not in the soma of cones (1.003 ± 0.003, n = 20, P = 1.7 × 10−6; Fig. 2c). Furthermore, we were able to block the fluorescence change by substituting the weak pH buffer bicarbonate with 20 mM of the strong pH buffer 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES) (0.996 ± 0.004, n = 21, P = 2.7 × 10−7; Fig. 2c) or the non-aminonosulfonate buffer Tris (1.005 ± 0.003, n = 21, P = 4 × 10−6; Supplementary Fig. 2c). Hence, light, which hyperpolarizes horizontal cells in the retina, alkalinizes the synaptic cleft selectively.

To quantify the light-elicited pH change, we calibrated the relationship between pH and fluorescence intensity. We superfused solutions with different pH values on CalipHluorin-expressing cone terminals in a retinal slice, which is a preparation that allows for faster equilibra- tion of buffer solutions than a retinal flat mount. Increasing the pH caused an increase in CalipHluorin fluorescence, and decreasing the pH caused a decrease in CalipHluorin fluorescence. From the fitted titration curve (Fig. 3b), we estimate that the pK₅ of CalipHluorin is 6.9, and the pH at cone terminals in darkness is about 7.4 (n = 4 retinas). Using this dark value as a starting point, we stepped the pH from 7.4 to 7.6 (Fig. 3c) and estimated that the alkalinization required to elicit a 5% increase in fluorescence from the pH in the dark was 0.14 ± 0.01 pH units (n = 6 retinas). Previous studies have shown that alkalinization of 0.1 pH unit hyperpolarizes the midport of voltage-dependent gating of cone Ca2+ channels by 1–3 mV (refs. 20, 21). Superimposing diffuse full-field illumination...
Figure 2 Light induces alkalinization of the cone synaptic cleft. (a) Timeline of the experimental protocol. Scans were collected at a constant interval (arrowheads). Frames that were acquired during the light flash (gray arrowheads) were confounded with the stimulation (stim.) light and were therefore excluded from the analysis routine. (b) Images of cone terminals collected before a light flash (1), immediately after the flash (2) and ~1 s after the flash (3). Fluorescence intensity (F; color coded according to the scale bar on the left) was brightest immediately after the light flash (as seen by an increase in red pixels). (c) Full-field illumination for 0.5 s caused a 5% increase in average pixel intensity at the terminals (n = 23) but not at the soma (n = 20, P = 1.7 × 10^{-6}). The increase in fluorescence was blocked by the addition of HEPES (n = 21, P = 2.7 × 10^{-2}). Data are shown as the mean ± s.e.m., and all statistical significance was calculated by two-tailed Student’s t test.

We next tested whether blocking postsynaptic AMPA receptors also prevents the CalipHluorin response. We found that the CalipHluorin response was blocked by 6,7-dinitroquinoxaline-2,3-dione (DNQX; 150 µM, 1.001 ± 0.005, n = 21, P = 2.1 × 10^{-6}) or GYKI-52466 (50 µM, 1.008 ± 0.004, n = 21, P = 1.3 × 10^{-5}), which are competitive and noncompetitive AMPA receptor antagonists, respectively (Fig. 5d and Supplementary Fig. 2d). AMPA receptors are absent from photoreceptors26,27, and therefore the CalipHluorin response is not cone autonomous. Taken together, the spatial, temporal and pharmacological properties of the CalipHluorin response are consistent with mediation by horizontal cells.

Although horizontal cells appear to be the primary source of the light-elicited CalipHluorin signal, events intrinsic to cones can also lead to changes in synaptic pH. Synaptic vesicles are acidic, and patch-clamp recordings from cones have indicated that phasic exocytosis can lower the pH near release sites, transiently inhibiting nearby cone voltage-gated Ca^{2+} channels20. We noticed that the initial relaxation rate of the light-elicited CalipHluorin signal was slightly less than that predicted from exponential decay of synaptic alkalinization (Fig. 4c, gray). This deviation might be a consequence of the transient acidification caused by vesicular protons.

To detect transient acidification at light offset, we added DNQX to block the contribution of horizontal cells to the CalipHluorin signal.
response, and we carried out line-scan analysis to improve temporal resolution. Solving the small signal from noisy fluorescence measurements required signal averaging from several retinal samples. By comparing lines of cone terminals images immediately after a light flash (0–40 ms) with those imaged 70–110 ms later, we could detect a small but significant delayed decrease in CalipHluorin fluorescence (n = 20 measurements for each time range, P = 0.009; Supplementary Fig. 3a,b), which was absent when we buffered the pH with HEPES (n = 20 measurements for each time range; Supplementary Fig. 3c). The response was transient and maximal at ~90 ms after light offset, which is slower than the transient acidification induced by abrupt depolarization of voltage-clamped cones (<10 ms)20. However, depolarization of cones after light offset was much slower than under voltage clamp, and the ensuing transient burst of vesicle release after light offset was maximal at ~100 ms27.

Depolarizing horizontal cells acidifies the cone synaptic cleft

To better understand how horizontal cells regulate synaptic cleft pH, we sought a tool that would enable us to manipulate horizontal cell voltage exclusively without affecting other retinal neurons. We generated a transgenic zebrafish line with horizontal cells expressing a receptor that is not normally found in zebrafish: the invertebrate FMRFamide (PheMetArgPhe-NH2)-gated Na+ channel (FaNaC)28. FaNaC has been used previously to depolarize genetically targeted neurotransmitter release owing to impaired vesicular glutamate loading. However, physiological and immunofluorescent studies on dissociated horizontal cells have suggested that V-ATPase is also present on the plasma membrane and can contribute to depolarization-elicited proton efflux30. We found that bafilomycin A1 (BFA1; 400 nM), a highly specific V-ATPase blocker, abolished the FMRFamide-elicited synaptic acidification in zebrafish retina (by ~91% of control; 1.02 ± 0.015, n = 5 retinas, P = 0.012; Fig. 6b, green). Washout of BFA1 restored the FMRFamide response, indicating that the V-ATPase blockade was rapidly reversible. BFA1 also reduced the light-elicited change in synaptic pH (by ~60% of control; 1.02 ± 0.02, n = 5 retinas, P = 0.001; Fig. 5d), which may be a consequence of reduced neurotransmitter release owing to impaired vesicular glutamate loading.

The invaginating tips of horizontal cell dendrites also possess connexin hemichannels10, which are proton permeable31. We found that treatment with the highly specific V-ATPase blocker, abolished the FMRFamide-elicited synaptic acidification in zebrafish retina (by ~91% of control; 0.963 ± 0.039, n = 5 retinas, P = 0.001; Fig. 5d). The pH response was transient and maximal at ~90 ms after light offset, which is slower than the transient acidification induced by abrupt depolarization of voltage-clamped cones (<10 ms)20. However, depolarization of cones after light offset was much slower than under voltage clamp, and the ensuing transient burst of vesicle release after light offset was maximal at ~100 ms27.

Hyperpolarization of horizontal cells with light causes synaptic alkalization, so we expected that depolarization of horizontal cells with FMRFamide would lead to acidification. Indeed, in fish possessing FaNaC-expressing horizontal cells, FMRFamide (10 μM) elicited a decrease in CalipHluorin fluorescence at cone terminals (0.585 ± 0.043, n = 5 retinas, P = 0.02; Fig. 6b, red), corresponding to acidification from a pH of 7.4 to a pH of about 6.8. In contrast, fish lacking FaNaC expression showed no change in pH from baseline (0.984 ± 0.015, n = 4 retinas, P = 0.88; Fig. 6b, black). The FMRFamide-elicited acidification was independent of cone neurotransmitter release, as the CalipHluorin response was unaltered by blocking voltage-gated Ca2+ channels in cones with nifedipine and blocking AMPA receptors in horizontal cells with GYKI-52466 (0.666 ± 0.034, n = 10 retinas, P = 0.30; Fig. 6c, gray). This result indicates that the FMRFamide-elicited pH change is a direct reflection of proton flux across the horizontal cell membrane without the involvement of photoreceptors.

Proton flux across the horizontal cell membrane may be mediated by proton-permeant ion channels and/or proton pumps. The vacuolar-type ATPase (V-ATPase) is a well characterized proton pump that is responsible for the acidification of synaptic vesicles and the consequent loading of neurotransmitters, including glutamate. However, physiological and immunofluorescent studies on dissociated horizontal cells have suggested that V-ATPase is also present on the plasma membrane and can contribute to depolarization-elicited proton efflux30. We found that bafilomycin A1 (BFA1; 400 nM), a highly specific V-ATPase blocker, abolished the FMRFamide-elicited synaptic acidification in zebrafish retina (by ~91% of control; 0.963 ± 0.039, n = 5 retinas, P = 0.012; Fig. 6c, green). Washout of BFA1 restored the FMRFamide response, indicating that the V-ATPase blockade was rapidly reversible. BFA1 also reduced the light-elicited change in synaptic pH (by ~60% of control; 1.02 ± 0.02, n = 5 retinas, P = 0.001; Fig. 5d), which may be a consequence of reduced neurotransmitter release owing to impaired vesicular glutamate loading.

The invaginating tips of horizontal cell dendrites also possess connexin hemichannels10, which are proton permeable31. We found that the hemichannel blocker carbenoxolone (CBX) almost completely...
Figure 6 Activation of an exogenously expressed ligand-gated Na\(^+\) channel in horizontal cells acidifies the cone synaptic cleft. (a) Double transgenic fish carrying CalipHluorin (green) in cones and FaNaC and mCherry (red) in horizontal cells. Shown are optical sections (left) and a three-dimensional reconstruction (right; width × depth × height = 60 × 60 × 55 µm\(^3\)) showing cone terminals adjacent to the horizontal cell layer. (b) FMRFamide (FMRFa) caused a decrease in CaliPfluorin fluorescence in retinas from the double transgenic fish (red, n = 5, P = 0.02) but not in the retina from the CalipHluorin-only fish (black, n = 4, P = 0.88). (c-f) The synaptic acidification mediated by activation of FaNaC in horizontal cells was significantly reduced by the V-ATPase blocker BFA1 (100 µM, green, n = 5, P = 0.012) but not by the hemichannel inhibitor MFA (100 µM, blue, n = 6 for d, n = 10 for e, P = 0.50). However, MFA significantly slowed the re-alkalinization of synaptic pH after washing out FMRFamide from the retina (d, f; blue, n = 6, P = 0.008). Adding nifedipine and GYKI-52466 did not significantly affect FMRFamide-elicted acidification (e, gray, n = 10, P = 0.30) or re-alkalinization after washing out FMRFamide (f, gray, n = 6, P = 0.24). The average decrease of CaliPfluorin fluorescence 5 min after FMRFamide is plotted in (e); the recovery of fluorescence 5 min after FMRFamide washout is plotted in (f). Scale bar, 10 µm (a). ∗P < 0.05, ∗∗P < 0.01. The data in b–d are shown as the mean ± s.e.m. The box and whisker plots in e and f represent the first three quartiles (25%, median and 75%) and values 1.5× the interquartile range below the first quartile (lower horizontal line) and above the third quartile (upper horizontal line); data points beyond the whiskers are displayed using +. Statistical significance was calculated by two-sided nonparametric Mann-Whitney U test (e, f).

block the pH response to FMRFamide by ~81% of control; 0.922 ± 0.047, n = 7 retinas, P = 0.004; data not shown). However, patch-clamp experiments on FaNaC-expressing HEK-293 cells showed that CBX directly blocked the FaNaC channel (by ~64% of control; from 4.070 ± 0.529 to 3.562 ± 0.341 nA, n = 7 pairs, P = 0.02), thereby interfering with the depolarizing trigger of the pH response. To circumvent this problem, we used meclofenamic acid (MFA; 100 µM), which is a gap junction inhibitor. MFA, like CBX, affects several other types of ion channels in addition to connexin hemichannels, but MFA has no effect on FaNaC channels (Supplementary Fig. 4) and should not interfere with the FMRFamide-elicted depolarization of horizontal cells. We found that MFA had no effect on the acidification of the synaptic cleft induced by adding FMRFamide (0.617 ± 0.033, n = 10 retinas, P = 0.50; Fig. 6d, blue). However, it substantially slowed the re-alkalinization when we washed away the FMRFamide (by ~78% of control; 0.224 ± 0.092, n = 6 retinas, P = 0.008; control: 1.019 ± 0.153, n = 5 retinas; Fig. 6d, blue). MFA greatly attenuated synaptic cleft alkalization in response to light (by ~80% of control; 1.01 ± 0.004, n = 26, P = 3.3 × 10\(^{-5}\), Fig. 5d). These results suggest that MFA selectively inhibits the mechanism that clears protons from the cleft while having no effect on the mechanism underlying proton efflux from horizontal cells.

DISCUSSION

In this study we examined the proton hypothesis of lateral inhibition by imaging an optogenetic pH indicator in the invaginating cone synapse. Previous experiments using HEPEs suggested that a change in synaptic pH is necessary for negative feedback to cones (ref. 12 contains a review of this concept). Other experiments involving artificial manipulation of pH showed that protons are sufficient for inhibiting cone Ca\(^{2+}\) channels and neurotransmitter release\(^{21}\). However, the possibility of nonspecific actions of HEPEs has left the role of protons in doubt\(^{13}\). HEPEs can acidify the cytoplasm of horizontal cells and may inhibit their connexin hemichannels, whose presence is required for at least a component of lateral inhibition\(^{13}\). However, although many aminosulfonate buffers can alter intracellular pH, lateral inhibition is blocked only by the subset of compounds that buffer pH in the physiological range (i.e., with a pK\(_a\) near 7.5)\(^{32}\). These findings are consistent with blockade being mediated by extracellular buffering per se rather than off-target chemical effects of aminosulfonate.

Meanwhile, the question of whether illumination naturally changes synaptic pH has remained unanswered. Our results indicate that illumination does indeed alter the extracellular pH, specifically in the synaptic cleft. Light causes synaptic alkalinization, which is the correct direction to mediate enhancement of cone synaptic output during lateral inhibition. Moreover, within our detection limits, the change in pH appears to be of sufficient magnitude and of appropriate kinetics to account for lateral inhibition.

Light-induced alkalinization can be eliminated with AMPA receptor antagonists, implicating postsynaptic cells as the signal generators of pH change. The spatial response properties of light-induced alkalinization point to horizontal cells specifically. Thus, alkalinization requires summated inputs from many cones and can occur on a long spatial distance from an illuminated annulus, which is consistent with the circuit properties of horizontal cells. Activation of FaNaC channels expressed exclusively in horizontal cells provides even more evidence that horizontal cells can modulate the pH of the cone synaptic cleft.

Cone photoreceptors also release protons into the synaptic cleft as vesicles fuse with the plasma membrane, but this is a cone-autonomous process that is distinct from lateral inhibition. In fact, because of the negative feedback synapse from horizontal cells to cones, illumination of the retina with an annulus results in an increase of vesicular release from cones in the unilluminated center. If the cone-autonomous process were dominant over the horizontal cell feedback process, one would expect to observe a net acidification of the synaptic clefts. However, we actually observed a net alkalinization,
indicating that the horizontal cell–mediated process is dominant, at least under our experimental conditions.

Light causes horizontal cells to hyperpolarize, which should drive protons down their electrochemical gradient into the cell, causing extracellular alkalization. Likewise, depolarization of horizontal cells, for example by FMRFamide activation of FaNaC, should lead to synaptic acidification, as we saw in the intact retina (Fig. 6b). However, pH-selective microelectrode measurements from enzymatically dissociated horizontal cells revealed the opposite phenomenon: depolarization of isolated horizontal cells can lead to extracellular alkalization. This effect must involve an active transporter that drives protons up their electrochemical gradient. The apparent discrepancy between these observations can be explained by active transporters and passive proton conductors coexisting in horizontal cells but distributed differentially across the cell. In this scenario, the horizontal cell is regionalized, with active proton transport occurring across much of the cell surface and passive proton conductors localized to the tips of horizontal cell dendrites near the cone synapse, creating microdomains of extracellular protons that are undetected by pH-sensitive microelectrodes. It is also possible that the distribution of active transporters and passive conductors was altered by the enzymatic dissociation procedure, contributing to the divergent findings.

Negative feedback from horizontal cells to cones persists indefinitely, so if protons serve as the negative feedback transmitter, their flux out of horizontal cells must also be continuous. Over time, this would alter the proton concentration gradient across the horizontal cell plasma membrane unless it was opposed by a compensatory active transport mechanism that maintained the gradient. A differential distribution of active transporters and passive conductors could maintain the gradient and establish a ‘dark current’ of protons circulating through a horizontal cell. By modulating this dark current, light-dependent hyperpolarization would change the flux of protons through open channels in invaginating synapses, thereby changing the pH of the synaptic cleft.

Our studies show that BFA1, a potent and highly specific inhibitor of V-ATPase, completely and reversibly blocks pH changes induced by changing voltage in horizontal cells in the intact retina (Fig. 6c). This finding is consistent with previous pH indicator measurements from isolated horizontal cells, which also showed immunocytochemical labeling for V-ATPase. V-ATPase is found in a wide variety of tissues, with functional roles ranging from solute reabsorption in kidney and bone to synaptic vesicle acidification and neurotransmitter loading in neurons. Our results indicate that V-ATPase is necessary for proton efflux from horizontal cells and is therefore crucial for horizontal cell feedback and lateral inhibition. V-ATPase has two voltage-sensitive paths that protons can use to traverse the membrane: the coupled transport machinery involving ATP hydrolysis and a parallel shunt that passively conducts protons down their electrochemical gradient. Flux through the shunt pathway is related directly to membrane potential, and because V-ATPase is an electrogenic pump, flux out of horizontal cells must also be continuous. Over time, this flux can alter the proton concentration gradient across the horizontal cell unless it was opposed by a compensatory flux out of horizontal cells which would alter the proton concentration gradient across the horizontal cell.

Although the V-ATPase is crucial for proton efflux and synaptic acidification, our results show that a separate mechanism that is sensitive to MFA is essential for proton influx and synaptic alkalization (Figs. 5d and 6f). MFA is an effective and reversible blocker of connexin hemichannels, which are proton permeant, highly concentrated in the dendritic tips of horizontal cells and implicated in mediating feedback using connexin-mutant zebrafish. MFA also affects several types of ion channels that are expressed in cones and in FaNaC-expressing fish is a direct signal from horizontal cells to the CaliPHiLuorin pH sensor, which is unaffected by inhibiting cone synaptic transmission (Fig. 6c, gray). Hence, although we cannot exclude contributions from other MFA-sensitive channels in horizontal cells, hemichannels most likely mediate the proton influx underlying synaptic alkalization.

The role of hemichannels in mediating proton influx and not efflux might be a consequence of the actions of intracellular protons on hemichannel gating. Depolarization leads to intracellular acidification of horizontal cells, and even a small increase in intracellular proton concentration can inhibit hemichannel opening. In this scenario, the pH-dependent closure of hemichannels would allow for accumulation of protons that are admitted into the synaptic cleft by V-ATPase during depolarization, whereas opening of the hemichannels after repolarization would allow protons to be retrieved from the synaptic cleft back into the horizontal cell. Horizontal cells express several different connexins and pannexins that can heteromultimerize to form hemichannels, so genetic removal of an individual type may not be sufficient to determine which is involved in proton uptake.

Although our results implicate protons as the primary mediator of horizontal cell feedback and lateral inhibition, other putative signals may have modulatory roles. It has been proposed that GABA serves as an autocrine neurotransmitter in horizontal cells and that activation of bicarbonate-permeant GABA_A receptors regulates extracellular pH indirectly. GABA_A receptor antagonists fail to block lateral inhibition in mammalian and nonmammalian retinas, indicating that GABA release is not necessary for mediating horizontal cell feedback. However, GABA may modulate the strength of feedback, for example, under different light adaptation conditions. Our results also do not exclude a role for ephaptic signaling. Nonselective ion channels that conduct protons, including hemichannels, may simultaneously conduct an electrical current carried by other permeant ions that are present at higher concentrations (for example Na^+, K^+ and Cl^-), thereby generating a change in extracellular voltage. In this scenario, the proton mechanism and the ephaptic mechanism would operate in parallel to regulate cone neurotransmitter release. However, at least under the light stimulation conditions that we used in this study, the change in pH appears to be sufficient to account for negative feedback.

METHODS

Methods and any associated references are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

T.-M.W. and R.H.K. conceived the project. T.-M.W. performed most of the experiments, analyzed the data and developed the software. I.C.H. made the FaNaC and the double transgenic fish lines, collected and analyzed the electrophysiological data and prepared Fig. 6a and Supplementary Figure 4. T.-M.W. and R.H.K. prepared the manuscript, and all authors edited the manuscript.

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**ONLINE METHODS**

**Solutions.** Unless indicated otherwise, the retinan was perfused continuously with bicarbonate-buffered saline solution containing (in mM) 100 NaCl, 2.5 KCl, 1 MgCl₂, 1 CaCl₂, 0.4 ascorbic acid, 20 glucose and 25 NaHCO₃, bubbled with 5% CO₂ and 95% O₂. For the pH and CaliHfluorin intensity-titration experiment, the calibration solution contained (in mM) 115 NaCl, 2.5 KCl, 1 MgCl₂, 1 CaCl₂ and 20 glucose together with 20 mM of one of the following buffers: 2-(N-morpholinio)ethanesulfonic acid (MES) for pH 5.5 and 6.5, HEPES for pH 7.5 and Tris for pH 8.5 and 9.5. The buffer used in the light-response experiments contained the same calibration solution plus 0.4 mM ascorbic acid and 20 mM HEPES (Fig. 2c) or Tris (Supplementary Fig. 2c), pH 7.3. The concentrations of the blockers used in Figure 5d were DNQX, 150 µM; GYKI-52666, 50 µM; nifedipine, 100 µM; baflomycin A1, 400 nM; meclofenamic acid, 100 µM; and carbamoyxolone, 100 µM.

**DNA constructs.** The α₁D subunit of the L-type Ca²⁺ channel was cloned from zebrafish retinal RNA using RT-PCR according to sequences in GenBank XM_691543. A tandem sequence containing the superecliptic pHluorin (cDNA kindly provided by R. Huganir, Johns Hopkins University School of Medicine), a protease recognition site and a 15-residue linker was inserted between the predicted signal peptide and the coding sequence of the α₁D subunit. The resulting construct was cloned into vector pDONR221 (Invitrogen) to produce pME-CaliHfluorin. Gateway recombination cloning (Invitrogen) with p3E-αTcP (kindly provided by S. Brockerhoff, University of Washington), pME-CaliHfluorin, and p3E-MTP-A and pDestTo12CG2 from the Tol2kit [44] was used for generating the CaliHfluorin construct for expression in zebrafish. For transfecting rat hippocampal neurons (Supplementary Fig. 1), a different plasmid was assembled from p5E-βactin2 (Tol2kit), pME-CaliHfluorin, p3E-MTP-A and pDestTo12pA2 (Tol2kit).

The invertebrate FMRFamide-gated Na⁺ channel (FaNaC) from Helix aspersa (EMBL accession number X92113) was subcloned from the vector pCMV-TnT/FaNaC [29] (kindly provided by A.R. McQuiston, Virginia Commonwealth University School of Medicine) into pDONR221 to obtain pME-FaNaC. For bicistronic expression of FaNaC with the red fluorescent protein mCherry, the coding sequence of the viral 2A peptide (GGATNFSLLQKGAVEENPGP) [35] was inserted at the 5' end of the mCherry coding sequence in p3E-McCherry[44] to produce p3E-2A-mCherry. To generate the FaNaC constructs, we recombined p5E-Cx55.5 (ref. 11) (kindly provided by M. Kamermans, Netherlands Institute for Neuroscience), pME-FaNaC, p3E-2AmCherry and pDestTo12CG2 from the Tol2kit for making transgenic fish and p5E-CMV (Tol2kit), pME-FaNaC, p3E-2AmCherry and pDestTo12pA2 (Tol2kit) for transfecting HEK-293 cells (Supplementary Fig. 4).

**Cell culture.** HEK-293 cells were cultured under standard conditions. Cells were plated on poly-l-lysine (0.1 mg ml⁻¹)-coated coverslips at a density of 13,000 cells cm⁻² for electrophysiological measurements. HEK-293 cells were transfected with the pCMV-FaNaC-2A-mCherry construct using calcium phosphate precipitation and measured 2–4 d after transfection. FaNaC-positive cells were identified by their mCherry fluorescence.

**Electrophysiology.** Patch-clamp recordings of HEK-293 cells (Supplementary Fig. 4) were performed at room temperature in voltage-clamp mode. The external bath solution contained (in mM) 138 NaCl, 1.5 KCl, 2.5 MgCl₂, 2.5 CaCl₂, 5 HEPES and 10 glucose. The pipette solution contained (in mM) 30 NaCl, 100 NaCl, 10 HEPES, 2 MgCl₂, 1 CaCl₂, 2 MgATP, 0.05 NaGTP, 0.8 ethylene glycol tetraacetic acid (EGTA) and 5 glucose. All solutions were adjusted to pH 7.4. Drugs were applied through a perfusion line, and the pipette tip was placed in close proximity to the recorded cell. Electrophysiological measurements were performed with a Patch-Clamp PC501A amplifier (Warner) and recorded using pClamp10.1 software (Molecular Devices). The pipette resistance was 3–6 MΩ. Voltage clamp recordings were low-pass filtered at 2 kHz. Data were analyzed using Clampfit 10.2 (Molecular Devices) and MATLAB (MathWorks).

**Transgenic fish.** One-cell-stage or two-cell-stage zebrafish (AB strain) embryos were microinjected with the CaliHfluorin or FaNaC DNA construct together with Tol2 mRNA for a higher germline transmission rate[44]. The transgene-positive F0 founders were selected by screening for green heart fluorescence in embryos at 2–4 days post fertilization[44] and raised at 28.5 °C in a 14 h light, 10 h dark cycle. The adult F0 fish were inbred, and their transgene-positive progeny were raised and used for imaging experiments. To generate CaliHfluorin and FaNaC double transgenic fish, the CaliHfluorin transgenic fish line was crossed with the FaNaC fish line. The genotype was verified by PCR.

**Tissue preparation.** All procedures were approved by the University of California, Berkeley Animal Care and Use Committee. After adult fish were euthanized in MS-222, the dark-adapted retinas were isolated from the eye cups with the retinal pigment epithelium removed and kept in darkness in bubbled bicarbonate-buffered saline. For flat-mount preparations, retinas were mounted onto a Biopore membrane (MILLIPORE) with photoreceptors facing the membrane[48]. For slice preparation, retinas were mounted onto nitrocellulose filter paper (pore size, 0.8 µm; MILLIPORE) with retinal ganglion cells facing the paper. Retinas were sliced to 200 µm in thickness using a tissue slicer[27]. Slices were counterstained with 2.5 µM sulforhodamine 101 for Fig. 1c. For Supplementary Figure 1, hippocampal neurons were prepared and transfected using standard protocols.

**Image acquisition.** The flat-mount retina was moved to the imaging chamber with the photoreceptor side facing the glass bottom of the chamber. We used a custom-built microscope based on a design from the Karel Svoboda lab (Janelia Farm Research Campus, Howard Hughes Medical Institute) equipped with a Ti:Sapphire pulse laser (Coherent) tuned to 910 nm. The microscope was controlled by ScanImage r3.6 software[37] with plugins developed in our lab. For light stimulation experiments, an area 30 × 30 µm² was imaged at a frame rate of 128 ms per frame and binned into 64 × 64 pixel images. The high-magnification image in Figure 1g was deconvolved using the Richardson-Lucy algorithm to enhance the sharpness of the image.

To image CaliHfluorin and mCherry fluorescence at the same time (Fig. 6a), we used a Zeiss LSM780 microscope equipped with a spectral analyzer. CaliHfluorin was excited at 488 nm, and mCherry was excited at 561 nm.

**Light stimulation.** The light sources were a LUXEON Rebel Blue light-emitting diode (LED) (PHILIPS) shortpass filtered at 460 nm and a LUXEON Rebel Amber LED longpass filtered at 550 nm. Light from the two LEDs was combined with a 505-nm longpass dichroic mirror and coupled to the projection optics with an optical fiber. The green portion of the spectrum (460–530 nm) was filtered out to protect the photomultiplier from photodamage. Except for Figure 5a (light intensity versus F/F₀), we used a light intensity of 10¹⁴–10¹⁸ photons m⁻² s⁻¹ (ref. 48) measured with a photometer at the specimen plane. A pattern mask (for example, a spot or an annulus) was placed in the projection light and projected onto the retina through the condenser. A program written in MATLAB (MathWorks) was used to command the shutter (T56R, UNIBLITZ) for controlling flash duration. Except for Figure 4a (light duration versus F/F₀), a flash duration of 508 ms was used. Light stimulation was given at ~1 s after the beginning of a scan series and was repeated three times (Supplementary Fig. 5b, black and yellow bar) in each experimental run.

For measuring the intensity versus response relation (Fig. 5a), we used a single blue LED shortpass filtered through a 460-nm filter rather than the mixed blue–amber LEDs. This enabled a more accurate estimation of the projected light intensity. The spectral sensitivity of zebrafish opsins is blue shifted[46], and thus this light should stimulate all except the long-wavelength cones. The intensity of the stimulation light was modulated by changing the brightness of the LED combined with different neutral density filters (THORLABS).

**Image analysis.** We first selected a region of interest (ROI) from the cone terminal image. The ROI was generated in ImageJ (rsweb.nih.gov/ij) using a thresholding-based technique (Supplementary Fig. 5a, red lines). We excluded from the ROI the first row of pixels (i.e., the first 2 ms of the scanned image), collected after terminating the light flash, to avoid detecting afterglow of the stimulation light. The background signal, measured at the end of the experiment in the absence of laser scanning, was subtracted from each image. To estimate photobleaching, the average pixel intensity within the ROI of each frame (termed F) was fitted with a single-exponential function y = a e⁻bx, where x is the frame number and y is the intensity (Supplementary Fig. 5b, red line). Fluorescence intensities acquired during the light flash were excluded from the photobleach fitting routine because they were confounded with the stimulus light (Fig. 2a, gray arrowheads). The CaliHfluorin response decays within six frames after a
light flash (~750 ms), so data from these time points were also excluded from the fitting routine. To compensate for photobleaching, the original intensity (I) of each frame was divided by its corresponding value in the fitted curve (y). The compensated light responses from the three lines were aligned, and the mean response was calculated and defined as n of 1 (Supplementary Fig. 5c).

For line scan analysis (Supplementary Fig. 3), because each image (30-μm square) is composed of 64 raster-scanned lines acquired at a speed of 2 ms per line, the fluorescence intensity in each line encodes the pH value of designated time points that are 2 ms apart from each other. As a result, the same line in two consecutive frames represents time points that are 128 ms apart. The photobleaching compensation and fluorescence normalization described above were applied to data grouped from each single line. The compensated and normalized fluorescence intensities from each line were assembled, which resembled the pH response at a 2-ms temporal resolution.

**Statistical analyses and curve fitting.** Statistical significance in Figure 6e,f and Supplementary Figure 4 (sample number less than ten) was determined by two-sided nonparametric Mann-Whitney U test, and significance in all other figures was determined by two-tailed Student’s t test. Data points and error bars represent the mean ± s.e.m. In Figure 3b, each data set was fitted with equation

\[ y = y_0 + y_{\text{max}} \left(1 + 10^{(pK_a - pH)}\right) \]

(ref. 14), where y is the fluorescence intensity, y_0 is the baseline, y_{\text{max}} is the magnitude of maximal response, and pK_a is the logarithm of the equilibrium constant for protonation. The data set was then normalized (i.e., \((y - y_0)/y_{\text{max}}\)) and pooled together with other sets of experiments. The combined data sets were averaged and fitted with the same equation again to yield the final fitted curve (Fig. 3b, gray).

For Figure 3c, the fluorescence intensity at pH 7.6 was normalized to the intensity at pH 7.4 (i.e., \(F_{\text{pH7.6}}/F_{\text{pH7.4}}\)) in each set of experiments. Linear interpolation of the average ratio of \(F_{\text{pH7.6}}/F_{\text{pH7.4}}\) (Fig. 3c, gray) was used to calculate the estimated pH values in the dark and light. The y axis of Figure 3c was converted to relative ΔF from the dark for clearer representation.

The decay of the CaliPHluorin signal in Figure 4c (0.5 s) was fitted with a single-exponential function, \(y = y_0 + y_{\text{max}} e^{-kx}\), where x is time after light flash, y is the magnitude of light response, y_0 is the baseline, y_{\text{max}} is the magnitude of maximal response, and k determines the speed of the decay. The first data point after the light flash, which deviated from the expected exponential decay, was excluded from the fit.

In Figure 5a, relative light responses (i.e., \(F/F_0\)) from data sets obtained at different light intensities were combined and fitted with equation \(y = y_0 + y_{\text{max}} x^{n}/(x^{n} + K^n)\) (ref. 50), where x is the light intensity, y is the magnitude of light response, y_0 is the baseline, y_{\text{max}} is the magnitude of maximal response, K is the intensity needed to elicit a half-maximal light response, and n is the slope factor.

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