

**Lateral Inhibition and Protons: The First
Level of Contrast in Stimulation of the Retina?**

BY

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THESIS

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TABLE OF ABBREVIATIONS

AC	Amacrine Cell
AMPA(R)	Alpha-amino-3-hydroxy-5methyl-4-isoxazolepropionic acid (receptor)
BC	Bipolar Cell
BCECF	2(‘,7’-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein, Acetoxymethyl Ester)
BFA1	Bafilomycin A1
CBX	Carbenoxolone
cGMP	Cyclic Guanosine 3’,5’-cyclic monophosphate
CHES	N-Cyclohexyl-2-aminoethanesulfonic acid
CNS	Central Nervous System
DAPI	(4’,6-diamidino-2-phenylindole
FMRFamide	FMRFamide (Phe-Met-Arg-Phe-NH ₂)-gate Na ⁺ channel
GABA(R)	γ-Aminobutyric acid (receptor)
HAF	5-hexadecanoylamino fluorescein
HC	Horizontal Cell
HEPES	2-[4-(2-hydroxyethyl)piperzin-1yl]ethanesulfonic acid
INL	Inner Nuclear Layer
IPL	Inner Plexiform Layer
IS/OS	Inner Segment/Outer Segment
MES	2-(N-morpholino)ethanesulfonic acid
MFA	Meclofenamic Acid
MOPS	(3-(N-morpholino)propanesulfonic acid)
ONL	Outer Nuclear Layer
OPL	Outer Plexiform Layer
PIPES	piperazine-N,N’-bis(2-ethanesulfonic acid)
PMCA	Plasma Membrane Ca ²⁺ ATPase
PR	Photoreceptor
RGC	Retinal Ganglion Cell

Tris

Tris(hydroxymethyl)aminomethane

V-ATPase

Vacuolar-type H⁺-ATPase

SUMMARY

Lateral inhibition mediated by horizontal cells in the outer plexiform layer of the retina has a large impact on perceived visual contrast. Much past work suggests that negative feedback from horizontal cells to photoreceptor synaptic terminals is important in generating this lateral inhibition, but the molecular mechanisms mediating this feedback remain unclear.

This thesis will survey work focusing on the hypothesis that proton release from horizontal cells is the key molecular player in generating negative feedback from horizontal cells to photoreceptors. According to this hypothesis, protons released from horizontal cells bind to calcium channels on photoreceptor synaptic terminals, leading to a decrease in calcium influx and a decrease in the release of neurotransmitter from photoreceptors. There is much evidence that changes in extracellular acidity significantly affect neuronal signaling in the retina, and these data have been taken as support for the proton hypothesis of lateral inhibition.

Recent experiments using optogenetic techniques to measure changes in extracellular acidity during light and chemical stimulation also strongly support the proton hypothesis. However, data measuring proton release from isolated horizontal cells using self-referencing H⁺-selective electrodes and fluorescent H⁺ sensors argue strongly against proton release from horizontal cells when stimulated. This thesis will summarize the current status of the proton hypothesis of lateral inhibition and suggest potential alternatives for the functional role that alterations in changes in extracellular acidity may play in retinal function.

I. INTRODUCTION

A. Signal Transduction in the Retina

The retina of the eye is a key point of experimentation in the CNS because of its accessibility and well-studied signal transduction mechanisms (1). The retina is a thin layer of nervous tissue located on the back of the eye that absorbs light and converts this into a neural signal that is translated by the brain into a visual image. The retina is composed of five main neural cells types that work together to create, refine, and send electrical signals to the brain: photoreceptors, horizontal cells, bipolar cells, amacrine cells, and retinal ganglion cells (Fig. 1A).

Light travels through the cornea, aqueous humor, lens, vitreous humor and other retinal cells before finally being absorbed by visual pigment molecules in photoreceptor outer segments. There are two main types of photoreceptors at the back of the eye – rod and cone photoreceptors. There are three main subtypes of cone photoreceptors in the human eye that absorb light waves in the 420 nm – 650 nm range and allow for color vision. These cells are highly concentrated in the fovea and allow for high visual acuity. Rod photoreceptors are more sensitive to light and can produce an electrically detectable response to the absorption of as little as one photon (3). This cell type is the primary source of visual information at night in part because of the high sensitivity of individual rod photoreceptors. (Fig. 1B & C).

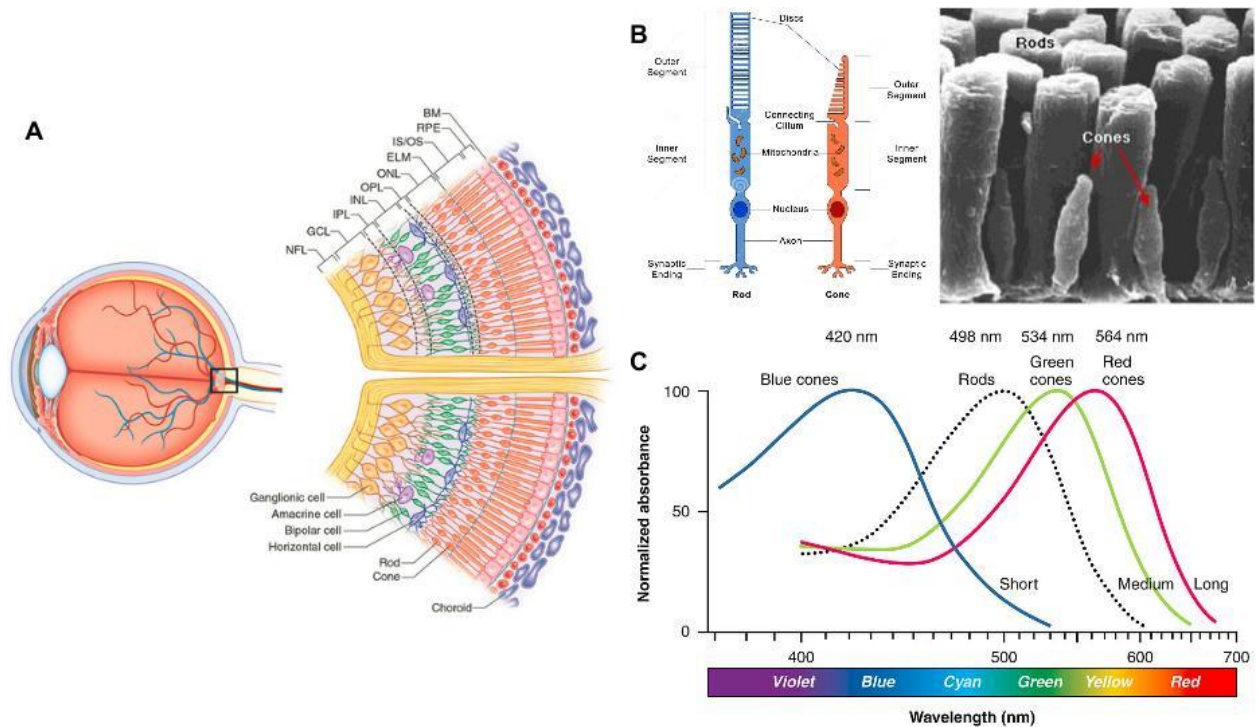


Figure 1. Retina and photoreceptor model. *A*, Retinal schematic showing cell types located in the fovea (2). *B*, Schematic showing rod and cone photoreceptors compared to electron image on the right (4) (5). *C*, Light absorbance by three different classes of cone photoreceptors and rod photoreceptors. Short cones absorb light at a peak of 420 nm. Rods absorb in the 498 nm wavelength. Medium absorb light peaking at 534 nm. Long absorb light around 564 nm (6).

Visual perception begins with the absorption of light by photoreceptive cells located in the back of the retina. In the dark, photoreceptors are depolarized (first shown by Tomita in 1965) (7) and release neurotransmitter, now believed to be glutamate. Visual phototransduction begins with a photon of light passing through the retina and hitting a photoreceptive pigment (iodopsin cones or rhodopsin in rods) within the outer segment of the photoreceptors. The photoreceptive pigment changes shape causing an activation of the G-protein transducin and in turn leading to the activation of cGMP phosphodiesterase. As cGMP is broken down, cGMP gated ion channels in the outer segment close. Positive ions no longer flow into the cell and the cell goes into a hyperpolarized state. The hyperpolarization generated in the outer segment travels passively down the cell to the synaptic terminals of the photoreceptors and ultimately causes a decrease in the release of neurotransmitter from the cells (8) (9) (10) (Fig. 2A). The first order synapse in the retina occurs in the outer plexiform layer, where the dendritic processes of horizontal and bipolar cells invaginate into the synaptic processes of the photoreceptors (11) (12) (13). Horizontal cells are depolarized by the neurotransmitter (glutamate) released from photoreceptors (14) and are believed to send negative feedback signals onto the terminals of photoreceptors when depolarized (8).

Bipolar cells are also responsive to glutamate released from photoreceptors, with some cells being depolarized and some hyperpolarized by the glutamate that is released. Changes in the voltage of bipolar cells are carried passively from dendrite to axon terminal resulting in a change in neurotransmitter release at the level of the inner plexiform layer (15). The inner plexiform layer is the location of more neural signal refinement (16). Amacrine cells have many properties similar to horizontal cells and have been postulated to also play a role in generating

negative feedback (17). Like horizontal cells, which make connections to many photoreceptors, amacrine cells have large receptive fields connecting to many bipolar cells (18). Once the signal reaches the inner plexiform layer, amacrine cells are believed to send negative feedback signals onto bipolar cells. The signals from bipolar cells are finally sent on to retinal ganglion cells which carry the signal to the brain for processing (19).

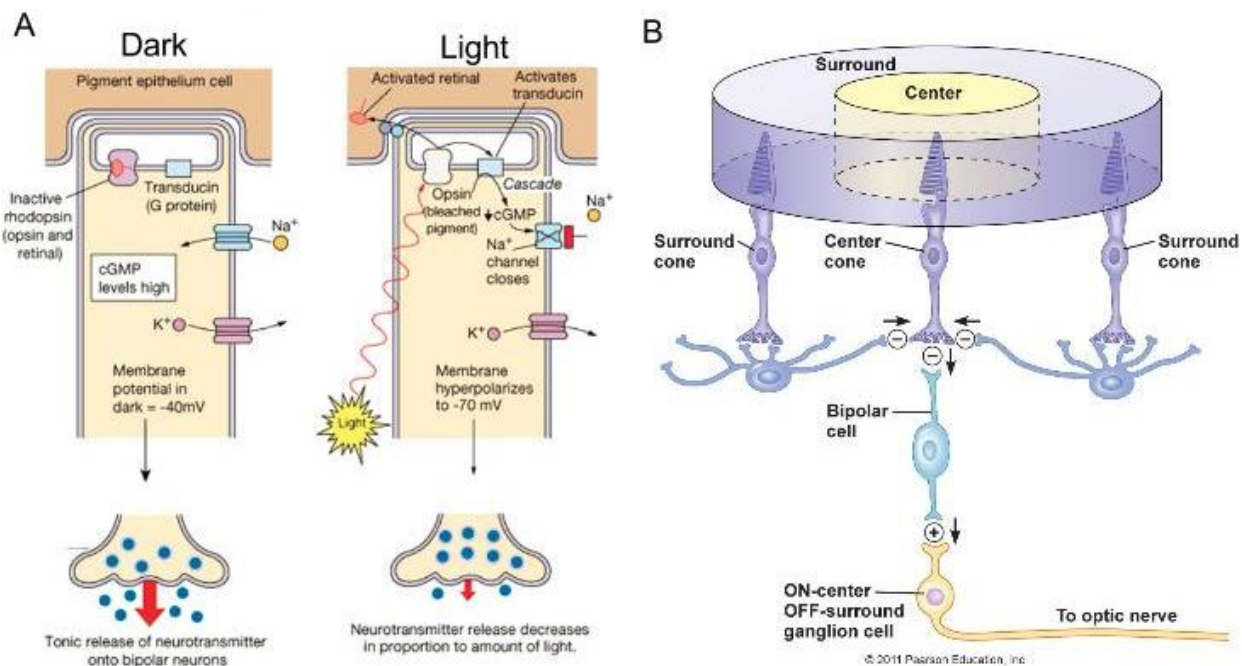


Figure 2. Phototransduction. A, Phototransduction pathway revealing the outer segment and the synaptic terminal. Light conditions are activated by a photon of light while the dark

conditions are the lack thereof (20). **B**, Center surround field schematic. Center field is under light stimulus and surround is in the dark (21).

B. Lateral Inhibition in the Retina

Bipolar cells in the retina possess what is called a center-surround receptive field organization (Fig. 2B). Small spots of light centered over what is called an On-bipolar cell will induce a depolarization, while large light annuli (rings of light with a dark center) will produce a hyperpolarization of the same cell. Photoreceptors respond well to small spots of light, but broadening the spatial extent of light of the same intensity does not significantly increase the response of a photoreceptor. Small spots of light are not very effective in stimulating horizontal cells; broadening the spatial extent of light of the same intensity produces a significantly larger response in the horizontal cells. This response characteristic has led to the hypothesis that the surround inhibition observed in bipolar cells by light annuli result from activation of the horizontal cells (8) (12) (13) (22).

Horizontal cells are interneurons that make direct synaptic connections with the photoreceptors and express AMPA receptors sensitive to glutamate released from photoreceptors (23). There is strong evidence that at least some classes of horizontal cells release the inhibitory neurotransmitter GABA (24). Originally a strong contender as the negative feedback messenger, it has since been shown that GABA is more than likely not the messenger involved in retinal feedback – not all classes of horizontal cells possess significant amounts of GABA, and the pharmacology of lateral inhibition does not appear to match GABA receptor pharmacologies (25) (26).

Lateral inhibition generated by horizontal cells is believed to play a role in enhancing visual contrast. Contrast sensitivity is the ability to distinguish between colors and brightness of

a static object in reference to other static objects (Fig. 3A). Because of the large impact that contrast has on how we see, it has been studied since the mid 1950's. Lateral inhibition is the ability of an excited neuron to decrease the signal coming from a laterally placed neighboring neuron. Lateral inhibition was discovered in 1956 on work done on the visual system of the horseshoe crab (27) for which H. Keffer Hartline was awarded the Nobel Prize in 1967. First observed in the visual system, lateral inhibition is also found in many other places and systems in the CNS, including somatosensation (28) (29).

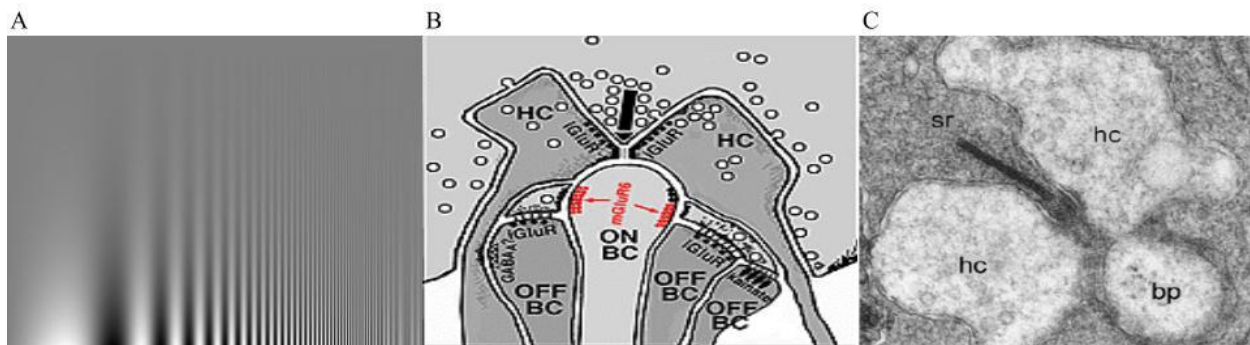


Figure 3. Contrast Inhibition. *A*, Image shows lines of varying widths and brightness. From left to right line width decreases and lines appear to become shorter. If retinal images were perceived without lateral inhibition all of the lines would appear the same height. With the effects of lateral inhibition the lines appear shorter towards the right side of the page (30) *B*, A photoreceptor synapse model showing a photoreceptor, horizontal cell, and both on and off bipolar cells. Central synaptic ribbon is the location of neurotransmitter release (31). *C*, Electron

microscopy showing the tight synaptic cleft between photoreceptor, horizontal cells and bipolar cells (32).

Although lateral inhibition has been studied for nearly five decades, the molecular mechanisms used by horizontal cells to establish the negative feedback loop are still unclear. One major challenge in examining these mechanisms is the extremely tight synaptic cleft between photoreceptors, horizontal cells and bipolar cells (Fig. 3B & C).

There are currently three major hypotheses in the field focusing on how horizontal cells may provide negative feedback onto photoreceptors (Fig. 4A-C). One, already mentioned and largely discarded, is that GABA released by horizontal cells may be the key player. A second hypothesis suggests that ephaptic pathways mediated by hemi-gap junctional proteins may alter the extracellular voltage of the tissue, affecting voltage-sensitive channels present in the photoreceptors (33). The third hypothesis, and the focus of this thesis, is the notion that horizontal cells when depolarized promote an increase in extracellular acidity, which depresses neurotransmitter release from photoreceptors. According to the proton hypothesis of lateral inhibition, glutamate released by photoreceptors activates AMPA receptors present on horizontal cells which depolarizes the horizontal cell and results in an increase in extracellular acidity in the area of the synaptic cleft. The protons released by horizontal cells bind to voltage-sensitive calcium channels present on the photoreceptor synaptic terminals and decreases calcium flux through these channels into the photoreceptors. This decrease in calcium influx results from two effects on the calcium channels – first, the maximal amount of calcium influx per unit time is decreased, and second, the voltage at which the channels are opened is shifted to more positive potentials (34). The decrease in calcium flux in the photoreceptor terminals depresses the

calcium-dependent release of glutamate from the photoreceptors, completing the hypothesized negative feedback loop.

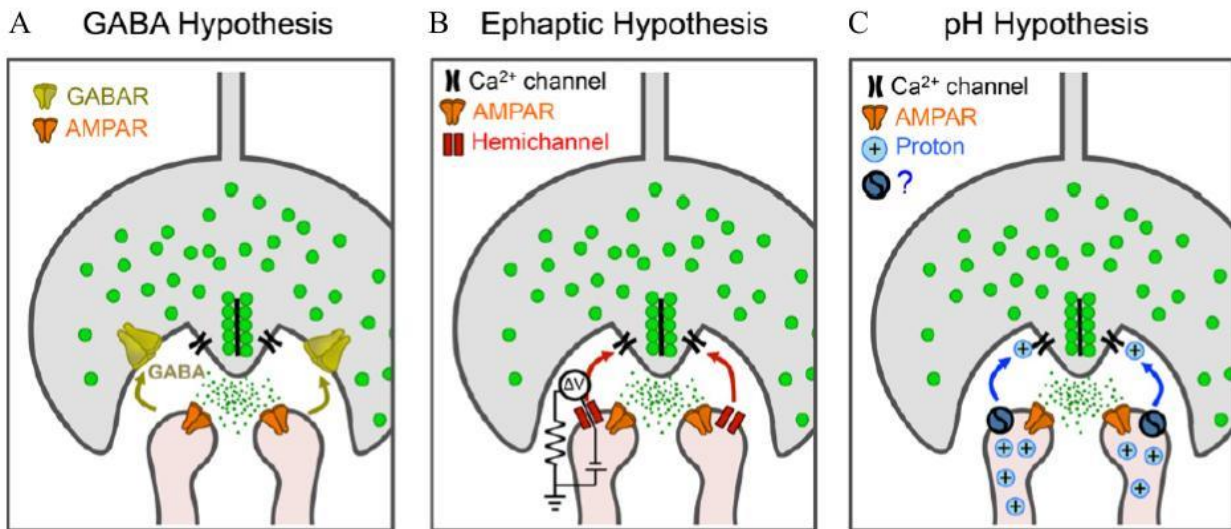


Figure 4. Negative feedback hypotheses. Photoreceptors release glutamate (large green circles) into the synaptic cleft in dark conditions. Horizontal cells feedback onto photoreceptors using GABA, ephaptic, or proton mechanisms, figure A, B, and C respectively (35).

II. EVIDENCE FOR AND AGAINST THE PROTON HYPOTHESIS OF LATERAL INHIBITION

A. Pro-proton studies

There is much evidence suggesting that small alterations in extracellular H^+ can play an important role in shaping the responses of retinal neurons in the retina. Decreasing the concentrations of protons in solutions bathing cells in the retina (that is, increasing levels of extracellular pH) increases photoreceptor calcium currents and shifts the activation of the calcium conductance to more negative voltages (34). In addition, enriching the pH buffering capacity of the extracellular solution blocks the ability of horizontal cells to induce shifts in the calcium currents in photoreceptors and alter calcium influx in photoreceptor synaptic terminals. Exogenous pH buffers test the proton hypothesis of lateral inhibition by blocking changes in pH in the external environment around the cell. A wide variety of buffers are used across experiments due to their differing pK_a values and buffering abilities: HEPES, MES, MOPS, PIPES and Tris. HEPES, MES, MOPS, and PIPES all have an aminosulfonate moiety in common but have different pK_a values. This means that each buffer has a particular range of pH over which they act effectively as pH buffers. Tris is structurally different from the amino sulfate buffers and has a pK_a close to that of the retina's physiological pH. Aminosulfates and Tris are structurally different but have similar buffering capacity and are both faster and stronger than the physiological buffer, bicarbonate.

Buffering experiments in the retina have been carried out many times in the past (36) (37) with evidence mainly in support of the proton model of feedback. The experimental tests show that horizontal cell feedback can be inhibited by high concentrations of pH buffers. If horizontal cell feedback is inhibited by a strong pH buffer then it would indicate that feedback is a product of pH change in the OPL synaptic cleft.

In one detailed examination of the effects of pH buffers on retinal function, Davenport and colleagues (38) monitored responses of horizontal cells and ganglion cells using high resistance sharp-tip electrodes. Horizontal cells were identified using DAPI staining of the horizontal cell nuclei as well as the location of the cell in the ONL. These investigators found that in normal conditions stimulating horizontal cells with a large field light stimulus caused an immediate and rapid hyperpolarization, which was followed by a slower “rollback” of the voltage to a more depolarized level (Fig. 5A). The authors argue that the rollback results from enhanced release of neurotransmitter from photoreceptors upon hyperpolarization of horizontal cells. According to the proton hypothesis for lateral inhibition, the hyperpolarization of horizontal cells by light decreases the release of protons from these cells, leading to the unblocking of voltage-sensitive calcium channels on the photoreceptors and enhanced release of glutamate from photoreceptors, and hence the depolarization rollback observed in the horizontal cell. Davenport et al. found that as the pH buffer concentration was increased, two things happened to the response of horizontal cells: the depolarizing rollback in voltage was eliminated, and the horizontal cells reached much deeper levels of hyperpolarization to the same light stimulus (Fig. 5B & C). This result occurred regardless of whether the pH buffer employed was HEPES, Tris, MOPS or PIPES, all of which are reasonably effective pH buffers at normal values of physiological pH (Fig. 5D). Addition of other pH buffers such as CHES or MES, which are similar in structure to HEPES but have differing values of pKa and hence are less effective buffers under physiological levels of pH, did not have the same potent ability of HEPES or Tris to block the signs taken for feedback inhibition. Recordings from retinal ganglion cells also revealed alterations in the surround portion of their receptive fields with varying levels of pH buffers.

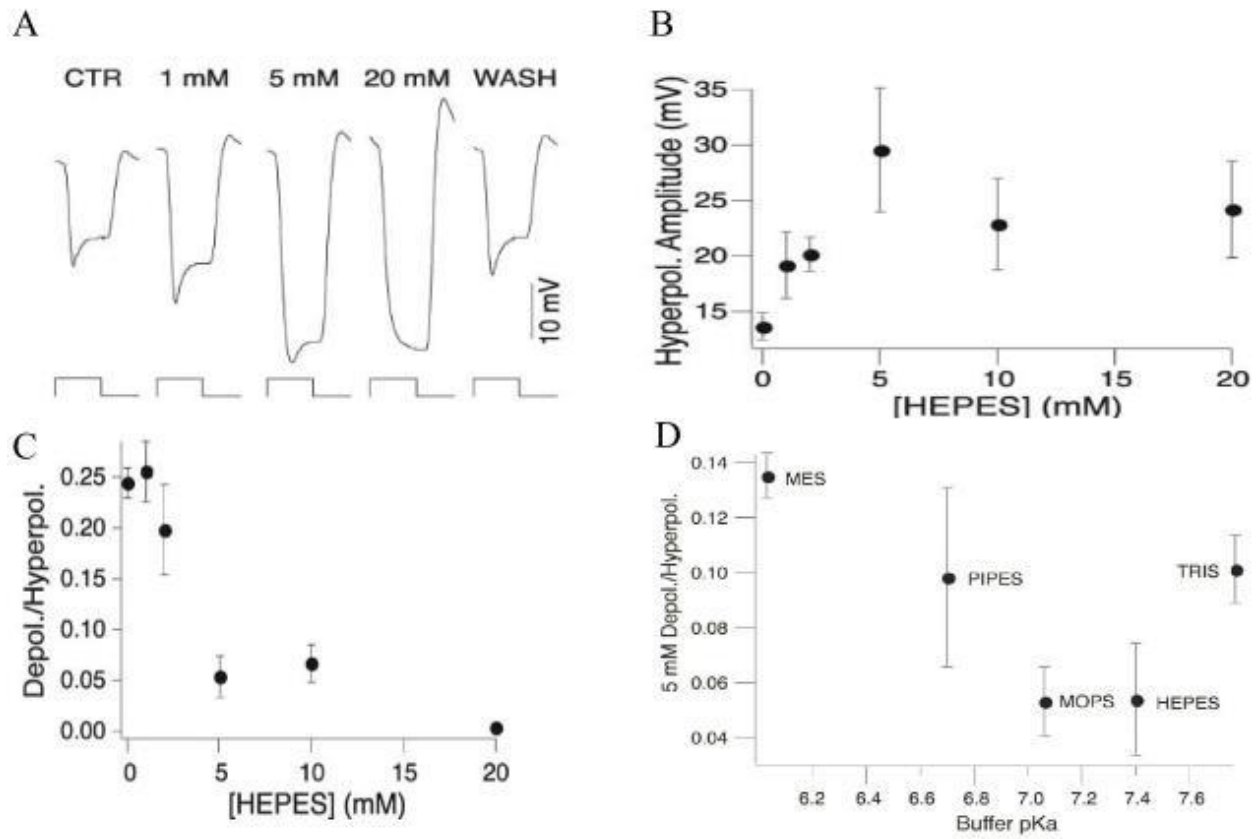


Figure 5: Horizontal cell light response and buffer capacity. *A*, Horizontal cell response to a 2000 μ m spot stimulus under increasing concentrations of HEPES. *B*, Horizontal cell hyperpolarization amplitude under increasing concentrations of HEPES. *C*, Ratio of depolarization to hyperpolarization as a function of HEPES concentration. Increased concentration of HEPES decreases horizontal cell depolarization. *D*, Ratio of depolarization to hyperpolarization. Buffers used were at a concentration of 5 mM. Buffers with pK_a values closer to physiological levels had stronger effects and thus had lower Depol./Hyperpol. Ratios. Buffers with pK_a values well outside of physiological buffers had very little to no effect (38).

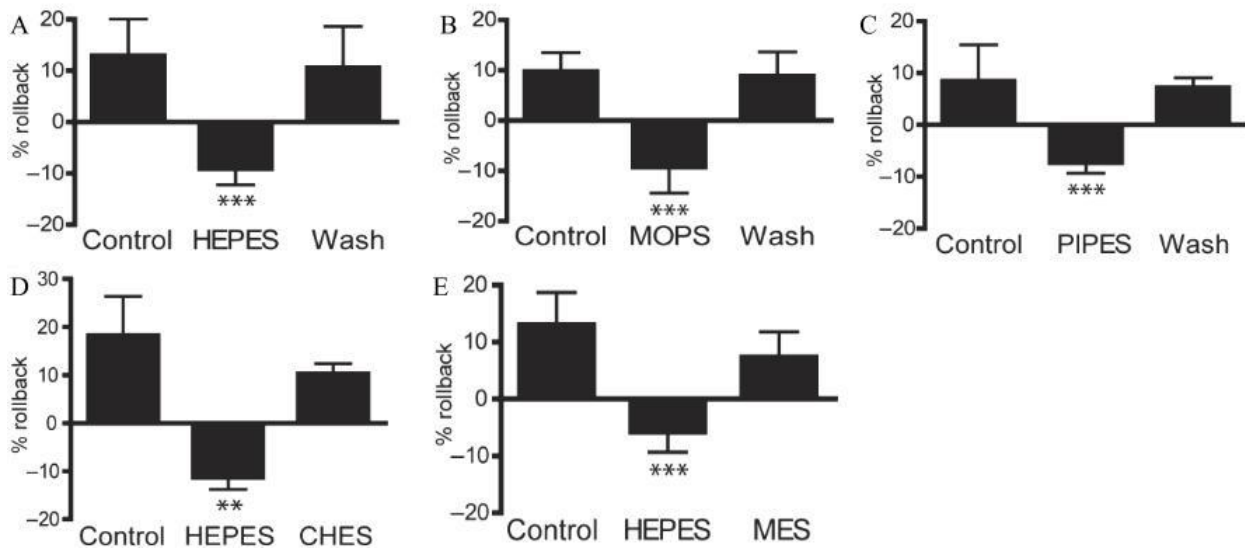


Figure 6. Buffer Capacities. Aminosulfonates with appropriate pK_a values block horizontal cell depolarization after light stimulus. Mean % rollback for each of the experimental buffers. **A**, 20 mM HEPES. **B**, 20 mM MOPS. **C**, 20 mM PIPES **D**, 20 mM CHES did not block horizontal cell depolarization (rollback) after light stimulus. **E**, 20 mM MES did not block horizontal cell rollback (39).

Work by Trenholm et al. 2010 (39) presented similar data supporting the proton model of negative feedback in the retina. In these experiments horizontal cells were identified for experimentation by their depth below the surface of the retina and by a hyperpolarization response due to light stimuli. In similar fashion to Davenport 2008, Trenholm used multiple aminosulfate buffers to test additional side effects the buffers could have on horizontal cells. One main cause of concern is that HEPES has been shown to directly affect gap junction hemichannel permeability

through acidification of the intracellular space (40). Horizontal cell gap junctions play a large role in the ephaptic model of negative feedback. To eliminate the possibility that HEPES could be affecting gap junctions as well, many different buffers were used in its place. Results were very similar to those shown above in that different aminosulfate buffers have graded effects on the feedback depolarization caused in horizontal cells. The graded effects come from the differing pK_a values of each buffer and not from non-buffering side effects. The buffers that had pK_a nearest to the pH of ringer solution had the highest effects on horizontal cell depolarization. HEPES, MOPS and PIPES all blocked horizontal cell depolarization. The further the pK_a value was away from the physiological pH the less effect the buffer would have on the horizontal cell depolarization (Fig. 6A-E).

Studies by Thoreson et al. 2006 (37) also show strong evidence for the proton model of negative feedback inhibition by recording simultaneously from photoreceptors and horizontal cells using two electrodes in retinal slice. This technique uses voltage clamp to change the membrane potential of horizontal cells while simultaneously recording changes in Ca^{2+} channel current in photoreceptors. These experiments show that depolarization of the membrane potential in a horizontal cell, in a manner similar to that seen in a graded response to glutamate depolarization, diminishes the calcium current in cone photoreceptors. The overall effect of horizontal cell voltage on the calcium current of photoreceptors is graded – over the range of -90 to 0 mV, the larger the depolarization of the horizontal cell, the greater the effect on the calcium current of the photoreceptor (Fig. 7A). In the presence of high extracellular concentrations of the pH buffer HEPES, the effect of alterations of horizontal cell potential on the Ca^{2+} current of the photoreceptors is abolished (Fig. 7B). After washing off the high HEPES buffer with a normal Ringer's solution, the Ca^{2+} current began to diminish with increased horizontal cell membrane potential (Fig. 7C). This is strong evidence showing that in the presence of high concentrations of

an extracellular buffer, Ca^{2+} current in the cone photoreceptor does not change even when horizontal cells are quite depolarized.

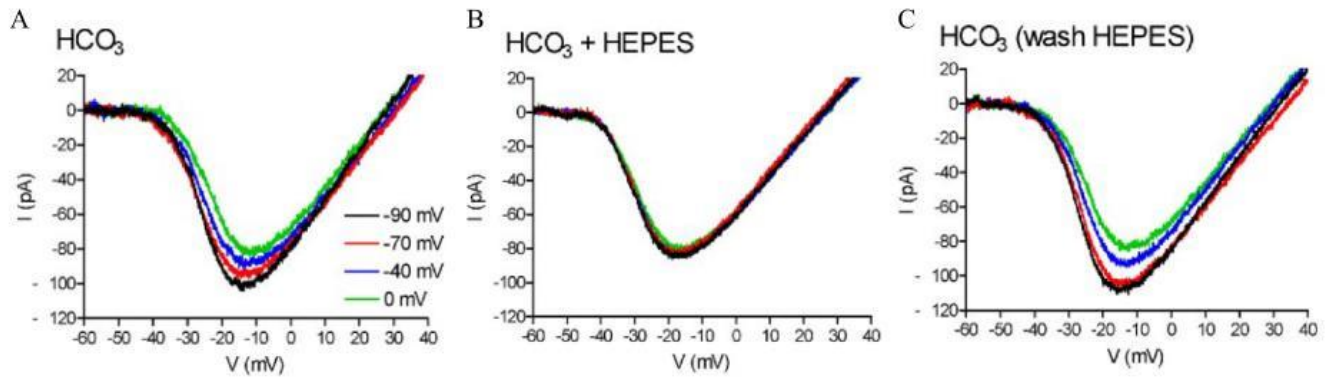


Figure 7. Two Electrode Recordings. Increased horizontal cell membrane potential using patch clamp techniques alter Ca^{2+} current in a simultaneously recorded cone photoreceptor. **A**, Cone Ca^{2+} current is progressively diminished and shifted to more positive potentials by voltage clamping the horizontal cell at increasing voltages. **B**, addition of 10 mM HEPES annihilated any changes in cone photoreceptor Ca^{2+} current under graded horizontal cell membrane potentials. **C**, After removal of HEPES cone Ca^{2+} current returned to control level (37).

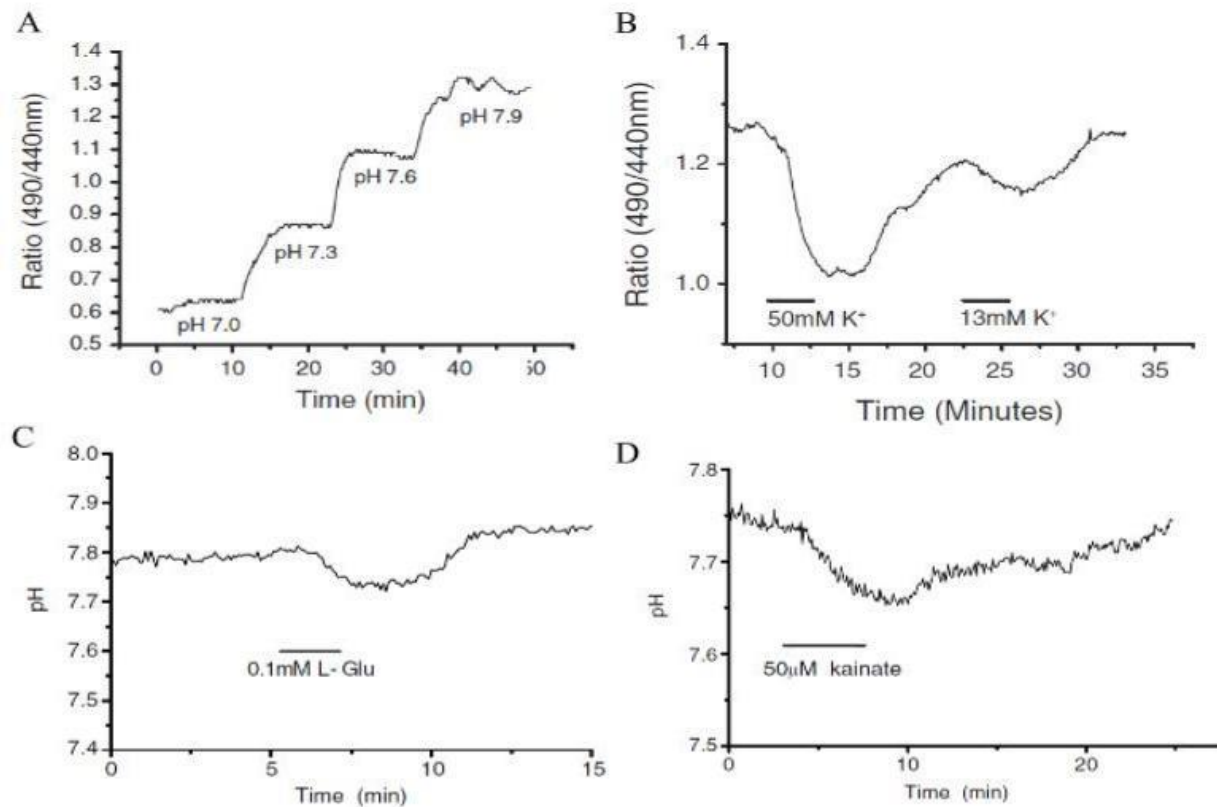


Figure 8. HAF-fluorescence. **A**, Graph showing the level of HAF fluorescence change that is pH dependent. Increased pH causes an increase in the ratio of 490/440 fluorescence-ratiometric. **B**, Also an alteration in the ratio, K⁺ depolarization leads to a decrease in emission. The level of emission is consistent with the concentration of depolarizing agent used. **C**, Glutamate has depolarizing effects on the HC causing an acidification. **D**, Kainate has depolarizing effect on the horizontal cell showing the presence of kainate receptors (41).

An alternative approach to test the proton hypothesis is to measure H^+ changes near the external surface of enzymatically isolated retinal horizontal cells maintained in primary culture using fluorescent pH indicators. Support in favor of the H^+ hypothesis using this method came from Jouhou et al. (41). Jouhou's experiments used both carp and goldfish isolated horizontal cells that were isolated using enzymatic break down and mechanical pipetting. Cells were stained using 5 μM 5-hexadecanoylamino-fluorescein (HAF), a pH-sensitive lipophilic dye, for 20 minutes (Fig. 8A). The fluorometric dye was excited using 488 nm light and HAF light emission monitored. Bath application of high- K^+ (a depolarizing agent) Ringer's solution caused a graded decrease in fluorescent emission that appeared to depend on the level of depolarization (Fig. 8B). The authors argued that this signified an acidification on the extracellular surface of the horizontal cell. 100 μM glutamate and 50 μM kainite also appeared to induce an extracellular acidification as indicated by the decrease in fluorescence (Fig. 8C & D). Similar results were obtained by Trenholm et al. 2010

a. CalipHluorin

Recently, a new experimental technique has been developed by Kramer and colleagues (42) to examine direct changes in extracellular pH in the outer plexiform layer synaptic cleft. Kramer developed a pH indicator integrated into Ca^{2+} channels and expressed exclusively on the cone synaptic terminals of zebrafish. The pH sensitive indicator pHluorin was genetically engineered to be part of a subunit on the N terminus of L-type Ca^{2+} channels, precisely where they need to be to measure changes in H^+ concentration that might affect the activity of the calcium channels. The authors dubbed this newly created pH sensor "CalipHluorin" (see Fig. 9A-C).

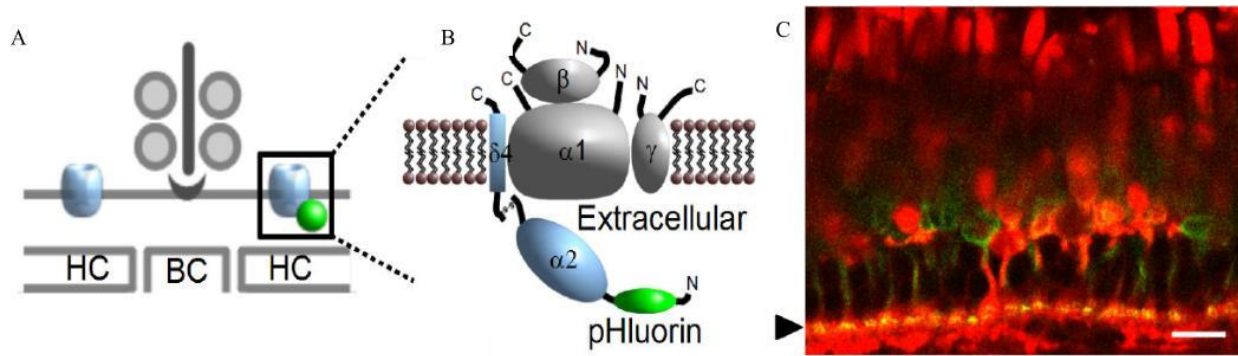


Figure 9. CalipHluorin. *A*, A model of genetically encoded pH indicator CalipHluorin located extracellularly on a cone photoreceptor Ca^{2+} channel. *B*, pH-sensitive GFP was fused to the extracellular N-Terminus of the Ca^{2+} channel. *C*, Retinal slice showing the presence of CalipHluorin in the photoreceptor synapse, and not in the soma. Slice was counter-stained with sulforhodamine 101 (red). The dye binds to basic amino acids of cellular proteins for overall neuronal cell morphology (42).

Imaging experiments were done using zebrafish retinal flat mounts and retinal slices. CalipHluorin was stimulated using a two photon microscope system using 910 nm wavelength light to avoid directly stimulating the light-sensitive photoreceptors, which do not absorb light of this wavelength, and alterations in fluorescence was taken as an indication of alterations in extracellular H^+ . Photoreceptors were directly stimulated using a blue light beam at 550 nm and an annulus was used in certain experiments to specifically stimulate horizontal cells. Baseline images were taken before the stimulus and compared to images at the same location after a stimulus was applied. This

allowed for changes to be quantitatively measured by taking the differential reading between the two time points.

Bright full field light stimulation onto cone photoreceptors caused an immediate increase in baseline fluorescence intensity that lasted approximately 0.5s (Fig. 10A). The increase in fluorescence indicates that the synaptic cleft is becoming alkaline, which the authors argue is due to what appears to be a rapid turnoff of horizontal cell negative feedback onto the photoreceptors. The alteration in pH, due to horizontal cell hyperpolarization, would allow for Ca^{2+} channels to open on photoreceptors. Similar experiments were then conducted in the presence of high concentrations (20 mM) of the pH buffers HEPES and TRIS; in this condition, CalipHluorin fluorescence did not change after stimulation. This suggests that the pH-sensitive indicator is in fact indicating a pH change in the synaptic cleft and not intracellularly.

Kramer and colleagues measured the magnitude of change of fluorescence and were able to create a titration curve to estimate the pH change within the synaptic cleft (Fig. 10B). Voltage gated Ca^{2+} channels have been shown to shift their voltage dependence by 1-3 mV during a change of 0.1 pH units in the immediate extracellular space (43). In order for the proton hypothesis of negative feedback to be correct, the pH shift in the synaptic cleft would have to be at least 0.1 pH unit during horizontal cell hyperpolarization. To test the overall pH change in the synaptic cleft, solutions of different pH values were superfused and CalipHluorin fluorescence measured to create a titration curve for the fluorescence intensity change due to pH changes. Alkaline conditions increased CalipHluorin fluorescence and acidic conditions decreased CalipHluorin fluorescence. Using dark conditions as a baseline, light conditions increase pH in the synaptic cleft by approximately 0.14 pH units (Fig. 10C). Since the recorded pH change induced by light was greater than 0.1 units, this suggests that the pH change in the synaptic cleft is sufficient in magnitude to alter Ca^{2+} channel function on the photoreceptors.

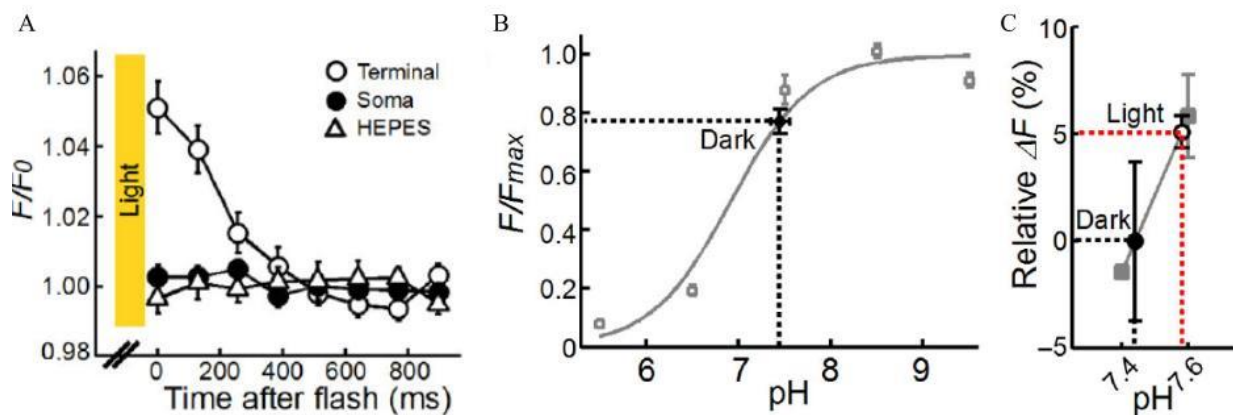


Figure 10. CalipHluorin emission recordings. *A*, Full field illumination for 0.5 s caused an increase in fluorescence in cone terminals but not in the soma. The increase in fluorescence was blocked by the addition of HEPES. *B*, Titration curve showing the relationship between fluorescence and pH. *C*, Showing a region more specific to cellular pH levels. Dark conditions in the cell have a higher pH than 7.4 (gray box) and light conditions are a little below pH buffers at 7.6 (gray box) (42).

Synapses in the outer plexiform layer contain processes from photoreceptors, horizontal cells and bipolar cells, and any of these cells could be involved in the changes in extracellular pH measured. Experiments examining the temporal and spatial characteristics of alterations in extracellular pH were done and found to match known horizontal cell characteristics. The authors noted that the kinetics seen using CalipHluorin under both long and short flash duration are similar to the time course of membrane potential responses in fish horizontal cells (Fig. 11A). The magnitude of the pH changes also depended on the spatial characteristics of the light stimulus in a fashion similar to that observed for horizontal cell responses to light: using larger diameter spots of

light caused larger changes in CalipHluorin fluorescence (Fig. 11B). In addition, annuli with diameters known to stimulate horizontal cells effectively also elicited a significant increase in CalipHluorin fluorescence (Fig. 11C). These results indicate that the temporal and spatial dependencies of changes in CalipHluorin fluorescence matched well with those of horizontal cell activation.

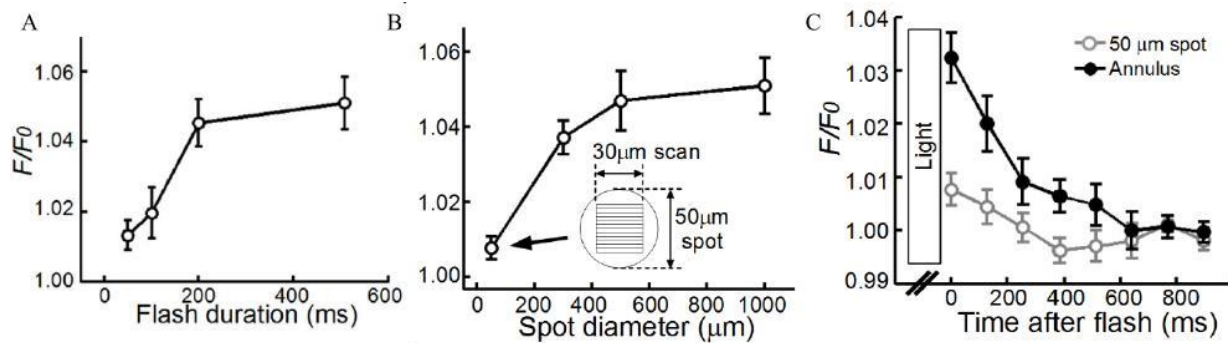


Figure 11. Dependence of CalipHluorin signals. *A*, A pH response as a function of flash duration. *B*, A pH response as a function of stimulus spot diameter. *C*, A pH response as a function of spot diameter. Use of an annulus (black line) increase pH response when compared to a 50 μm spot (gray line) stimulus. (42).

To better target horizontal cells and their role in synaptic cleft acidification, a new technique was developed to selectively depolarize only horizontal cells in the retinal slice. A transgenic zebrafish line was generated that expressed invertebrate FMRFamide gated Na⁺ channels (FaNaC) (44) selectively in the horizontal cells. This technique has been used in the past to specifically target neurons in brain slice (45). To make sure FMRFamide targeted only horizontal cells, a

horizontal cell-specific connexin 55.5 promoter was used as the target for placement of the channel (46). These transgenic zebrafish were crossed with CalipHluorin zebrafish to create a wildtype zebrafish with both FMRFamide and CalipHluorin. This allows for horizontal cells to be directly targeted by applying FMRFamide and depolarized without altering any other cell type and subsequently measure the acidification in the synaptic cleft by CalipHluorin.

Fish horizontal cells expressing FaNaC were shown to depolarize upon application of FMRFamide, and a decrease in CalipHluorin fluorescence at cone terminals was also detected following addition of FMRFamide. Horizontal cells lacking FaNaC were not depolarized by FMRFamide and CalipHluorin emission did not change from control conditions (Fig. 12A). This suggests that acidification within the synaptic cleft can occur with selective depolarization of horizontal cells.

Proton movement across the horizontal cell membrane could come from many different transporters and ion channels. One molecule known to play a vital role in transporting protons across membranes is the vacuolar ATPase, or V-ATPase for short. V-ATPases play a central role in acidifying the interior of vesicles in neurons, a process associated with the accumulation of neurotransmitter into the vesicles. The protons packed into the vesicles by V-ATPases flow out through additional proteins that selectively allow the accumulation of specific neurotransmitters. There is also evidence that V-ATPases may be present in the plasma membrane of neurons. Previous studies have suggested that retinal horizontal cells contain V-ATPase in their membrane as well (41) and could play a large role in extracellular acidification after cell depolarization. The presence of V-ATPase was tested using bafilomycin A1, a highly specific V-ATPase antagonist. In the presence of bafilomycin A1, and after horizontal cell stimulation by addition of FMRFamide, Kramer and colleagues found that the acidification in the synaptic cleft did not happen (Fig. 12B).

Washout of BFA1 restored the extracellular acidification showing that V-ATPase plays some role in extracellular acidification by horizontal cells.

Horizontal cells are known to possess hemi-gap junctional channels (47) that are proton-permeable (48). To test whether hemi gap-junctional channels (also known as hemichannels) were involved in the pH changes observed, Kramer and colleagues sought to measure alterations in CalipHluorin fluorescence in the presence of blockers of hemichannels. Initially, they used carbenoxolone, a hemichannel blocker in concert with FMRFamide stimulation. Carbenoxolone blocked acidification completely. However, in experiments done using patch clamp of cells expressing FMRFamide receptors, it was found that carbenoxolone was directly blocking the FaNaC channel. Since FaNaC channel was blocked the cell was not able to depolarize. To get around this problem the compound MFA was used to block the connexin hemichannels. MFA is an effective hemichannel blocker and does not have an effect on FaNaC channels but it affects several other types of ion channels besides the hemichannels as well. MFA had no effect on the extracellular acidification of the synaptic cleft using the FMRFamide stimulus. Although the connexin hemichannels do not appear to have a role in acidification, re-alkalization after FMRFamide was significantly slowed compared to control. Also under light stimulus MFA significantly slowed alkalization of the synaptic cleft (Fig. 12C). This indicates that MFA is decreasing the movement of H^+ plus into the cell and thus inhibiting the mechanism that move H^+ into the cell while having no effect on H^+ moving out of the cell.

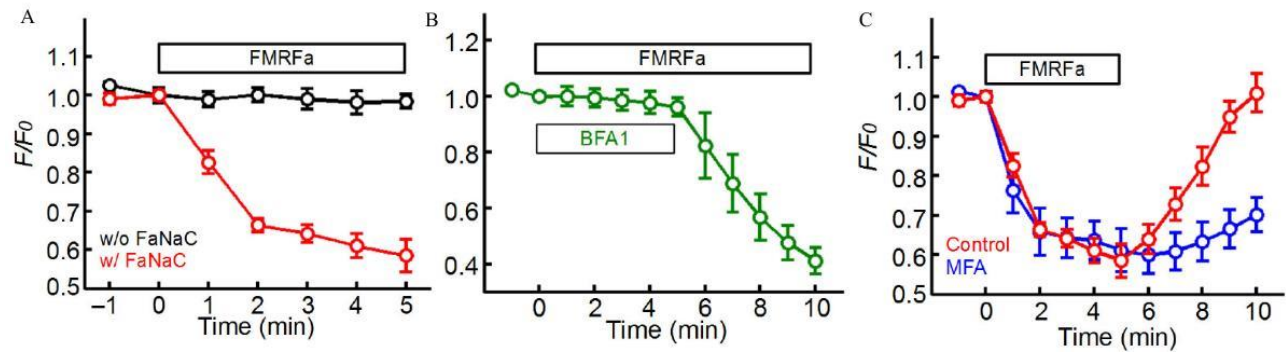


Figure 12. FMRFa horizontal cell stimulation. **A**, FMRFamide caused a decrease in CalipHluorin fluorescence in double transgenic fish but not in fish lacking the FMRFa gene. **B**, Acidification of the synaptic cleft was annihilated in the presence of BFA1 (a V-ATPase block) **C**, Addition of MFA did not have an effect on the feedback signal (42).

B. The Anti-Proton Spin: Evidence Against the Proton Hypothesis for Lateral Inhibition

The evidence cited above provides strong evidence that protons may play an important role in shaping the responses of retinal neurons, and implicate horizontal cells as a possible source of those protons. However, work by other laboratories has also resulted in data that argue strongly against the hypothesis that horizontal cells release protons when they are depolarized. In this section, studies focusing primarily on responses from acutely isolated horizontal cells will first be discussed. In these studies, two methods were used to measure levels of acidity adjacent to the extracellular surface of horizontal cells. One method involved the use of H^+ -selective microelectrodes employed in what is called a self-referencing fashion to increase the level of sensitivity of the sensors. The second method examined alterations in fluorescence of the pH-sensitive dye HAF. The description of the results of these studies will be followed by other work bringing into question some of the studies done using high concentrations of extracellular pH buffers.

The synaptic space in the outer plexiform layer where photoreceptors, horizontal cells and bipolar cells meet is a very small region that precludes accurate measurement of changes in pH with such methods as H^+ -selective microelectrodes. However, such sensors can be very useful in examining levels of H^+ adjacent to the cell membranes of isolated cells in culture. A standard technique used for many years to study the physiological properties of retinal cells has been to use an enzyme-based dissociation protocol to separate the cells and keep them for several days in primary culture. In the experiments to be described, isolated horizontal cells were

obtained using a papain-based digestion protocol followed by mechanical separation, a protocol very similar to that used in the studies of Jouhou and Trenholm mentioned above. Once the cells are pulled apart the cells sink to the bottom of a plastic culture dish and adhere to it. Once attached, these cells appear to possess normal physiological functions and characteristics and can go through the process of depolarization (49) (50).

The trials completed by Kreitzer et al., 2007 were completed in isolated catfish horizontal cells. In these experiments both cone- and rod- driven horizontal cells were used. These cell types were distinguished by their morphological shapes - cone horizontal cells had a star-like shape while rod horizontal cells were much larger and did not appear star shaped, as can be seen in Fig. 13A.

Highly selective H^+ electrodes used in a self-referencing fashion were used to measure changes in extracellular acidity directly adjacent to the external plasma membranes of isolated cells (49). The electrodes were created by first backfilling with a 100 mM KCl solution adjusted to a stable pH with 10 mM of the pH buffer HEPES. Then, a small column of a highly selective hydrophobic H^+ resin was placed in the tip of the electrode (51). H^+ selective microelectrodes generate a voltage signal whose magnitude depends on the difference in concentration of protons in the outside solution compared to that on the inside of the recording pipette. The voltage changes in a Nernstian, logarithmic fashion, with electrode voltage changing about 58 mV for each 10-fold difference in H^+ concentration at normal room temperatures. The electrode is placed within 1-2 μm (Fig. 13B) from the cell and a voltage reading taken at this point. The electrode is then moved 30 μm away from the cell and a second voltage reading taken. The reading obtained 30 μm away is subtracted from the reading taken when the electrode was positioned within 1-2 μm of the cell; this simple subtraction removes slow electrical drift inherent in all ion selective electrodes and enhances the useful sensitivity of H^+ electrodes by

~ 1000 X. Using this unique differential recording methodology, these experiments were able to see very small changes in H⁺ concentration in very close proximity to the external surface of horizontal cells.

Kreitzer and colleagues (49) showed that isolated catfish cone and rod horizontal cells have a standing H⁺ flux prior to stimulation (Fig. 13C). A positive standing flux indicates that more H⁺ ions are located near the cell membrane compared to the point 30 μ m away. In these circumstances the solution directly adjacent to the cell would be more acidic. This means that without stimulation, when horizontal cells are typically sitting at -70 mV or so, the cell is constantly extruding H⁺ ions. Depolarization of the horizontal cells using a bath application of 300 μ M glutamate to produce a final concentration of 100 μ M glutamate induced a decrease in the differential signal of the H⁺-selective electrode, often reversing the polarity of the signal (Fig. 13D). Depolarization by glutamate thus caused the immediate area around the cell to become more alkaline, meaning H⁺ ions were lower in concentration near the horizontal cell compared to 30 μ m away. This indicates the cell is bringing in large quantities of H⁺ ions upon depolarization by glutamate.

Horizontal cells of catfish are known to contain both NMDA and AMPA type glutamate receptors. Upon binding glutamate, AMPA receptors cause an influx of positive ions into the cell; in horizontal cells thus far studied, both sodium and calcium can permeate through the channels and enter the cell. The depolarization and presence of glutamate also promotes the opening NMDA receptors which are both voltage and glutamate dependent (15). To check for the presence of both receptor types they were individually targeted using selective receptor agonists. Kainate is an AMPA receptor agonist. Bath application of 20 μ l kainate revealed an overall decrease in extracellular voltage indicating an influx of H⁺ into the cell (Fig. 14A). Similar results were seen when specifically targeting NMDA receptors (Fig. 14B).

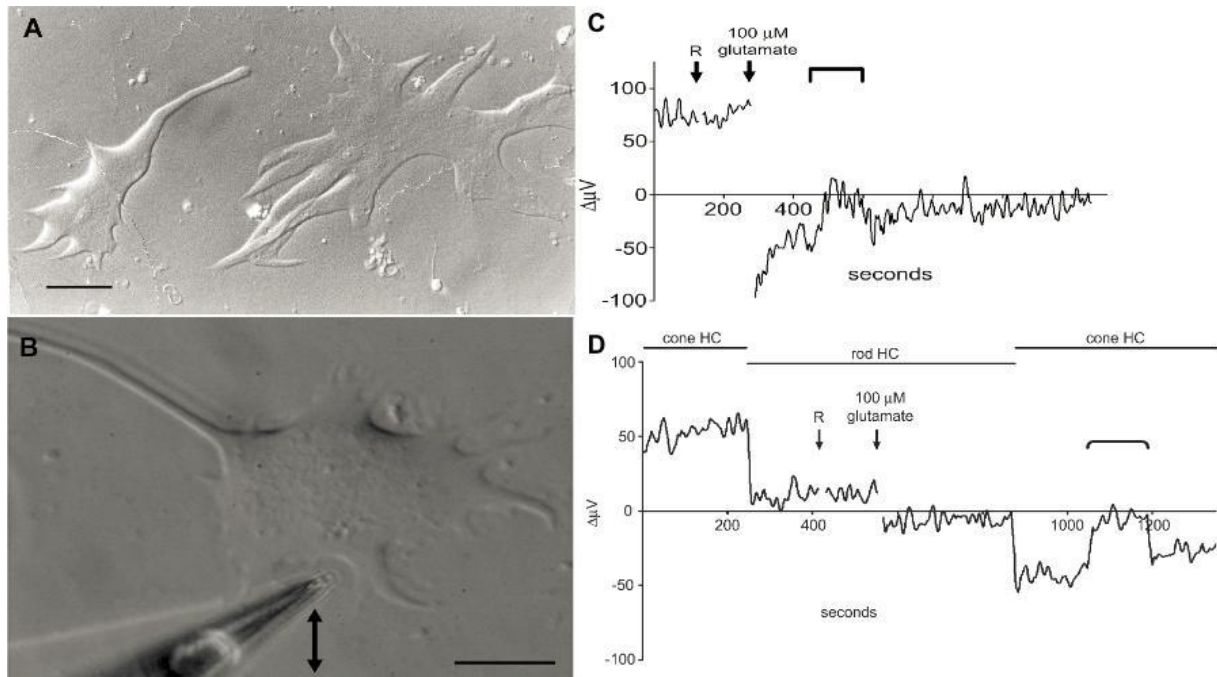


Figure 13. Catfish horizontal cell recordings. **A**, Light microscope image showing catfish cone horizontal cell on left and catfish rod horizontal cell on right. Rod horizontal cell is much larger and does not include a noticeable axon. **B**, Image showing the location of the microelectrode used during self-referencing. The electrode is placed within a few micrometers of the cell and then moved back and forth to create a differential. **C**, H^+ flux before and after stimulation by glutamate. Positive standing flux around 70 μV indicates an acidic environment directly outside of the cell. R is a Ringer's solution bath control. At approximately 300 s 100 μM final concentration glutamate is added into the culture dish. The cell then moves to a negative standing flux after depolarization. This shows a more alkaline environment next to the cell. **D**, Shows the standing flux for both a cone horizontal cell and a rod horizontal cell. The cone horizontal cell

has a much greater standing flux compared to the rod horizontal cell but they both have positive values. At approximately 600 s 100 μ M final concentration glutamate is added into the culture dish. Cell depolarization causes a negative standing flux in both the rod horizontal cell and the cone horizontal cell. Again this indicates that depolarization by glutamate causes and extracellular alkalinization (49)

This data shows the presence of both AMPAR and NMDAR on isolated HC and that activation of these channels creates an extracellular alkalinization. These results are opposite of that expected by the proton theory of lateral inhibition in the retina – activation of the horizontal cells by these agents should have induced an extracellular acidification. Kreitzer et al. (52) showed very similar voltage data results using isolated goldfish horizontal cells, and similar results were also found using horizontal cells isolated from the skate retina (53).

Kreitzer et al. also examined the molecular mechanism likely responsible for the observed changes in extracellular acidity. A key observation was that the change in extracellular acidity was abolished when extracellular calcium was eliminated from the Ringer's solution (Fig. 14C). This experiment points to the following mechanism used to move H^+ into horizontal cells. AMPA receptors in horizontal cells are glutamate gated channels that once opened allow the positive ions Na^+ and calcium into the cell (15). NMDA receptors are both glutamate and voltage gated. Increased intracellular voltage due to the AMPA channel opening allows NMDAR to open and allow in large quantities of Na^+ and calcium. Ca^{2+} enters the horizontal cells through both AMPA and NMDA receptors. In addition, the depolarization induced by glutamate, kainate or NMDA also induces the opening of voltage gated Ca^{2+} channels. Ca^{2+} thus enters the cell in large amounts through all three pathways.

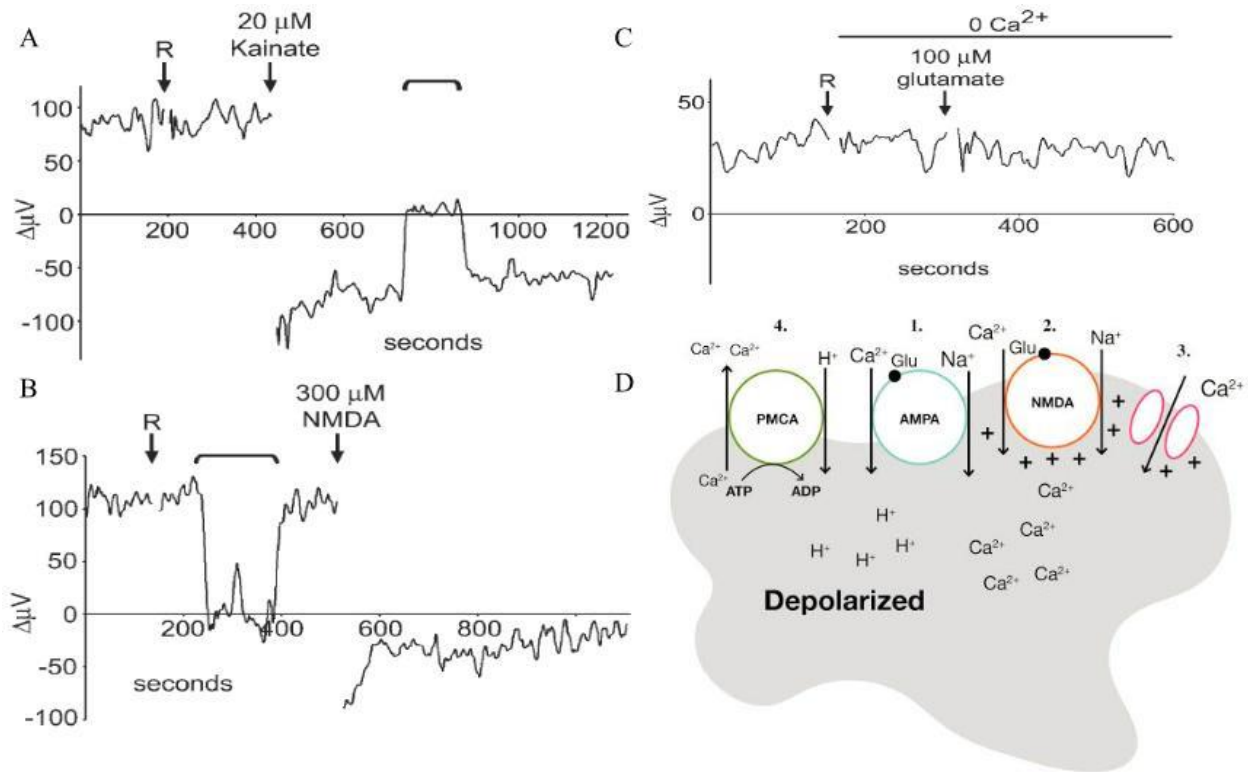


Figure 14: Isolated horizontal cell depolarization hypothesis: **A**, Isolated horizontal cell voltage recordings. 20 μM kainate causes a decrease in cell voltage signifying an alkalization. **B**, 300 μM NMDA see the same results as kainate. **C**, 0 Ca^{2+} ringer solution shows no depolarization with 100 μM Glutamate (49). **D**, Isolated horizontal cell alkalization hypothesis.

This leads to activation of a plasma membrane calcium pump (PMCA), which actively transports Ca^{2+} out of the cell. PMCA pumps are believed to transport two H^{+} into the cell for every calcium ion extruded. This mechanism explains why upon depolarization the extracellular space directly adjacent to the horizontal cell becomes alkaline. Kreitzer and colleagues provided further evidence for this mechanism by examining the effects of carboxyeosin, an inhibitor of PMCA pumps, on changes in extracellular acidity. They found that carboxyeosin abolished the

changes in extracellular acidity normally observed upon depolarization by high external potassium or addition of glutamate (Fig. 14D).

Experiments with isolated horizontal cells using self-referencing microelectrodes indicated that depolarization by high K^+ or glutamate and its analogues lead to an extracellular alkalization, precisely opposite of what would be expected from the proton hypothesis of lateral inhibition. However, studies using isolated horizontal cells treated with the fluorescent pH indicator HAF indicated an extracellular acidification (41). To address this puzzling difference, Jacoby et al (54) used both self-referencing microelectrodes and HAF fluorescence to examine alterations in extracellular acidity for cells from the same species in identical conditions. In this study, horizontal cells were stained with 5-hexadecanoylamino fluorescein (HAF), a pH-sensitive ratiometric dye that alters its fluorescence with pH (54). Using the same loading protocol as used by Jouhou et al. (41). Jacoby et al. found changes in fluorescence very similar to that observed by Jouhou et al. and Trenholm et al. – that is, high extracellular K^+ and stimulation by glutamate led to changes in fluorescence suggestive of an extracellular acidification and supporting the proton hypothesis of lateral inhibition. However, measurements using self-referencing microelectrodes from isolated horizontal cells under the same experimental conditions consistently revealed an extracellular alkalization, in disagreement with the proton hypothesis of lateral inhibition.

Jacoby et al. then examined the distribution of the HAF dye using confocal microscopy. They found that, rather than being restricted to the exterior face of the plasma membrane of the horizontal cells, the loading of the cells using the protocol used by Jouhou led to extensive accumulation of HAF into the interior membranes of the horizontal cells. Jacoby et al. concluded that the dye distribution was such that it was likely measuring changes in intracellular pH, not extracellular pH, as had tacitly been assumed.

In further studies examining this issue, Jacoby et al. altered the loading protocol for the HAF dye to find conditions in which HAF staining could be largely restricted to the plasma membrane. They confirmed that HAF staining using the original protocol used by Jouhou et al. led to extensive dye localization in the interior of horizontal cells. However, by lowering the concentration of the dye and the duration and temperature of application, the degree of intracellular contamination could be significantly reduced, with dye now accumulating largely in the plasma membrane of the cells. Cells were stained with concentrations of 5 μ M, 2.5 μ M, and 500 nM HAF for 20, 15, and 10 minutes, and the condition of low HAF and short application led to staining primarily in the extracellular membrane of the horizontal cell as indicated by confocal microscopy (Fig. 15A, B & C). Longer duration incubation and or higher concentration HAF caused intracellular staining and did not give optimal results. To insure only extracellular membrane staining, 500 nM HAF was used and incubation time was limited to 10 minutes. The ratiometric dye was stimulated alternately at 488 and 440 nm. Drug delivery was either superfused over the cells or were applied as small bolus additions in close proximity to the cell. Under these new loading conditions, application of 50 μ M kainate led to an increase in the fluorescent emission, signifying an alkalization in the extracellular space directly surrounding the cell (Fig. 15D). Cells depolarized by 300 μ M glutamate also showed an extracellular alkalization (Fig. 15E). These results go hand in hand with the measurements obtained using self-referencing H⁺-selective microelectrodes (49), with both methods now indicating that stimulation of isolated horizontal cells causes an extracellular alkalization in place of an acidification, a result opposite to that predicted by the proton hypothesis of lateral inhibition.

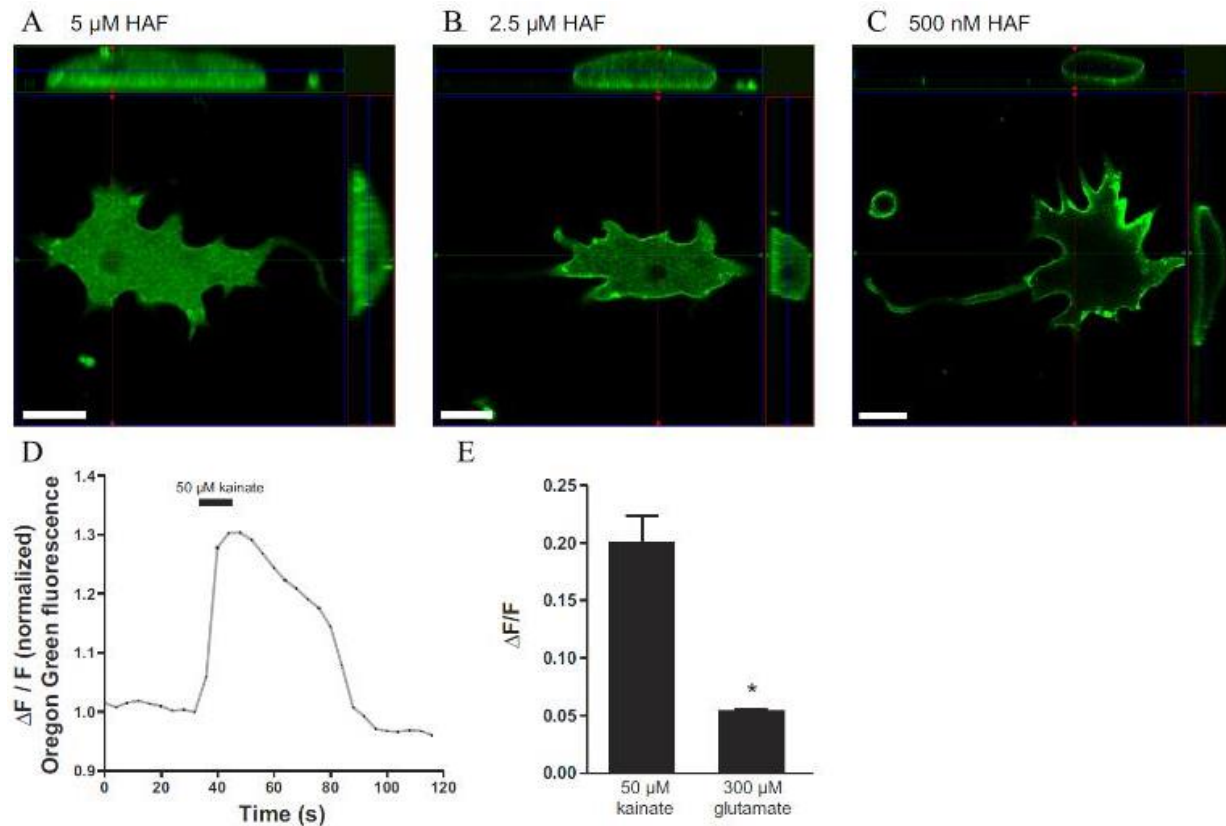


Figure 15. HAF concentration dependence and cell alkalization. *A*, confocal slice showing intracellular HAF staining on cone horizontal cell. 5 μM HAF for 20 minutes. Cell is stained both intra and extracellularly. *B*, 2.5 μM HAF staining for 10 minutes shows less intracellular staining but it is still present. *C*, 500 nM HAF staining for 10 minutes shows only extracellular membrane staining. *D*, Fluorescence staining of cone horizontal cell. AMPA agonist kainate used to depolarize the cell. Depolarization causes an increase in emission showing an extracellular alkalization. *E*, Bar graph showing horizontal cell fluorescence emission after depolarization with kainate and glutamate. Both show an increase in emission after depolarization (54).

Thus, data from isolated horizontal cells using both self-referencing H⁺ microelectrodes and HAF argue against the proton theory: both indicate an extracellular alkalization after depolarization. Although this is strong evidence to the contrary, it is also possible that isolation of the cell can cause irregular physiological tendencies. When the cells are removed from retina they are forcibly removed and dendritic appendages get removed in the process. Negative feedback would occur in the small spaces at the dendritic ends and thus recordings from the cell body might not show real physiological traits. Also after the cell is removed the normal distribution of channels, transporters and receptors could be altered throughout the cell. The movement of channels could also cause the cell to act in a non-physiological manner. All that in mind, all indications in isolated horizontal cells indicate that the proton theory of lateral feedback inhibition is incorrect.

A lynchpin of many of the studies reporting to support the proton hypothesis of lateral inhibition is the reliance of high concentrations of extracellular pH buffers such as HEPES and TRIS to block characteristics associated with feedback. In a set of experiments investigating the mechanisms of feedback inhibition, Fahrenfort et al. (55), found some surprising additional effects of high concentrations buffers, complicating the interpretation of many experiments. Fahrenfort used goldfish retinal slice to examine initially the effects of the endogenous buffer system, bicarbonate/carbonic anhydrase. This was done to avoid the use of non-physiological buffers and avoid overlapping results between the proton model of feedback and the ephaptic feedback model. Unlike previous experiments that were carried out in horizontal cells, indirectly measuring the feedback response on photoreceptors (56) (57), these experiments were completed by taking measurements directly from cone photoreceptors, allowing negative feedback effects due to horizontal cells to be directly measured. The recordings were done using a glass microelectrode to patch into the photoreceptor and record intracellular voltage changes.

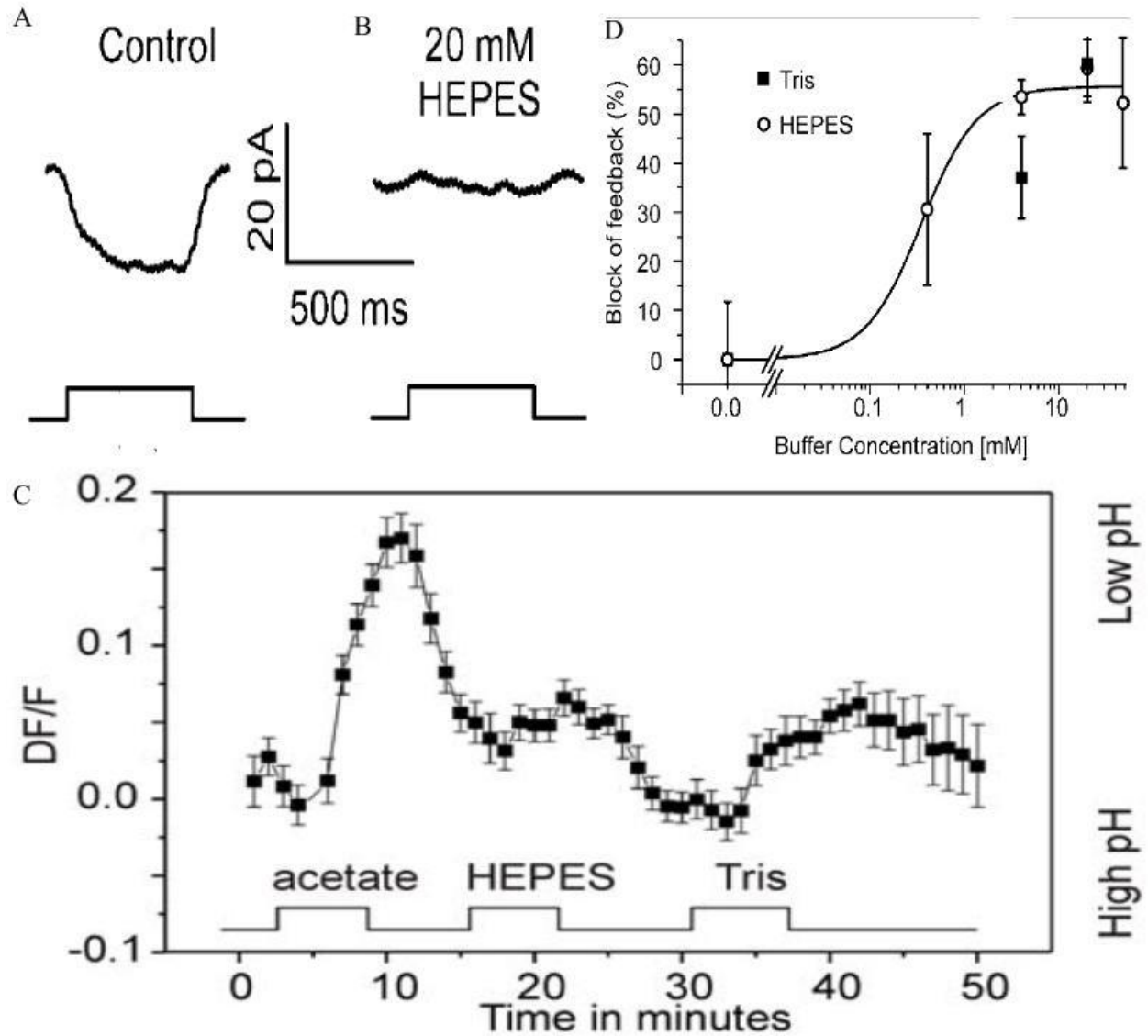


Figure 16. Photoreceptor feedback response. *A*, Feedback response of a photoreceptor under light stimulus. Normal current response shown in control. *B*, Feedback response in 20 mM HEPES response. *C*, Percent of feedback blocked by Tris and HEPES. Solid line is a fitted Hill equation through the HEPES data points. *D*, BCECF emission of horizontal cells as a function of time. Increase in fluorescence signal indicates acidification. Tris, HEPES, and acetate all lead to intracellular acidification (55).

Recordings from photoreceptors showed that in normal physiological conditions light stimuli induced an inward rectifying current (Fig. 16A). An inward rectifying current in these conditions signifies positive ions moving into the cell. Upon stimulation by light, photoreceptors become hyperpolarized, with positive ions leaving the cell. The authors argue that the inward current was due to negative feedback from horizontal cells. In the presence of a 20 mM HEPES solution the inward current of photoreceptors was decreased but not completely nullified (Fig. 16B). In similar fashion high concentrations of Tris decreased the inward photoreceptor current but does not completely stop it (Fig. 16C). Tris and HEPES have very similar values for their pKa values and buffering capacity; however, they had different magnitudes of effect on the photoreceptor inward current. HEPES had a greater effect, more fully blocking the inward current, when compared to the effects of Tris. If feedback was solely due to pH changes in the cleft then a strong extracellular buffer should completely eliminate the feedback current in photoreceptors. Also buffers with similar buffering capacity and pKa values should affect the inward current of photoreceptors in the same magnitude. Since HEPES seems to have larger effects than Tris it appears to be acting on other physiological mechanisms.

In addition, Fahrenfort et al. made the surprising finding that high extracellular concentrations of pH buffers led to significant changes in intracellular acidity. To test for intracellular acidification in horizontal cells and cone photoreceptors, two-photon microscopy was used in concert with the pH-sensitive dye BCECF, which can be localized exclusively to the interior of cells (58). Application of high concentrations of HEPES and Tris produced an increase in BCECF fluorescence which indicates a significant increase in acidity inside of the cell (Fig. 16D). Thus, contrary to the results found by Davenport 2008, these results show that the extracellular pH buffers HEPES and Tris change acidity inside of the cell. Increases in intracellular acidity can significantly alter the activity of many intracellular processes, such as

blocking gap junctions in the horizontal cells, which could also interfere with negative feedback if it were being mediated by hemi-gap junctional channels as hypothesized by the ephaptic model (32). The increase in intracellular acidity by exogenous pH buffers observed by Fahrenfort suggests that interpretation of past experimental results in other studies may be complicated indeed. These results were disputed by Trenholm 2010.

To avoid using high concentrations of artificial extracellular buffers, Fahrenfort used the body's physiological buffer system to test the proton hypothesis. Bicarbonate-based pH buffer has a very high buffer capacity due to the speed of the catalyzing enzyme carbonic anhydrase. Using immunochemistry of a known membrane bound isoform of carbonic anhydrase Fahrenfort was able to locate high concentration of the catalyst in the synaptic cleft of the OPL. The next step was to block its buffering capacity using benzolamide, a membrane impermeable carbonic anhydrase inhibitor (59). It decreases the buffering capacity of carbonic anhydrase by about three times. With three times less buffering capacity, proton mediated feedback should have greater effect on downstream signals.

To test for a pH change in the synaptic cleft they use a technique described by Palmer (60). They found that Ca^{2+} channels were transiently inhibited by the increase in concentration of H^{+} in the synaptic cleft. This directly shows that Ca^{2+} current in cone photoreceptors decreases as acidity in the synaptic cleft increases. Cone photoreceptor Ca^{2+} channels were activated using whole cell patch clamp to depolarize the photoreceptors. The authors argue that as glutamate is released from photoreceptors, H^{+} located in the same vesicles as glutamate, is released as well, shutting down Ca^{2+} channels. In Fahrenfort's experiments baseline Ca^{2+} currents were recorded and subtracted from experimental Ca^{2+} to find the current change due to protons in the synaptic cleft. In the presence of benzolamide photoreceptor depolarization induced a smaller Ca^{2+} current that was also inhibited more quickly than the control. The

authors argue that this makes sense because less buffer means H^+ will increase in concentration faster so the acidity will increase at a faster rate without the presence of buffer.

To test the effects of benzolamide on horizontal feedback, kainate was used as an AMPA agonist to depolarize the horizontal cells and Ca^{2+} current in the cone photoreceptors again measured. Under these conditions no significant difference was seen between control and trials with benzolamide (Fig. 17A&B). These results do not correlate with the proton hypothesis. In the presence of benzolamide the buffering capacity of carbonic anhydrase was severely decreased. If the proton hypothesis for lateral inhibition was correct, the Ca^{2+} current should have decreased at a much faster rate than in control due to the increase in acidity in the synaptic cleft from the horizontal cell depolarization.

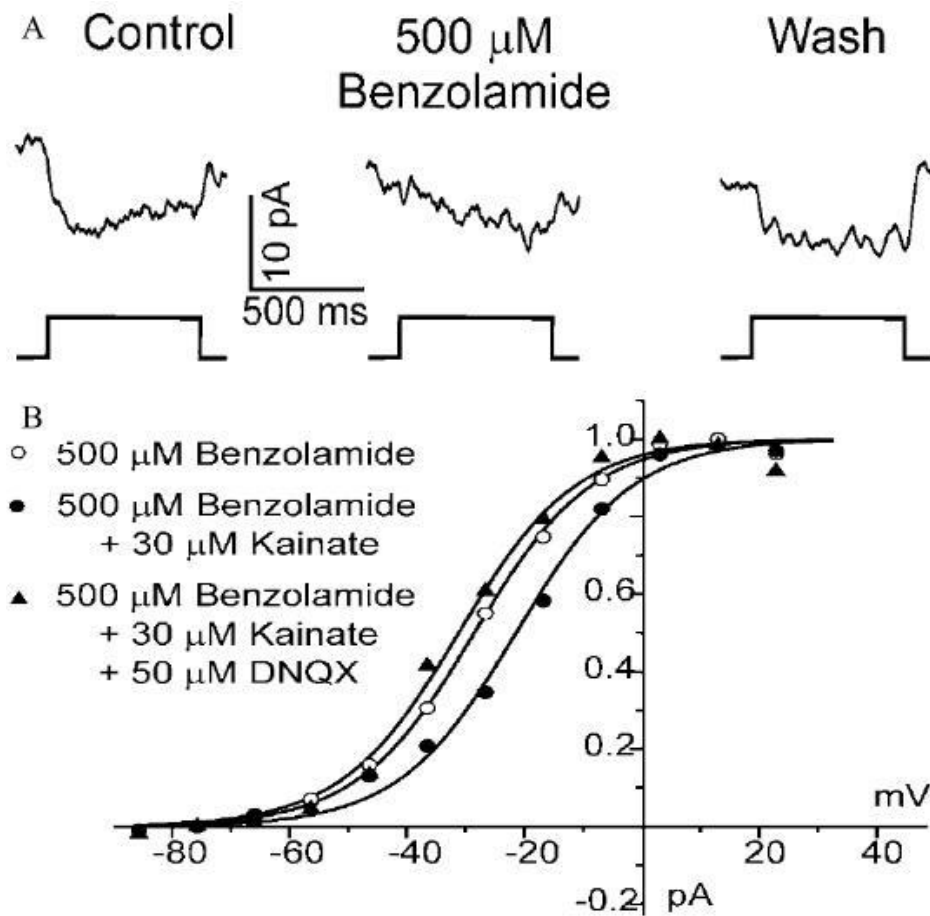


Figure 17. Endogenous pH Buffer blocker. *A*, 20 μ m spot stimulus centered over photoreceptor clamped at -43 mV. Response under control, benzolamide, and wash conditions. *B*, Cone photoreceptor Ca^{2+} current response after bath drug application. Benzolamide + Kainate increase potential. Benzolamide + kainate + DNQX cause a more negative potential. (55).

III. Discussion

Since the discovery of lateral inhibition, scientists have debated the molecular mechanism by which it occurs. Much evidence supports a model in which protons released by horizontal cells play the central role. However, there is additional strong evidence against the proton hypothesis of lateral inhibition.

Experiments employing high concentrations of buffer are advantageous in that they allow the pH in the synaptic cleft to be locked in place. This makes testing for the proton hypothesis fairly easy in that feedback should not occur when pH remains constant. Two key experiments found that high concentrations of pH buffers completely stop feedback signals when recording from horizontal cells. Although buffers have key advantages in these types of experiments, they also have limits as to what information they can provide. One major limit of extracellular buffers, brought up in all three articles, was the possible pH effects these buffers may have intracellularly. It appears that strong extracellular buffers may cause alterations in the internal pH of cells. This may alter the physiology of cells, and can also alter proteins suggested to be involved in the ephaptic mechanism, a serious contender for causing negative feedback. This means that the extracellular buffer could be blocking both the proton feedback as well as ephaptic feedback making it impossible to distinguish between the two. It appears that buffering experiments do not offer definitive results at this time as both sides of the argument are supported by evidence.

Thoreson's simultaneous recordings from horizontal cells and photoreceptors are a technical tour de force that also show strong support for the proton model of feedback. In these experiments horizontal cells were activated by directly changing their membrane potential. This allowed for direct stimulation of horizontal cells and removed possible side effects of drug application. These experiments also measured Ca^{2+} current directly from cone photoreceptors in

the in the intact retinal slice. Their recordings showed that horizontal cell depolarization caused Ca^{2+} current changes in cone photoreceptors, strongly indicating the role of horizontal cells in pH acidification. CalipHluorin is a new technique offered through genetic modification of zebrafish. It provides a close up look at pH changes within the synaptic cleft of intact retina that have never been seen before. Also because of the genetically modified horizontal cells created in zebrafish retina, they can specifically stimulate one cell type, which rules out some of the effects of other cell. This technique provides an extensive amount of data in favor of the proton model of feedback.

This system has many advantageous due to the specific nature of the genetically modified cells. Arguably most important is that pH change can be viewed in the synaptic cleft of the intact retina. These experiments provide data that has only been hinted at by high concentration extracellular buffers in the past. Another advantage of these experiments are the Na^+ placed into horizontal cells so that very specific cell stimulation can occur. Since this technique is highly specific to horizontal cells it makes it unlikely that other cell types are being activated with a broad stimulus. This is important in a retinal slice because as stated before many different cell types could play a role in the negative feedback and this eliminates that possibility. These experiments strongly suggest that extracellular acidification occurs after horizontal cell depolarization; however, since they are done in the intact retina it is too early to say if the full response seen is due solely to horizontal cell activation.

In contrast to the above experiments, experiments completed by Kreitzer and colleagues using self-referencing H^+ microelectrodes and HAF fluorescence from isolated horizontal cells provide strong evidence against the proton hypothesis of lateral inhibition. This evidence suggests that rather than extruding H^+ after depolarization, horizontal cells actually bring H^+ into the cell

and alkalinize the extracellular fluid. Although some dismiss these trials since they are not in intact retina, this provides strong evidence that HC's do not behave as once suspected.

Isolated cells have many advantages over retinal slices that may be a contributing factor in the different experimental results. The accessibility of isolated cells allow for different methods of experimentation and also provide an environment that can be easily altered to test different stages of the hydrogen mechanism. Isolated cells also have an advantage in that they ensure all experimental results are coming specifically from only the cell type being tested. In retinal slices it is virtually impossible to remove the effects of all cell types other than the ones under examination. This means that in slice preparations other cells could be contributing to the final recordings being measured. Work on isolated cells also insures that no other cell can provide upstream effects. In contrast to retinal slices, this allows for seclusion from other cell types that might be playing a role in any feedback mechanism. It is important to keep in mind that many cell types are within the retina including glial cells and many other types of neurons. Isolating the cell is the only true way to eliminate all outside factors and it would be unwise to push aside the possible role of other cells in the lateral inhibition pathway.

Although isolated cells carry many advantages it is also possible that cells being removed from their anatomical locations alters their cell physiology. When horizontal cells are removed from tissue, connections with other cells are removed using enzymatic solutions. After this they are mechanically agitated to separate the cells. Both of these events have been hypothesized to change some of the cell's normal physiology and possibly alter the output of the cell. Removal of the cell has the negative effect of removing many of the cells' dendritic tips. Dendritic tips are the specific areas where ion movement is being studied and thus removal of the tips could lead to a loss in the synaptic cleft physiology. Lastly, isolated cells may relocate channels and pumps after

isolation. Cell plasticity could alter the channels and pumps locations so that they are no longer where they would be in an intact cell. Although these factors contribute to experiments in isolated horizontal cells it would be imprudent to disregard the results found in these trials.

As shown by differing experimental results, regulation of pH is a complicated process undertaken by cells in the body. All cells have a variety of mechanisms for regulating pH making it difficult to tease out the role of each. Bicarbonate/bicarbonate transporters, plasma membrane Ca^{2+} ATPase, and $\text{Na}^{+}/\text{H}^{+}$ exchangers all have possible roles in extracellular pH. Because of the complexity of pH regulation, the mechanism behind pH changes in the OPL remain opaque.

Differing theories behind feedback inhibition leave us guessing as to what the true mechanism may be. In some experiments horizontal cells cause extracellular acidification while in similar conditions other experiments can show an alkalinization. This controversy is a good indication that perhaps another explanation is needed. Recent experiments done by the Malchow lab show possible implications of glial cells in extracellular pH change in the retina. Muller cells are large glial cells that span the width of the retina. Up to this point they have not been implicated in direct neuronal signal transduction. Experiments in the Malchow laboratory performed primarily by Boriana Tchernookova show that application of ATP (a known glial cell stimulant) causes a large extracellular acidification from isolated Muller cells, and also produces an extracellular acidification at the level of the outer plexiform layer in retinal slices (these are unpublished observations currently submitted for publication). The acidification by Muller cells is about ten times greater than the size of the alkalinizations seen from horizontal cells using self-referencing H^{+} microelectrodes. The large acidification could have implications on the feedback model of lateral inhibition. This suggests that Muller cells may cause the extracellular acidification seen in the OPL synaptic cleft after photoreceptor hyperpolarization. Although much more

experimentation needs to be done before this alternative hypothesis takes hold, it offers a worthy alternative to the current proton model of feedback inhibition, and suggests that alterations in extracellular levels of acidity in the retina may be important in regulating neuronal responses, but not in the manner originally envisioned by the early H^+ hypothesis of lateral inhibition.

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