Thermal Excitation of the Mechatransduction Apparatus of Hair Cells

Highlights

- Hair cells respond mechanically and electrically to ultraviolet light
- These responses involve the opening of mechatransduction channels
- Temperature measurements demonstrate that the responses stem from local heating
- Heat both softens gating springs and opens mechatransduction channels

Authors

Julien B. Azimzadeh, Brian A. Fabella, Nathaniel R. Kastan, A.J. Hudspeth

Correspondence

hudspaj@rockefeller.edu

In Brief

Azimzadeh et al. show that hair cells of the inner ear can be stimulated by light without external or genetic manipulation. Chromophores in mitochondria convert light to heat, which subsequently acts on elements of the hair bundle to stimulate it.
Neuron Article

Thermal Excitation of the Mechatransduction Apparatus of Hair Cells

Julien B. Azimzadeh,1,2 Brian A. Fabella,1,2 Nathaniel R. Kastan,1,2 and A.J. Hudspeth1,2,3,*
1Howard Hughes Medical Institute
2Laboratory of Sensory Neuroscience
The Rockefeller University, 1230 York Avenue, New York, NY 10065, USA
3Lead Contact
*Correspondence: hudspaj@rockefeller.edu
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SUMMARY

Although a hair bundle is normally deflected by mechanical stimuli, we found that irradiation of a hair cell from the bullfrog’s saccus with ultraviolet light causes rapid motion of the hair bundle toward its tall edge. This movement is associated with opening of mechatransduction channels and disappears when tip links are disrupted. We localized the absorptive element responsible for the motion to the region directly below the hair bundle and measured an action spectrum similar to the absorption spectra of mitochondrial constituents. Temperature measurements revealed heating around the site of absorption; direct heating of the hair bundle confirmed that the response to light is mediated through heat. Although mechanical offsets of the hair bundle revealed that heat softens gating springs, it also acts directly to open transduction channels. This study identifies an unconventional method of hair-cell stimulation and clarifies the previously unexplained sensitivity of auditory organs to thermal stimulation.

INTRODUCTION

A hair cell of the auditory, vestibular, or lateral-line system transduces mechanical energy into electrical signals by means of its hair bundle. Each bundle is an upright cluster of actin-filled protuberances called stereocilia, which increase in length from one edge of the hair cell to that opposite to produce a staircase (Figure 1A). Along the same axis, each shorter stereocilium is attached to its tallest neighbor by a cadherin-based strand, the tip link. Moving the hair bundle in the positive direction, toward its tall edge, increases the tension in each tip link, which in turn opens mechanically sensitive ion channels at the link’s lower insertion. The ensuing influx of cations initiates an electrical response that propagates by means of a chemical synapse to an afferent nerve fiber. A myosin-based motor at the upper end of each link ascends or descends a stereocilium to maintain the tip-link tension within a narrow range, thus effecting adaptation that restores the hair bundle’s mechanical sensitivity during protracted stimulation.

Hair cells are not only passive detectors, but also active amplifiers (Ashmore, 1987; Brownell et al., 1985; Hudspeth, 2014; Martin and Hudspeth, 1999). Their capacity to amplify sound stems from a combination of somatic motility and active hair-bundle motility, the ability of a bundle to produce force that augments a mechanical stimulus. One mechanism by which the hair bundle might supplement the energy in a stimulus is fast adaptation, the rapid reclosure of mechatransduction channels after stimulation (Benser et al., 1996; Howard and Hudspeth, 1987). The concerted closure of channels produces a rapid mechanical twitch that augments mechanical stimuli (Benser et al., 1996; Choe et al., 1998; Hudspeth, 2008; Kennedy et al., 2005). Although its mechanism in mammals remains a subject of debate (Bozovic and Hudspeth, 2003; Cheung and Corey, 2006; Corns et al., 2014; Howard and Hudspeth, 1988; Howard and Spudich, 1996; Peng et al., 2013; Tinevez et al., 2007; Wu et al., 1999), fast adaptation is believed to arise from the direct activity of Ca2+ on the transduction channel, an associated relaxation element, or the motors responsible for slow adaptation.

The initial aim of this study was to elucidate the role of Ca2+ in fast adaptation by generating intracellular surges in Ca2+ concentration without altering the state of the mechatransduction channels or the associated components. We used a light-sensitive, Ca2+-loaded cage compound to release Ca2+ in hair cells by ultraviolet irradiation (Ellis-Davies and Kaplan, 1994). To our surprise, irradiation in either the presence or the absence of the caged Ca2+ compound caused rapid motion of hair bundles. Because this unexpected sensitivity to light provides insight into the transduction process and might be useful in stimulating hair cells, we undertook a detailed analysis of its mechanism.

RESULTS

Light-Evoked Hair-Bundle Motion

We studied the responses of hair bundles from the bullfrog’s saccus to pulses of ultraviolet irradiation at a wavelength of 375 nm. When placed within or directly adjacent to a 6 × 12 μm oval beam of ultraviolet light, a hair bundle moved rapidly toward its kinocilium for the duration of the light pulse and relaxed to its original position upon light offset (Figure 1B). In healthy preparations, this phenomenon was observed in over
90% of irradiated hair cells. Light-evoked motion was always directed toward the kinocilium, regardless of a bundle’s orientation within the epithelium (Movie S1). When we irradiated a hair bundle from a direction orthogonal to its plane of symmetry, hair-bundle motion was no different from that elicited by apical mechanical stimulation, the mechanotransduction current adapted as well, ranging from negligible to 300 nm. However, all hair bundles exhibited a linear power-displacement relation at low power densities (Figure 1C). There was no apparent threshold for the movements; instead, a bundle’s displacement response fell into the noise floor as the irradiation decreased.

The structure and transduction mechanism of the hair bundle are highly conserved throughout the vertebrates. Although we conducted most of our experiments on hair cells of the bullfrog’s sacculus owing to the large size and robustness of their hair bundles, we also tested hair cells from the murine utricle as representatives of mammalian hair cells. Irradiation with ultraviolet light produced hair-bundle motions similar to those observed for frog bundles (Figure S1B).

As depicted in a video capturing both a cell’s soma and its hair bundle, light-evoked motion was confined to the bundle (Movie S2). This observation was confirmed by visualizing the light-evoked motion after image subtraction (Figure S1C). Because movement was confined to the hair bundle and restricted to the bundle’s direction of mechanical sensitivity, we suspected that the mechanoelectrical-transduction apparatus was involved in the response. To test this, we examined a hair bundle’s response before and after and after the iontophoretic disruption of tip links by the application of ethylene glycol-bis (β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid (EGTA). After tip-link rupture, a hair bundle no longer moved in response to ultraviolet irradiation, indicating that the mechanotransduction apparatus was required for the response (Figure 1D). To determine whether kinocilia were involved in light-evoked motion, we irradiated three hair bundles before and after microdissection of their kinocilia. We observed no significant changes in the bundles’ responses to ultraviolet illumination (Figure S2).

We next sought to determine whether ultraviolet irradiation opens mechanotransduction channels. Microelectrode recordings revealed that each of 31 hair cells with intact tip links depolarized in response to irradiation, whereas all 18 cells with EGTA-disrupted links did not (Figure 2A). To confirm that depolarization originated from the mechanotransduction current, we performed tight-seal whole-cell recordings from 12 hair cells. Irradiation elicited an inward current that was interrupted by gentamicin, a blocker of mechanotransduction channels (Figures 2B and 2C). The average maximal current evoked by irradiation of 12 cells was 68 pA, as compared to typical values of 150 pA evoked by mechanical stimulation (Assad et al., 1989). As it does with mechanical stimulation, the mechanotransduction current adapted in response to light stimulation (Figure S3). These results confirmed that mechanotransduction channels were the source of the light-evoked electrical activation of hair cells.

Gating springs slacken upon the opening of mechanotransduction channels, resulting in a positive movement of the hair bundle (Howard and Hudspeth, 1988), so the bundle motion evoked by illumination might result from channel gating. To examine this possibility, we simultaneously recorded a hair cell’s membrane potential and its hair bundle’s position while iontophoretically releasing gentamicin to block channel gating. Although the light-evoked receptor potential vanished during a large release of gentamicin, indicating complete blockade of mechanotransduction channels, the corresponding hair-bundle displacement did not decline to zero (Figures 2D and 2E). The
gating of mechanotransduction channels evidently underlay slightly more than half of the light-evoked motion of the hair bundle, with the balance contributed by another process. The displacement evoked by this second process might be enhanced by gentamicin treatment, resulting in a decrement in the decay of hair-bundle displacement relative to the decay of the voltage response. Such a mismatch appears in our data, for the receptor potential decays to zero with 5 nA of iontophoretic current, whereas the displacement is only mildly reduced at that level. We interpret this effect in more detail in the Discussion.

**Intracellular Chromophores**

To identify the molecular underpinnings of light-evoked motion, we sought to localize the cellular components responsible for the conversion of light into hair-bundle motion. We confined ultraviolet irradiation to subcellular regions with a custom-built patterned-illumination system based on a digital micromirror device (Figure 3A). We then positioned an isolated hair cell with its plane of symmetry orthogonal to the beam of light and irradiated distinct areas of the cell while measuring the displacement of its hair bundle. To our surprise, a cell responded maximally to light directed at the apex of its soma but just below its hair bundle (Figures 3B and 3C). Furthermore, the response was minimal when irradiation was confined to the hair bundle. Results from five cells show that irradiation of the cuticular plate produced 70% ± 22% of the motion elicited by total irradiation, whereas irradiation of the hair bundle yielded only 24% ± 14%. The absorbers responsible for hair-bundle motion therefore lay at the hair bundle’s base.

To characterize the molecular absorbers underlying the photo-stimulation of hair cells, we obtained a coarse action spectrum of hair-bundle displacement. We recorded the motion of bundles in response to light in six different wavelength ranges, each encompassing the same stimulus power. The resulting action spectrum revealed that illumination was most efficient near a wavelength of 400 nm (Figure 3D). This sensitivity resembles the absorption spectra of nicotinamide adenine dinucleotide (NADH), flavoproteins, and hemoproteins, the principal intracellular absorbers of ultraviolet light (Liu et al., 2010; Butt and Keilin, 1962; Siegel et al., 1959). Because these molecules fluoresce, we were able to localize them in hair cells through fluorescence microscopy. The chromophores were found to occur primarily near the cuticular plate and the base of a cell, but were absent from the hair bundle (Figure 3E). The two regions of strong fluorescence were dense with mitochondria and lesser numbers of intracellular absorbers of ultraviolet light.
peroxisomes, organelles filled with NADH, flavins, and cytochromes (Figure 3F). These organelles are required to satisfy the heavy metabolic demands of the hair bundle and synaptic machinery. Hair-bundle motion thus appeared to be linked to light absorption by intracellular chromophores in mitochondria and peroxisomes.

**Thermal Pulses**

A portion of the energy delivered to intracellular chromophores by ultraviolet light might be released as heat. By measuring the temperature with the calibrated resistance of a glass micropipette (Yao et al., 2009), we sought to quantitate this effect and determine whether it underlay light-evoked hair-bundle motion (Figure S4). As expected from water’s low absorption of ultraviolet light, irradiation of saline solution did not generate a measurable temperature increase. Irradiation of 25 hair cells, however, resulted in detectable local increases in temperature (Figure 4A). The action spectrum of this effect matched that of the hair bundle’s displacement, suggesting that the same molecular source mediated both bundle motion and heat production (Figure 4B).

Measurements of the light-evoked temperature changes along the sides of three dissociated hair cells revealed that the locale of maximal heat production was near the cellular apex, adjacent to the cuticular plate (Figure 4C). Furthermore, the temperature gradient above a hair cell irradiated in the sensory epithelium matched a model of heat diffusion from mitochondrial sources (Figure 5). If heat arises from the irradiation of intracellular absorbers, we might expect other cell types to experience temperature changes as well. Indeed, ultraviolet illumination of red blood cells and extramacular saccular cells also resulted in local temperature increases (Figure S5A). Although the temperature increase produced by irradiation varied between cell types, the action spectrum of the effect was similar among all cells,
supporting the conclusion that the absorbers responsible for heating were ubiquitous (Figure S5B).

To determine whether the temperature increase elicited by ultraviolet irradiation caused hair-bundle motion, we recorded the response of a hair cell to temperature pulses generated by infrared heating of water. A hair cell was placed in the paths both of an ultraviolet beam traversing the objective lens and of an infrared beam emanating from a 100 μm diameter optical fiber positioned about 100 μm from the hair bundle (Figure 4D). In response to either ultraviolet or infrared irradiation, we simultaneously recorded hair-bundle motion and the temperature at a distance of 2 μm from the hair bundle. With the infrared power adjusted to generate a temperature increase at the hair bundle similar to that caused by the ultraviolet pulse, we observed nearly identical hair-bundle displacements for both wavelengths (Figure 4E).

We confirmed that heating effects hair-bundle motion by exchanging a saline solution based on ordinary water (H2O), the primary absorber of infrared light in our system, with a solution containing deuterium oxide (D2O). Because the absorption coefficient (Bayly et al., 1963) of D2O at 1,470 nm is only 0.5% that of H2O, a smaller temperature increase ensued. For three hair cells in D2O-based saline solution, we measured a reduction of 63% in hair-bundle displacement and a corresponding 60% decrease in the infrared light-evoked temperature increase (Figure 4E). The residual temperature increase in D2O likely stems from the heating of intracellular H2O that was not replaced by the exchange of the extracellular solution. For ultraviolet light, which traverses H2O and D2O about equally well, neither the displacement response nor the temperature increase was altered by the exchange of liquids. This observation confirms that heat and not light was the primary effector of hair bundle motion and that the relevant site of ultraviolet absorption was intracellular.

To further illustrate that a local pulse of heat could evoke hair-bundle motion, we situated the tip of a 5 μm diameter carbon fiber next to a dissociated hair cell. When the fiber’s shaft was heated by illumination with ultraviolet light, the direct thermal stimulus produced hair-bundle motion that disappeared upon retraction of the fiber (Figure 6).

**Effects of Heat**

Heat pulses exert a variety of effects on excitable biological tissue. Because the heat-evoked stimulation of hair cells required intact tip links, it was unlikely to have been mediated by thermally gated channels, changes in membrane capacitance, or other processes independent of the hair bundle. We therefore investigated whether heat pulses stimulated hair bundles by mechanically altering components of the transduction apparatus.

The position of a hair bundle is determined by the forces in two sets of opposing elastic elements, gating springs and stereociliary pivots. Given this arrangement, positively directed motion might ensue either from loosening the gating springs or from stiffening the stereociliary pivots. These alternatives could be differentiated with the help of offset displacements provided by a mechanical-stimulus fiber. If a decrease in tip-link stiffness were to underlie heat-mediated hair-bundle motion, positive offsets would increase the amplitude of motion evoked by the stiffness change, whereas negative offsets would reduce it (Figure 7A). The opposite result would be expected if the thermal motion were to result from an increase in the stiffness of the stereociliary pivots.
To determine which elastic element underlay heat-mediated hair-bundle motion, we irradiated ten hair cells whose bundles had been offset by either a flexible glass fiber or a fluid jet. Because the amplitude of light-evoked motion consistently increased with positive offsets (Figure 7B), heat apparently softened the gating springs, which probably include the tip links and other components in series with them, such as the insertional plaques and adaptation motors. To verify that heat did not alter the stiffness of the stereociliary pivots, we severed the tip links and measured the stiffness of stereociliary pivots before and during irradiation (Figure S6). No significant change in stiffness was detected in any of four experiments.

If a hair bundle were prevented from moving toward its tall edge, relaxation of the gating springs should close the mechano-transduction channels that are open at rest. In support of this hypothesis, when the motion of five hair bundles was arrested by a stiff glass fiber, their light-evoked responses changed from depolarization to hyperpolarization (Figures 7C and 7D).

**DISCUSSION**

The responses presented in this study reflect an unexpected mechanism of hair-cell stimulation by light-evoked heat pulses. During illumination by ultraviolet light, heat is generated by the excitation of intracellular chromophores, the preponderance of which resides in mitochondria (Berns et al., 1970; Salet, 1972). The thermal pulses alter the mechanical properties of the hair bundle, simultaneously opening the transduction channels and displacing the bundle toward its tall edge. This response mechanism relies on the softening of gating springs and is thus specific to hair cells. The transformation of light into thermal energy, however, is a ubiquitous property of cells that depends on their concentration of chromophores. Hair cells may be particularly prone to photothermal stimulation owing to their high concentration of mitochondria. We nonetheless anticipate that other mitochondrion-rich cells, such as myocytes, hepatocytes, and neurons, can respond to irradiation-evoked heating, especially if the cells contain highly temperature-sensitive molecules. Heterogeneity in the density and distribution of mitochondrial absorbers from one hair cell to another likely accounts for much of the variability that we observed in the amplitude and time course of responses.

The ability of a hair cell to respond to thermal pulses hinges upon the high sensitivity of its mechanical antenna, the hair bundle. A bundle’s transduction channels lie in parallel, an arrangement that divides tension equally among the gating springs (Kozlov et al., 2007). When a fraction of these springs relax, the tension is redistributed among the remainder, increasing the open probability of the associated channels. These open channels represent only a fraction of a bundle’s total, as supported by our observation that the maximal current evoked by irradiation is only 45% of that evoked mechanically. The relaxation of gating springs themselves is enough to generate hair-bundle motion. The contribution of gating springs to thermally induced motion can be increased by treatments that raise the baseline tip-link tension, such as the blockage of mechanotransduction pores with gentamicin. The reduction of Ca2+ influx when pores are blocked enhances the activity of myosin motors and increases the tip-link tension. The overall hair-bundle displacement accordingly decays less with gentamicin treatment than does the hair cell’s voltage response (Figure 2D).

Although heat appears to relax the gating springs, the present results are not readily explicable on the basis of that effect alone. Prolonged relaxation of the gating springs should cause a net decrease in channel opening in the steady state. It is thus likely that thermal pulses alter an additional property of the hair bundle. One possibility is that heating both softens the gating springs and promotes channel opening, so that the thermal responses reflect partly antagonistic influences. The amount of heating in these experiments is by itself insufficient to open channels, for severing the tip links abolishes thermal responsiveness. When
probability contraction of actin could relax gating springs. Differences in length between adjacent stereocilia, thermal shrink owing to the negative thermal expansion coefficient of filaments; and Hudspeth, 2003). Finally, the actin cores of stereocilia might grow more compliant, for example, by the release of stabilizing Ca$^{2+}$ ions from protocadherin 15 and cadherin 23 or by partial unfolding of extracellular cadherin domains. Fewer myosin molecules might bind at the insertional plaques atop tip links, thus reducing the stiffness of the assembly (Berger and Hudspeth, 2017). The relaxation elements hypothesized to explain fast adaptation might display thermal softening (Bozovic and Hudspeth, 2017). The relaxation elements hypothesized to explain fast adaptation might display thermal softening (Bozovic and Hudspeth, 2003). Finally, the actin cores of stereocilia might shrink owing to the negative thermal expansion coefficient of filamentous actin (Rosin et al., 2014). As a result of the systematic differences in length between adjacent stereocilia, thermal contraction of actin could relax gating springs.

It is also unclear how a temperature step would alter the open probability $P_o$ of the transduction channels. For a channel considered in isolation, this probability is a function of the difference $\Delta G$ in Gibbs free energy between the channel’s open and closed states:

$$P_o = \frac{1}{1 + e^{\Delta G/kT}} = \frac{1}{1 + e^{\Delta S/kT} e^{-\Delta H/kT}},$$

in which $\Delta H$ and $\Delta S$ are, respectively, the enthalpic and entropic components; $k$ is the Boltzmann constant; and $T$ is the absolute temperature. In the simplest analysis, the temperature dependence of the open probability may be found by differentiating $P_o$ with respect to $T$:

$$\frac{dP_o}{dT} = \frac{-1}{(1 + e^{\Delta S/kT} e^{-\Delta H/kT})^2} (e^{\Delta H/kT} e^{-\Delta S/kT}) \left( -\frac{\Delta H}{kT^2} \right).$$

For a resting open probability of 0.2, the free energy of an open channel exceeds that of a closed one (Corey, 1980) by about 1 $kT$. If this value were entirely enthalpic, the thermal sensitivity of the open probability at room temperature would be only 0.0007 K$^{-1}$. A temperature increase of 10°C, near the largest observed in this study, would raise the open probability by less than 0.01, a value well below that needed to explain our results.

The calculation above does not take into account the intrinsic energy difference between the closed and open states of a channel when it is disconnected from its tip link. This value, $\Delta G^0$, is estimated at 10 $kT$ per molecule (Hudspeth, 1992). Even if this change were entirely enthalpic, the change in open probability would remain less than 0.1 for a 10°C temperature change. Only if the opening transition were to involve a substantial increase in entropy might the difference in intrinsic enthalpy exceed that in free energy. Unfortunately, though, there are no measurements bearing on this issue.

Light-based stimulation of the peripheral auditory system is of clinical interest owing to its potential use in cochlear prostheses (Hernandez et al., 2014; Richardson et al., 2017; Richter et al., 2013). Although infrared light has been used to stimulate a variety of peripheral nerves, the power density required to activate the auditory and vestibular nerves is only a fraction of that required for others (Goyal et al., 2012; Wells et al., 2005). This distinction likely reflects the generation during optical stimulation of optoacoustic pulses that hair cells can detect (Kallweit et al., 2016; Teudt et al., 2011). That ablation of hair cells abolishes the auditory-nerve response to infrared pulses accords with this hypothesis, suggesting that most responses to optical stimulation have been mediated primarily by optoacoustic phenomena (Kallweit et al., 2016; Teudt et al., 2011; Thompson et al., 2015; Verma et al., 2014).

The present results, however, cannot be explained by optoacoustic stimulation. Optoacoustic waves are generated under conditions of thermal confinement, a regime in which heat is deposited in a region faster than it can be removed by diffusion (Paltaluf et al., 1998). The longest irradiation pulse permitting thermal confinement is given by

$$\tau_m = \frac{d^2}{4k},$$

where $d$ is the diameter of the fiber.
in which $\delta$ is the beam’s radius or the optical penetration depth of the irradiating light, whichever is smaller, and $\kappa$ is the thermal diffusivity of water (Paltauf et al., 1998), $1.43 \times 10^{-7}$ m$^2$·s$^{-1}$. The primary absorber in mitochondria is cytochrome $c$, with a molar absorption coefficient (Butt and Keilin, 1962) of 0.06 M$^{-1}·$cm$^{-1}$ at 405 nm. The absorption coefficient for cytochrome $c$ at a concentration of 100 $\mu$M in mitochondria (Gupte and Hackenbrock, 1988) is $6 \times 10^{-6}$ cm$^{-1}$. For a mitochondrion-filled cylinder of diameter 10 $\mu$m and height 5 $\mu$m at the apex of a hair cell, $\delta$ is 5 $\mu$m. $\tau_\text{rel}$ is therefore 43 $\mu$s, or only one-thousandth our typical pulse duration of 40 ms. Thermal confinement should not be possible and no optoacoustic wave should be generated. This analysis is supported by our observation that hair bundles do not respond to light after their tip links have been ruptured. If hair-bundle motion arose from optoacoustic stimulation, the response would persist after that treatment.

Two alternative mechanisms have been proposed for hair-cell stimulation by irradiation. In the first, light evokes a rapid heat pulse that alters the membrane’s capacitance and depolarizes the cell (Rabbitt et al., 2016). The change in capacitance stems from asymmetric movement of ions present on the two sides of the plasma membrane, a general mechanism that does not require ion channels (Liu et al., 2014; Shapiro et al., 2012). The second proposition is that illumination releases Ca$^{2+}$ from mitochondrial stores (Rajguru et al., 2011). Although light-induced Ca$^{2+}$ release has not been observed in hair cells, it occurs in cardiomyocytes as well as vestibular- and spiral-ganglion neurons (Dyttami et al., 2011; Lumbbreras et al., 2014). Neither of these two mechanisms can explain the results presented in this study. If a change in cellular capacitance elicited the response to light, neither tip-link rupture nor transduction-channel blockage would eliminate the response. Similarly, if Ca$^{2+}$ release from intracellular stores were responsible for hair-cell stimulation, the electrical response would persist after tip-link rupture. We observed instead that the thermal response requires the integrity of tip links and the potency of the transduction-channel pores.

Although we have focused on the effects of heating on mechanoelectrical transduction, we anticipate that illumination of the mitochondria elsewhere in hair cells also has effects. In particular, irradiation of the mitochondria clustered at the basal ends of these cells might cause heating and Ca$^{2+}$ release in the region of the ribbon synapses. The resultant enhancement of

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Figure 7. Effect of Heating the Gating Springs

(A) A hair bundle can be modeled as a pair of opposing elastic elements: the gating springs that tug the bundle in the negative direction and the stereociliary pivots that pull in the opposite direction. When a hair bundle undergoes a positive movement in response to illumination, the distinct effects of offsets imposed by a flexible fiber of stiffness $K_{\text{FIBER}}$ (upper traces) distinguish a decrease in the stiffness of the gating springs ($K_{\text{GS}}$, middle traces) from an increase in the stiffness of the stereociliary pivots ($K_{\text{SP}}$, lower traces). Arbitrary parameter values: $K_{\text{GS}} = 100; X_{\text{GS}} = -1; K_{\text{SP}} = 100; X_{\text{SP}} = 1; K_{\text{FIBER}} = 100$; fiber-base offset commands $-2, -1, 0, 1$, and 2; au, arbitrary unit. To model a reduction in gating-spring stiffness during the laser pulse, we diminished $K_{\text{GS}}$ by 50%; to represent an increase in stereociliary-pivot stiffness, we augmented $K_{\text{SP}}$ by 50%.

(B) While exposed to iontophoretically applied gentamicin to block transduction, an actual hair bundle of a hair cell in the saccular epithelium was offset with a flexible glass fiber (upper traces). Fifty milliseconds after the initiation of the offset, the bundle was irradiated with ultraviolet light for 40 ms. The resultant hair-bundle motion (lower traces) accorded with the expectation for a heat-induced decrease in gating-spring stiffness. Iontophoretic current, 70 nA; fiber stiffness, 280 $\mu$N·m$^{-1}$; wavelength, 405 nm; power density, 83 MW·m$^{-2}$.

(C) A pulse of ultraviolet light depolarized a hair cell in the saccular epithelium whose hair bundle was free to move (black). When the same bundle was prevented by a stiff glass fiber from moving toward its kinocilium, identical irradiation instead produced hyperpolarization (red).

See also Figure S6.
transmitter release could make an additional contribution to the eighth-nerve responses to light.

The phenomenon described here, which permits stimulation of hair cells without genetic manipulation or mechanical contact, allows previously impossible studies. For example, irradiation might be used to study the activity of mammalian cochlear hair cells without the generation of a traveling wave. In addition, the ability to rapidly and synchronously stimulate groups of hair cells would facilitate studies of processes subsequent to the receptor potential, such as synaptic transmission. Finally, irradiation could be employed to verify that a hair cell’s transduction apparatus is intact.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- CONTACT FOR REAGENT AND RESOURCE SHARING
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
- METHOD DETAILS
  - Hair-cell isolation
  - Folded-epithelium preparation
  - Microscopic apparatus
  - Photometric recording
  - Electrophysiology
  - Mechanical stimulation
  - Signal production and acquisition
  - Kinociliary dissection
  - Ultraviolet and infrared stimulation
  - Patterned illumination
  - Action spectra
  - Fluorescence imaging
  - Temperature measurement
  - Heat application by carbon fiber
  - Modeling of the thermal pattern around an irradiated hair bundle

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes six figures and two movies and can be found with this article online at https://doi.org/10.1016/j.neuron.2018.01.013.

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**AUTHOR CONTRIBUTIONS**

J.B.A. and A.J.H. designed the experiments, B.A.F. wrote the software, B.A.F. and J.B.A. prepared the specialized apparatus, J.B.A. and N.R.K. conducted the experiments, and J.B.A. and A.J.H. wrote the paper and prepared the figures.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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**REFERENCES**


STAR METHODS

KEY RESOURCES TABLE

<table>
<thead>
<tr>
<th>REAGENT or RESOURCE</th>
<th>SOURCE</th>
<th>IDENTIFIER</th>
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<tbody>
<tr>
<td>Chemicals, Peptides, and Recombinant Proteins</td>
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</tr>
<tr>
<td>Protease XXIV</td>
<td>Sigma-Aldrich</td>
<td>P8038</td>
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<tr>
<td>Fish-oil-based lubricant</td>
<td>WD-40 Company</td>
<td>WD-40</td>
</tr>
<tr>
<td>Experimental Models: Organisms/Strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>American bullfrogs (Rana catesbeiana)</td>
<td>Rana Ranch</td>
<td>N/A</td>
</tr>
<tr>
<td>Mice (Mus musculus)</td>
<td>Charles River Laboratories</td>
<td>C3H/HeNCl</td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td></td>
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<td>Electron Microscopy Sciences</td>
<td>G100-Au</td>
</tr>
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<td>Prizmatix</td>
<td>UHP-LED-630</td>
</tr>
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<td>Charge-coupled-device camera</td>
<td>Olympus</td>
<td>OLY-150</td>
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<td>Redlake</td>
<td>Motionscope 2000S</td>
</tr>
<tr>
<td>Bessel electronic filter</td>
<td>Kemo</td>
<td>BenchMaster 8</td>
</tr>
<tr>
<td>Heterodyne interferometer</td>
<td>Polytec</td>
<td>OFV 501</td>
</tr>
<tr>
<td>Piezoelectric actuator</td>
<td>Piezosystem Jena</td>
<td>PA 120/14 SG</td>
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<tr>
<td>Power amplifier</td>
<td>Piezosystem Jena</td>
<td>ENV 300SG</td>
</tr>
<tr>
<td>Electrophysiological amplifier</td>
<td>Axon Instruments</td>
<td>Axoclamp 2B</td>
</tr>
<tr>
<td>Electrode puller</td>
<td>Sutter Instruments</td>
<td>P-80/PC</td>
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<tr>
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<td>World Precision Instruments</td>
<td>1B120F-3</td>
</tr>
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CONTACT FOR REAGENT AND RESOURCE SHARING

Requests for information and resources should be directed to the Lead Contact, A.J. Hudspeth (hudspaj@rockefeller.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

All procedures were approved by the Institutional Animal Care and Use Committee of The Rockefeller University. Sacculi were extracted from adult male and female bullfrogs (Rana catesbeiana) as described (Azimzadeh and Salvi, 2017). Dissections were performed in artificial perilymph containing 114 mM Na⁺, 2 mM K⁺, 2 mM Ca²⁺, 118 mM Cl⁻, 5 mM HEPES, and 3 mM D-glucose. The pH was 7.2-7.3 and the osmolality was 230 mOsm·kg⁻¹. Once isolated from the inner ear, each saccular macula was digested in
medially and the roof of the liberated utriculus was removed. Following a 20 min incubation in 100 mg
ulus was extracted from the skull into ice-cold Hank’s balanced salt solution (HBSS, Thermo Fisher). The otic capsule was opened
were anesthetized with isoflurane, euthanized by cervical dislocation, and decapitated. Under a dissecting microscope, each utric-
measured by a heterodyne interferometer (ENV 300SG, Piezosystem Jena; OFV 501,
image in 10
filtered at 4 kHz with a Bessel filter (BenchMaster 8, Kemo). The photodiode signal was calibrated by translating a bundle’s
Hair cells were visualized with differential-interference-contrast optics with a 60X objective lens on an upright microscope (BX51WI,
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Electrophysiology
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### METHOD DETAILS

#### Hair-cell isolation

Frog maculae destined for experiments on isolated hair cells were dissected in perilymph containing 100 μM free Ca\(^{2+}\). After removal of the inner ear, the semicircular canals were trimmed to the bases of their ampullae, which were sealed with cyanoacrylate adhesive expelled from a fine glass pipette. The pipette was fabricated by pulling a glass capillary to an external diameter of 100-200 μm. Before being filled with cyanoacrylate adhesive, the pipette’s tip was transiently filled with lubricant (WD-40 Company). Adhesive was delivered to the trimmed ampullae by positive pressure.

After all the ampullae had been glued shut, the perilymphatic cistern was removed and the inner ear was placed for 12 min at 22°C in Ca\(^{2+}\)-free solution consisting of 114 mM Na\(^+\), 2 mM K\(^+\), 1 mM EGTA, 1 mM Mg\(^{2+}\), 120 mM Cl\(^-\), 3 mM D-glucose, and 5 mM HEPES at pH 7.2. Following the return of the inner ear to 100 μM Ca\(^{2+}\) solution, the saccular macula was isolated and digested in 67 mg·L\(^{-1}\) protease XXIV (P8038, Sigma) for 30 min at room temperature.

For experiments with mechanically isolated hair cells, the saccular macula was held with pins in a chamber with a concanavalin A-coated slide at its bottom. Hair cells were gently flicked from the sensory epithelium with a fine eyelash and allowed to settle for 10 min.

When extruded hair cells were sought, the macula was secured in a chamber by two mounting pins, which applied a slight tension that encouraged hair cells to gradually extrude from the epithelium. Cellular extrusion commenced approximately 20 min after mounting and continued for several hours. The liberated hair cells could be used in situ or individually held in a polished glass pipette. The pipette was prepared by pulling a pair of fine borosilicate microelectrodes, one of which was used to scratch and cleave the other at a location where its inner diameter was 9 μm. The broken pipette was fire-polished smooth, filled with saline solution, and mounted in an electrode holder. Hair cells were picked up and held by pipettes under gentle negative pressure.

#### Folded-epithelium preparation

The saccular macula was dissected and digested as above. By careful probing with the tip of a fine eyelash, the saccular epithelium was separated from the underlying connective tissue and folded along its plane of mirror symmetry. A gold electron-microscopy grid (G100-Au, Electron Microscopy Sciences) was rested upon the folded epithelium to stabilize it and maintain the crease (Kozlov et al., 2007). Hair cells were visualized from a direction orthogonal to their axes of sensitivity.

#### Microscopic apparatus

Hair cells were visualized with differential-interference-contrast optics with a 60X objective lens on an upright microscope (BX51WI, Olympus). For recordings of hair-bundle displacement, preparations were illuminated with a 630 nm light-emitting diode (UHP-LED-2007). Hair cells were visualized from a direction orthogonal to their axes of sensitivity.

#### Photometric recording

When the sample was illuminated as described above, light piping through stereocilia resulted in a high-contrast image of a hair bundle’s tip that was imaged on the photodiode at a magnification of 1250X. Output from the photodiode was low-pass filtered at 4 kHz with a Bessel filter (BenchMaster 8, Kemo). The photodiode signal was calibrated by translating a bundle’s image in 10 μm steps with a mirror mounted on a piezoelectric actuator (PA 120/14 SG, Piezosystem Jena). This actuator was driven by a 300 mA amplifier and calibrated by a heterodyne interferometer (ENV 300SG, Piezosystem Jena; OFV 501, Polytec).

#### Electrophysiology

Hair-cell currents were recorded with tight-seal, whole-cell electrodes filled with potassium gluconate internal solution containing 117 mM K\(^+\), 2 mM Na\(^+\), 4 mM Mg\(^{2+}\), 117 mM gluconate, 1 mM ATP, 2 mM Cl\(^-\), 5 mM EGTA, and 5 mM HEPES at pH 7.2-7.3. Electrodes were pulled to a tip resistance of 3-8 MΩ with an electrode puller (P-2000, Sutter Instruments) and were coated with

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Neuron 97, 586–595.e1–e4, February 7, 2018 e2
nail polish to decrease the transmural capacitance. Hair cells were held at –70 mV with a voltage-clamp amplifier (Axopatch 200B, Molecular Devices) without compensation for the diffusional tip potential.

Sharp microelectrodes were pulled with an electrode puller (P-80/PC, Sutter) and their tips were bent through an angle of 60° to allow vertical insertion into the hair-cell apex (Hudspeth and Corey, 1978). When filled with 3 M KCl, tip resistances were 100–300 MΩ. Intracellular voltage was recorded by a DC-coupled amplifier (Axoclamp 2B, Axon Instruments) set to pass 0–3 kHz.

We used iontophoresis to release EGTA and gentamicin onto hair bundles. An iontophoretic electrode was fabricated from a glass capillary pulled with an electrode puller (P-80/PC, Sutter Instruments) and filled with 500 mM EGTA in 1 M NaOH or with 500 mM gentamicin sulfate. The electrode’s tip was placed approximately 3 μm from the top of a hair bundle. An amplifier was used to pass currents of up to ±100 nA through the electrode to elicit release of its contents (Axoclamp 2B; Axon Instruments). A holding current was used between current pulses to prevent leakage of the electrode’s contents onto the hair bundle.

**Mechanical stimulation**

Mechanical stimuli were delivered by flexible or stiff glass fibers fabricated from borosilicate capillaries (1B120F-3, World Precision Instruments). A flexible probe was made by first thinning a capillary with an electrode puller (P-2000, Sutter Instruments) and subsequently pulling its tip laterally with a 120 V solenoid to form a 90° angle with the capillary shaft. Each probe was 0.5–0.8 μm in diameter and no greater than 100 μm in length. To increase optical contrast, a probe was sputter-coated with gold-palladium (Hummer 6.2, Anatech); its stiffness was 250 μN·m⁻¹. A stiff probe was made by pulling a borosilicate capillary to a tip diameter of 1–2 μm. The tip was fire-polished to a diameter of 1 μm and attached to a kinociliary bulb with light suction.

Flexible probes were calibrated by imaging their Brownian motion on the dual photodiode. A Lorentzian fit to the power spectrum of this motion yielded estimates of the probe’s stiffness and drag coefficient (Howard, 2001; Salvi et al., 2015). Probes were displaced by a piezoelectric actuator (PA 4/12, Piezosystem Jena) driven by a 800 mA amplifier (ENV 800, Piezosystem Jena). The actuator was mounted on a micromanipulator (MP-285, Sutter Instruments) to control the fiber’s position. The control signal sent to the amplifier was digitally low-pass filtered at 2 kHz.

**Signal production and acquisition**

All stimuli were controlled with and data were acquired by programs written in LabVIEW (version 16.0; National Instruments). Data were digitized by an analog-to-digital converter and sampled at 50 μs intervals (PCIe-6353, National Instruments). Stimulation and control signals were generated with a digital-to-analog converter (PCI-6733, National Instruments).

**Kinociliary dissection**

We separated the kinocilium from a hair bundle by first severing the links attaching it to the tallest stereocilia and then forcing it away from the bundle (Hudspeth and Jacobs, 1979). These manipulations were performed with a fine glass pipette pulled with an electrode puller (P-80/PC, Sutter Instruments). The kinociliary links were severed by first sliding the pipette tip between the kinocilium and the tallest stereocilia at a height of 2 μm from the cell surface and then moving the pipette upward through the kinociliary links.

**Ultraviolet and infrared stimulation**

We used a 375 nm diode laser (DM-375, Oxxius) or a 405-nm diode laser (DL-405-100, Crystalaser) to irradiate hair cells with ultraviolet light. Laser beams were focused to the back focal plane of the objective lens to create a collimated beam exiting the objective lens.

We irradiated cells with infrared light from a 1470 nm diode laser (MDL III 1470, Changchun New Industries Optoelectronics Technology Company). The light was delivered with a 105 μm-diameter optical fiber with a flat-cleaved tip (FG105LCA, Thorlabs).

**Patterned illumination**

We irradiated hair cells with customized illumination patterns by reflecting the light of a 405 nm laser diode (DL-405-100, Crystalaser) from a digital micromirror device (DLP2010, Texas Instruments). Light reflected from micromirrors in the “on” position was collected behind the epifluorescence port of the microscope by a lens (XFLuor 4X, Olympus) to form an image of the surface of the digital micromirror device. This image was relayed and demagnified onto the sample plane through an aspheric lens (ASL-10142-A, Thorlabs) and the objective lens (LumPlanFLN60X, Olympus). Micromirror patterns were loaded on a digital controller (DLPC3435, Texas Instruments) with LabVIEW software (version 16.0, National Instruments).

The micropatterns were visualized by projecting them onto a fluorescein-coated slide and imaging the resulting fluorescence. During an experiment key landmarks such as the boundaries between adjacent rectangles of irradiation were marked on a video screen bearing an image of the sample plane. Each hair cell was then aligned with these landmarks prior to stimulation. In addition, images of the micropattern location and hair cell position were taken in each experiment for post hoc verification of the positioning.

The responses to stimulation with the digital micromirror device were relatively small owing to our use of a mercury lamp, the luminance of which was lower than that of the lasers. In addition, some light was lost upon reflection from the micromirrors.
Action spectra

Action spectra were elicited with six illumination bands filtered from the emission of a mercury lamp (X-Cite exacte, Excelitas Technologies). The mercury lamp was mounted on the epifluorescence port of the microscope and focused with a lens (AC254-250, Thorlabs) placed 250 mm from the objective lens. Broadband light was filtered and reflected into the sample with six filter and dichroic-mirror sets to give rise to the following wavelength bands (in nanometers): 350-390, 395-415, 426-446, 480-520, 543-557, and 568-590. The filters and dichroic mirrors used in each set were:

- FF01-370/36-25, T425lpxr, ET425lp
- ET405/20x, T425lpxr, ET430lp
- ET436/20x, T470lpxr, ET460lp
- ET500/40x, T525lpxr, ET542lp
- ET550/15x, T570lpxr, ET570lp
- ET580/25x, T605lpxr, ET610lp

All filters and mirrors were obtained from Chroma Technology except the first, which was from Semrock.

To ensure that each wavelength band passed the same intensity of light, the power output in each band was measured using a photodiode-based power meter (S120VC and PM100A, Thorlabs) at all input-command levels of the mercury lamp. The highest power achievable in every band, 5.9 mW, was chosen as the irradiation level. The input command to the mercury lamp was then adjusted to the appropriate value before irradiation with each wavelength band.

Fluorescence imaging

Intracellular chromophores were imaged by epifluorescence excited with a mercury lamp (X-Cite exacte, Excelitas Technologies). NADH was excited at 350-390 nm (FF01-370/36-25, Semrock) and imaged at 425-475 nm (ET450/50 m, Chroma). Flavins and hemoproteins were excited at 426-446 nm (ET436/20x, Chroma) and imaged at 510-560 nm (ET550/50 m). Images were captured with a CCD camera (ORCA-R2, Hamamatsu).

Temperature measurement

The calibrated resistance of an electrode was used to measure temperature (Yao et al., 2009). Electrodes were pulled (P-2000, Sutter Instruments) and filled with saline solution identical to that being used in the experiment. Tip resistances ranged from 4-10 MΩ as determined by measuring the current required to elicit a 5 mV voltage drop across the pipette tip with a voltage-clamp amplifier (Axopatch 200B, Molecular Devices). Each electrode was calibrated by recording its resistance at several temperatures. The electrode tip was placed in approximately 2 mL of saline whose temperature was controlled with a chilling and heating dry bath (Echotherm IC20XT, Torrey Pines). A linear fit to the relation of the logarithm of resistance to the reciprocal of temperature yielded the relation between temperature and resistance.

Heat application by carbon fiber

To rapidly heat cells without irradiating them, we applied local heating pulses with a carbon fiber. Because carbon is an efficient ultraviolet absorber and heat conductor, thermal pulses generated by irradiating a fiber’s shank rapidly propagated to its tip. We mounted a single 4.8 µm-diameter carbon fiber (C 005731, Goodfellow) in a pulled glass pipette and positioned its tip 2-5 µm from a hair bundle. The fiber was irradiated approximately 20 µm from its tip with 405 nm light from a laser diode (DL-405-100, Crystalaser). We adjusted the power density of irradiation to elicit the desired temperature increase at the fiber’s tip.

Modeling of the thermal pattern around an irradiated hair bundle

For comparison with the measured pattern of temperature changes around a hair bundle, we modeled the steady-state temperature change expected from sources in the mitochondria at the cellular apex. For modest temperature changes, the flow of heat follows the diffusion equation, whose linearity implies that the response at each point in space can be obtained by summing the individual contributions of multiple sources. In an aqueous medium of thermal conductivity $k = 0.60 \text{ W} \cdot \text{m}^{-1} \cdot \text{K}^{-1}$, the temperature increment $\Delta T$ owing to a point source emitting an energy flux $f$ at distance $r$ is

$$\Delta T = \frac{f}{4\pi kr}.$$

We calculated the steady-state temperature change expected for an annular array of 476 point sources, each of 250 nW, surrounding the base of the hair bundle on a 0.25 µm square lattice between radii of 2.5 µm and 4.0 µm (Figure 5B). The offset of approximately 6°C in the recorded temperatures (Figure 5A) presumably reflects the background of heat production by irradiated mitochondria in the lower portions of the illuminated hair cell as well as in the adjacent hair and supporting cells.
Neuron, Volume 97

Supplemental Information

Thermal Excitation of the Mechanotransduction Apparatus of Hair Cells

Julien B. Azimzadeh, Brian A. Fabella, Nathaniel R. Kastan, and A.J. Hudspeth
Figure S1. Ultraviolet stimulation of hair bundles, Related to Figure 1.

(A) A hair cell that had been extruded from the epithelium was irradiated orthogonal to its plane of symmetry. The resulting hair-bundle motion along the axis of sensitivity, which was recorded on a dual photodiode, confirmed that photonic force did not underlie the light-evoked response. Wavelength, 375 nm; power density, 106 MW·m⁻². (B) A hair bundle from the murine utriculus moved in response to ultraviolet illumination. The bundle remained in the utricular epithelium and was irradiated from a direction orthogonal to the plane of the utriculus. Wavelength, 405 nm, power density, 57 MW·m⁻². (C) Subtraction of a video frame acquired during laser irradiation of a hair bundle (center) from that before irradiation (left) reveals light-evoked motion (right) that was maximal at the top of the hair bundle. The soma did not move detectably. Irradiation was orthogonal to the hair bundle’s axis of sensitivity. Frame rate, 250 Hz; scale bar, 5 μm; wavelength, 375 nm; power density, 106 MW·m⁻².
Figure S2. Absence of a contribution from the kinocilium, Related to Figure 1.

(A) The movement of a hair bundle was recorded in response to pulses of ultraviolet light at two power densities, 35 MW·m⁻² and 71 MW·m⁻² (upper traces). The bundle's motion was similar after its kinocilium had been carefully separated from the stereociliary cluster with a sharp glass microelectrode (lower traces). (B) Held against the epithelial surface, the kinocilium's bulbous end (arrowhead) lay immediately to the right of the dissection electrode's tip. Wavelength, 375 nm. This cell remained in the saccular epithelium and was irradiated from a direction orthogonal to the plane of the sacculus.
Figure S3. Adaptation of the light-evoked mechanotransduction current, Related to Figure 2.

(A) Representative light-evoked currents from three hair cells showed adaptation during protracted illumination. Wavelength, 375 nm; power density, 106 MW·m⁻². (B) During a simultaneous recording of light-evoked current (black) and hair-bundle displacement (red), the bundle moved 54 nm and the transduction current reached -109 pA. To allow comparison of time courses the current trace has been inverted and both records have been normalized to their maxima. Wavelength, 375 nm; power density, 106 MW·m⁻². The cells were extruded from the epithelium and were irradiated from a direction orthogonal to the axis of sensitivity of their hair bundles.
Figure S4. Calibration of a temperature-sensing microelectrode, Related to STAR Methods.

The glass electrode’s tip resistance was measured in saline solution at various temperatures. A plot of the logarithm of the resistance divided by the reference value $R_0 = 77.62$ kΩ against the reciprocal of the temperature yields the linear relation $\log_{10}(R/R_0) = 662.83/T$ with coefficient of determination $r^2 = 0.998$. 
Figure S5. Heat production through ultraviolet irradiation of other cell types, Related to Figure 5.

(A) Irradiation of saline solution did not lead to a measurable increase in temperature. However, irradiating extramacular cells, hair cells, or red blood cells generated temperature increases. Temperature was measured using a calibrated electrode at a distance of 2 μm from the cellular surface. Wavelength, 375 nm; power density, 106 MW·m⁻². The lowest calibration bar pertains only to the erythrocyte. (B) Thermal action spectra show that the temperature increases (∆T) measured above a supporting cell and an extramacular cell are similar in shape to that of a hair cell, indicating that the responsible absorbers are ubiquitous.
Figure S6. Negligible effect of ultraviolet irradiation on stereociliary pivots, Related to Figure 7.

A hair bundle with tip links disrupted by iontophoresis of EGTA was displaced with a calibrated glass fiber. The force required to move the bundle is plotted against its displacement. In this hair bundle, the stiffness of the stereociliary pivots was 224 μN·m⁻¹ without irradiation (black) and 221 μN·m⁻¹ during ultraviolet irradiation (red). The same experiment was repeated in four cells and yielded an average stiffness of 289 ± 64 μN·m⁻¹ without irradiation and 291 ± 76 μN·m⁻¹ during ultraviolet illumination, confirming that light did not alter this value. The hair cells for this experiment remained in the saccular epithelium and were irradiated from a direction orthogonal to the plane of the sacculus. Wavelength, 405 nm; power density, 70 MW·m⁻².