A Population Representation of Absolute Light Intensity in the Mammalian Retina

Graphical Abstract

Highlights

- M1 ipRGCs code a broad range of light intensities as a population
- Depolarization block constrains the range of single cells
- Dynamics of block onset and reversal confer response flexibility and efficiency
- Distortion of spike output during partial block is removed by axonal propagation

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In Brief

M1 photoreceptors sense environmental light intensity, but individual cells have ambiguous outputs and limited ranges. Differential tuning across cells, arising from intrinsic depolarization block and varied sensitivity, allows them to divide labor and code a broad range of intensities collectively.
A Population Representation of Absolute Light Intensity in the Mammalian Retina

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SUMMARY

Environmental illumination spans many log units of intensity and is tracked for essential functions that include regulation of the circadian clock, arousal state, and hormone levels. Little is known about the neural representation of light intensity and how it covers the necessary range. This question became accessible with the discovery of mammalian photoreceptors that are required for intensity-driven functions, the M1 ipRGCs. The spike outputs of M1s are thought to uniformly track intensity over a wide range. We provide a different understanding: individual cells operate over a narrow range, but the population covers irradiances from moonlight to full daylight. The range of most M1s is limited by depolarization block, which is generally considered pathological but is produced intrinsically by these cells. The dynamics of block allow the population to code stimulus intensity with flexibility and efficiency. Moreover, although spikes are distorted by block, they are regularized during axonal propagation.

INTRODUCTION

The intensity of illumination changes over many log units with each rotation of the earth, driving differences in environmental parameters such as temperature and the types of species that are active. Tracking irradiance therefore provides a survival advantage, and mammals do so in order to control functions that are as diverse as sleep regulation, hormonal adjustment, neuromodulation, and pupillary constriction (Lucas et al., 2014). Irradiance is also the principal cue for synchronizing the circadian clock with the solar day and thereby helps to maintain appropriate patterns of gene expression throughout the body; clock dysregulation is linked to disorders that range from metabolic to psychiatric in nature (Takahashi et al., 2008). Despite the importance of coding irradiance over its broad span, the underlying mechanisms have remained unclear even within the well-characterized circuitry of the retina. Indeed, most investigations of dynamic range in the visual system have focused on the opposite question: how irradiance information is omitted, such that objects appear invariant across lighting conditions (Rieke and Rudd, 2009).

The neural representation of irradiance became more tractable with the discovery of intrinsically photosensitive retinal ganglion cells (ipRGCs) (Berson et al., 2002). Like conventional RGCs, they integrate signals originating with the rod and cone photoreceptors and transmit information to the brain using electrical spikes. IpRGCs also possess a phototransduction cascade that is initiated by the visual pigment, melanopsin. When the melanopsin gene is deleted, ipRGCs no longer produce their own responses to light. As a result, functions such as circadian and pupillary control are abnormally transient and have a truncated dynamic range, failing to reach their maxima in bright light (Keenan et al., 2016; Mrosovsky and Hattar, 2003; Panda et al., 2002). Conversely, these functions can be driven steadily and maximally by the intrinsic responses of ipRGCs alone, even if rods and cones are inoperative and there is no visual perception (Freedman et al., 1999; Keenan et al., 2016; Lucas et al., 1999). When ipRGCs are ablated, these functions are essentially blind (Güler et al., 2008; Hatori et al., 2008). Thus, there is compelling evidence at the level of the whole organism for irradiance coding by ipRGCs (reviewed further in Do and Yau, 2010).

The view from direct examination of ipRGCs has been less clear. These cells have been reported to spike at a rate that is proportional to irradiance (Berson et al., 2002; Dacey et al., 2005; Do and Yau, 2013) and to do so continuously (Wong, 2012). On the other hand, suppression of spiking has been observed at higher irradiances (Do and Yau, 2013; Wong et al., 2005)—which is where ipRGCs are thought to have the largest impact on behavior (Keenan et al., 2016; Panda et al., 2002). This suppression is reminiscent of depolarization block, in which the excitation level is so high that the mechanism of spike generation fails. Block is generally considered to accompany abnormal states (Bragin et al., 1997). However, there are hints that ipRGCs produce it with their own phototransduction cascade (Do and Yau, 2013; Wong et al., 2005). Experiments have not been done to study this phenomenon systematically or to reconcile it with the notion that ipRGCs signal continuously throughout the day. We have developed methods to study the axonal output of ipRGCs and provide an understanding of how irradiance is coded in the mammalian visual system. Depolarization block does indeed limit the dynamic range of individual cells but unexpectedly allows the population to represent a broad range of irradiances with flexibility and efficiency.
RESULTS

Defining the Spike Outputs of M1 IpRGCs through Axonal Recordings

To identify ipRGCs for electrophysiological recording, we used a transgenic mouse line in which the melanopsin gene locus drives expression of the fluorescent protein, tdTomato (Do et al., 2009). We focused on ipRGCs of the M1 type because they are key mediators of circadian regulation, pupillary control, and other functions that are driven by irradiance (STAR Methods) (Chen et al., 2011); the brain projections and response properties of the other types appear specialized for different roles (Ecker et al., 2010; Schmidt et al., 2014). Somatic recording is customary but provides a limited view of spikes in the axon that carry information to the brain. Accessing the soma also requires disruption of supporting cells and direct exposure of the photosensitive somatodendritic compartment to imaging light. Therefore, we developed a method of recording from axons in the isolated retina, one inspired by work in brain slices (Shu et al., 2006). We placed a shallow cut near the head of the optic nerve to produce a field of axon endings and drew one into the electrode for loose-patch recording (Figure 1A). Unless otherwise noted, we examined one cell per retina for strict control over light history.

Figure 1. Irradiance Tuning of M1 IpRGCs

(A) Left: Schematic of the experimental configuration for recording from distal axons (loose-patch mode, 35°C). Right: A single M1, electroporated with Alexa 488 through the axonal recording site at the conclusion of an experiment and imaged live by epifluorescence. Arrowheads show the trajectory of the axon (810 μm in length). Scale bar, 100 μm.

(B) Histogram of spike rate measured from the axon of a monotonically tuned M1, evoked by ascending irradiances (stimulus schematic at top). 1 s bins (blue) and 5 s moving average (black). Online estimate of firing threshold, I_F, was 3.7 (in units of log photons μm^2 sec^-1, with a log base of 10, unless otherwise specified).

(C) Excerpts of traces from the last 30 s of each step.

(D and E). As in (B) and (C), respectively, but for a unimodally tuned M1, I_F = 1.3.

(F) Axonal irradiance-firing (IF) relations of the representative monotonic (blue) and unimodal (red) M1s for ascending irradiance. Plotted are firing rates averaged over the last 30 s of each step. See Figure S1 for the stability of IF relations over repeated stimulations, and simultaneous axonal recordings from M1s that displayed similar or different tuning curves.

(G) Overlaid spike waveforms from (D) during sustained (i) and falling (ii) firing rates.

(H) Steady firing rate at the peak of the irradiance pyramid (10^4-fold I_F) in recordings with synaptic antagonists excluded (n = 27 cells, top) or included (n = 26 cells, bottom). Synaptic antagonists included for (B)–(G). See Figure S2 for additional data obtained without antagonists.
equivalent to moonlight and the brightest to direct sunlight (STAR Methods). The responses of M1s develop slowly, necessitating long (2 min) steps of light to approach steady state. They also sensitize and desensitize over extended timescales (Do and Yau, 2013; Emanuel and Do, 2015; Wong et al., 2005), and we examined the impact of this light adaptation by following the ascending staircase of irradiances with a descending one, forming a pyramid.

**M1 IpRGCs Display Distributed and Intrinsic Tuning to Irradiance**

Some M1s responded to ascending light intensity in a simple manner: the rate of spike firing rose to a plateau that was relatively stable, thus describing a monotonic irradiance-firing (IF) relation (Figures 1B, 1C, and 1F). Most M1s responded differently. The spike rate rose and then sharply fell as irradiance increased, yielding a unimodal relation (Figures 1D–1G). We consider a cell to show monotonic tuning if its firing rate remains within a quarter of its maximum, both at the apex of the irradiance pyramid and during subsequent exposure to 10-fold brighter light. By this criterion, unimodal M1s composed the majority of our sample, with most cells showing complete silencing of axonal spikes at the apex irradiance. Both monotonic and unimodal tunings are cell autonomous, because they are found in similar proportions whether antagonists of synaptic transmission are included or not (Figure 1H; n = 19/26 cells with antagonists and 20/27 cells without). Unless otherwise noted, subsequent experiments are performed with antagonists to focus on the intrinsic properties of these cells.

Within the monotonic and unimodal variants, IF relations have conserved shapes (Figures 2A and 2B). For example, given the irradiance that produces one aspect of the IF curve, those that produce others can be predicted with some accuracy (e.g., threshold activation and half-maximal activation have a correlation coefficient of 0.9; Figures 2C–2E). Across variants, they are similar in their rising phases and diverge only near their maxima, with monotonic relations approaching their plateaus and unimodal relations initiating their descents. Hence, the mechanisms that distinguish these tuning curves appear to manifest when firing rates are high.

In contrast to their conservation of shape, the IF relations of M1s are widely dispersed across the irradiance axis (Figures 2A and 2C–2E). The most sensitive cell in our sample was unimodal; it activated in the equivalent of moonlight (~1.1 log photons μm⁻² sec⁻¹), fired maximally at the threshold of twilight (~3.1), and was suppressed completely in brighter twilight (~4). The least sensitive cell was monotonic, activating in twilight (~4.1) and saturating in daylight (~5.8). Cells with intermediate tunings covered the range between these extremes, with unimodal and monotonic cells tending to populate lower

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**Figure 2. Variation in Shape and Position of M1 IpRGC Tuning Curves**

(A) Axonal IF relations, normalized to their peaks, for all M1s recorded with antagonists of synaptic transmission (red, unimodal; black, monotonic). Relations are ordered by the half-saturating irradiance on the positive-slope limb of the IF relation (I₁/₂) and offset vertically for clarity; the position on the abscissa is absolute.

(B) Top: Relations overlaid and aligned by I₁/₂ for comparison of shape. Bottom: Average unimodal and monotonic IFs (solid) with ± SD shown (dotted).

(C–E) Tuning-curve parameters of single M1s (C: half-saturating irradiance, I₁/₂; D: saturating irradiance, Iₘₐₓ; and E: half-saturating irradiance on the negative-slope limb of unimodal relations, I₁/₂ₜₙ) plotted against activation threshold (I₉₉). All units are log photons μm⁻² sec⁻¹. Dashed lines are linear fits and correlation coefficients (r) are shown. See Figure S2 for data from recordings without antagonists.

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Cell 171, 865–876, November 2, 2017 867
and higher irradiances, respectively (Figure 2A). Therefore, M1s
distribute their tuning curves to cover an extended range of envi-
ronmental irradiances as a population.

Robustness of Unimodal Tuning to Irradiance

Unimodality has not been found in prior studies of M1s and is not
intuitive because it makes spike rate an ambiguous measure of
irradiance. The range of sensitivities displayed within a single
type of photoreceptor is also unexpected, being larger than the
separation between rods and cones. One concern is that unim-
odality and the lower sensitivities of some cells arise from an
inability of the isolated retina to support normal responses to illu-
mination. However, we found that relations were quite stable in
shape and position across a second stimulus presentation
(n = 7/8 cells; Figures S1A–S1D). We also controlled for
particular experimental histories and global modulatory states
by recording from pairs of axons, which gave simultaneous
observation of unimodal and monotonic relations (Figures
S1E–S1H) that differed in sensitivity by 0.3–1.5 log units. Another
potential concern is that these diverse relations result from
blocking synaptic transmission. However, M1s studied without
antagonists spanned a similar range of irradiances (Figure S2).
Unimodal tuning and sensitivity variation appear to be robust
features of M1s.

We asked whether unimodal tuning was detectable in vivo by
presenting mice with different irradiances and then immuno-
staining for a marker of neuronal activation, the phosphorylated
S6 ribosomal subunit (pS6) (Knight et al., 2012). This approach
also allows hundreds of M1s to be examined at each irradiance.
It requires that pS6 reflect spiking, which should be the case for
ipRGCs because blocking spikes makes rises in intracellular cal-
cium undetectable even with ongoing phototransduction (data
not shown; see Hartwick et al., 2007). To promote development
of the pS6 signal, we used long (90 min) stimuli. Few melanopsin
cells had detectable pS6 in darkness (Figures 3A, 3B, and S3).
When exposed to light, the level of pS6 immunofluorescence
rose with irradiance over the dimmest three levels tested
(3.0, 4.1, and 5.0 log photons μm⁻² s⁻¹ at the retina) and then
decreased (at 6.9). That is, the distribution of activated cells at
the highest irradiance collapsed to resemble that at a much
lower irradiance (Figures 3C, 3D, and S4A–S4D). The majority
of cells analyzed for pS6 were M1s (>91%, based on dendritic
stratification; STAR Methods) and were therefore comparable
to those studied electrophysiologically. Indeed, pS6 fluores-
cence and recorded firing rates showed broadly similar depen-
dencies on irradiance (Figure 3D).

Examining animals that lack melanopsin and thus the intrinsic
light responses of all ipRGCs, we found little pS6 labeling in M1s
at the tested irradiances, which should activate their rod- and
cone-driven inputs (Figures S4E–S4G) (Keenan et al., 2016).
This finding is expected from behavioral evidence that melanop-
sin signaling is dominant over long timescales and high irradi-
ances (Keenan et al., 2016; McDougal and Gamlin, 2010;
Morosovsky and Hattar, 2003) and from our observation that IF
relations were similar whether synaptic antagonists were
included or omitted (Figures 2 and S2). We also controlled for
any nonspecific decline of pS6 labeling under intense illumina-
tion by examining the M4 ipRGCs (also known as sustained
ON-β RGCs). These cells encode visual contrast but also fire
steadily at a rate that increases with irradiance (Schmidt et al.,
2014). We found that their pS6 expression increased monotoni-
cally with irradiance, with no sign of a reduction at the highest
level (Figures S4H–S4J). In summary, pS6 expression reflects
the melanopsin-driven responses of M1s, and its dependence
on irradiance indicates that many of these cells are unimodally
tuned in vivo.

Adaptation of Response Tuning

Light history has profound effects on individual ipRGCs (Do and
Yau, 2013; Emanuel and Do, 2015; Wong et al., 2005), and we
investigated how it shapes the population of M1s by comparing
IF relations obtained from single-axon recordings during the ris-
ing and falling staircases of the irradiance pyramid (Figures 4A–
4E). Both staircases comprised identical irradiances, but the
descent began with the cell in a light-adapted state. All M1s
maintained their tuning variant (monotonic versus unimodal) dur-
ing these descents, though most showed lower peak firing rates
(Figure 4F). They also shifted position on the irradiance axis. The
shift was orderly: cells with higher sensitivity shifted leftward
to lower irradiances, while those with lower sensitivity did the oppo-
site. Shift magnitude was practically zero for cells with tuning
near the center of the population and increased in roughly linear
fashion with progressively more eccentric sensitivities (r = 0.9,
maximum displacement of ~2 log units; Figure 4G). The magni-
tude of the leftward shift was proportional to the difference in
dark firing rate before and after the irradiance pyramid (Figure 4H
and text below). The overall effect of light adaptation was that the
population of M1s sampled a broader range of irradiances more
sparsely (Figures 4D and 4E). Omitting antagonists of synaptic
transmission had little effect (Figures S2D, S2H, and S2I).
Thus, M1s show intrinsic and systematic adaptation to light his-
tory at the population level.

Unimodal Tuning Confers Firing in Response to Light
Decrements

IpRGCs are understood to be pure ON cells, with their activation
level being directly proportional to irradiance. However, unimo-
dal M1s exhibited the opposite behavior on the descending
limbs of their IF relations, firing less rapidly as irradiance rose.
Moreover, as irradiance decreased below the threshold of
silencing, cells increased their firing rates (Figures 4A, 4C, and
5A). Such “inverse” responses were absent in monotonic M1s.
Even those that showed partial suppression at high irradiances
only decreased firing during subsequent darkening (Figure 4A).
A hallmark of the inverse response is its slow time course.
Following the step that allowed resumption of firing, the first spike
occurred only after 50 ± 59 s (n = 22 cells that had been completely
silenced). For most cells, this slowness was also evident in the
smooth changes in firing rate that followed from further decre-
ments in irradiance (Figures 5A and 5B). Hence, unimodal M1s
impose a strong low-pass filter on declining irradiance.

The preceding analysis considered inverse responses to be a
function of irradiance, but irradiance changed alongside the time
elapsed since firing was suppressed. We dissociated these vari-
ables by measuring the firing of unimodal M1s at a fixed time in-
terval after stepping from a silencing irradiance to different probe
irradiances (Figures 5B and 5C; step duration doubled to accommodate the slow kinetics of unsilencing). All cells showed pure inverse behavior, increasing their spike rates following darkening and decreasing them after brightening (n = 9 of 9 cells). IF relations constructed from this protocol were coarsely unimodal (with 2 cells firing only at one irradiance and thus lacking full relations; Figure 5D). Consequently, unimodality is revealed under diverse stimulus regimes, and it broadens the signaling repertoire of M1s to include inverse responses with unique kinetics.

Unimodal Tuning Arises from Phototransduction Overdriving Spike Generation

To investigate the mechanism of intrinsic response tuning, we used somatic, perforated-patch electrophysiology to observe the phototransduction current, subthreshold voltage, and spiking of unimodal M1s during irradiance pyramids (remaining at 35°C; Figures 6A–6D). These experiments necessitated two presentations of the pyramid (for current- and then voltage-clamp recording), and we accommodated them by covering the dynamic range of each cell in fewer steps. For all cells tested, steady phototransduction current and subthreshold depolarization were directly proportional to irradiance (Figures 6D, S5A, and S5B; n = 4 and 11 cells). These intensity-response (IR) relations were monotonic whether synaptic antagonists were included or not (n = 9 and 2 of the aforementioned 11 cells). By contrast, IF relations were unimodal, being indistinguishable in shape to those measured from the axon (Figures 6D, S5A, and SSB; n = 4 and 11 cells, respectively). These intensity-response (IR) relations were monotonic whether synaptic antagonists were included or not. Unimodality therefore arises from the interaction of phototransduction and spike generation.

As irradiance increases and phototransduction generates a larger depolarization, the firing rate should decline in large part...
because of sodium channel inactivation. Indeed, the maximum upstroke velocity of the spike ($dV_{max}$), a conventional measure of sodium channel availability (Bean, 2007), diminished with each increase in irradiance (Figure 6B). Following the peak of the IF relation, availability fell together with the firing rate until spikes became short, broad, and difficult to distinguish from noise (Figures 6B and 6C). The opposite occurred when irradiance decreased. Phototransduction current declined, the membrane hyperpolarized, and availability rose in tandem with firing rate (Figures 6A–6C). The change in $dV_{max}$ with irradiance was paralleled by a change in the maximum downstroke velocity ($dV_{min}$; the two parameters were correlated with $r = 0.90 \pm 0.1$, $n = 11$ cells), suggesting that the inactivation of sodium channels is accompanied by reduced activation of outward currents (e.g., from potassium channels) that would otherwise promote recovery from inactivation. Therefore, phototransduction activates and then hyperactivates the voltage-gated channels that produce spikes. The tuning curve peaks at the transition between activation and hyperactivation, then descends into silence as hyperactivation intensifies into complete depolarization block; as irradiance decreases, the reversal of this process drives the inverse response.

Phototransduction, being a G protein cascade, might also promote block through the modulation of voltage-gated channels. We reasoned that evidence for modulation may be found in the dependencies of spike parameters ($dV_{max}$ and $dV_{min}$) on the voltage preceding the spike ($V_{pre}$), when comparing activation by illumination to activation by electrical current delivered in darkness. We detected no difference between these dependencies ($n = 4$ cells; Figures S5C and S5D). Block appears to arise in large part from the depolarization produced by phototransduction.

If unimodal tuning arises from block, a reduction in photocurrent may be sufficient to produce monotonic tuning. To test this idea, we screened an additional set of M1s to obtain a sample of monotonic cells and measured their saturated, steady photocurrents. They were smaller than those of unimodal cells (19–42 pA for 3 monotonic cells and 41–76 pA for 8 unimodal M1s; $p = 0.01$; Figure 6E). Furthermore, on a background of saturating light ($10^{4}$-fold $I_0$), block could be obtained with an injection of electrical current whose size (5–20 pA, $n = 3$ cells; Figure 6F) was comparable to the difference in saturated photocurrent between monotonic and unimodal cells. A given current should evoke comparable responses in both variants due to their similar input resistances ($0.6–1 \, G\Omega$ and $0.5–2 \, G\Omega$ for 3 monotonic and 8 unimodal M1s, respectively; $p = 0.4$). Thus, the parsimonious explanation for monotonic tuning is that it exists when the

Figure 4. Dependence of Irradiance Tuning on Light History
(A) Histograms of axonal firing rate (as plotted in Figure 1, for two different cells) during a complete irradiance pyramid for a representative monotonic (blue, $I_{sat} = 2.7 \, \log \, \text{photons} \, \mu\text{m}^{-2} \, \text{s}^{-1}$) and unimodal (red, $I_{sat} = 1.7$) M1.
(B) IF relations for the cells in (A) obtained for ascending irradiance.
(C) As in (B) but for descending irradiance. Ascending relations (dashed) are plotted for comparison.
(D) Heatmaps representing all ascending IF relations measured. Each bar is one cell, color corresponds to normalized firing rate, and cells are ordered by ascending $I_{sat}$. Blue and red arrows indicate monotonic and unimodal M1s from (A), respectively.
(E) As in (D) but for descending IF relations, ordered by descending $I_{sat}$. Representative cells are marked.
(F) Difference in 95% peak firing rate between ascending and descending IF relations, plotted against $I_{1/2}$. Negative values indicate lower rates following light history.
(G) Difference in $I_{sat}$ between ascending and descending IF relations ($\Delta I_{sat}$) plotted against ascending $I_{1/2}$. Negative and positive values indicate leftward and rightward shifts due to light history, respectively.
(H) Difference in firing rate between the dark period that led the irradiance pyramid or followed it (measured 90 s after illumination ceased, with rates in both periods averaged over 30 s), plotted against displacement ($\Delta I_{sat}$). Leftward-shifting cells (negative $\Delta I_{sat}$ values) showed larger persistent changes in firing. Units in (F)–(H) are log photons $\mu$m$^{-2}$ s$^{-1}$. See Figure S2 for recordings made without antagonists of synaptic transmission.
See also Figure S6.
Mechanisms of Adaptation

Our perforated-patch recordings also provide insight into the basis of adaptation. For cells that sensitize, the key observation is that they were more depolarized during a given irradiance when it was preceded by a brighter step rather than a dimmer one (Figures 6A–6D). This additional depolarization would be predicted to promote block. Consistent with this idea, during the descending limb of the irradiance pyramid, the firing of these cells resumed at lower irradiances than those that initially activated them. Their IF relations were shifted leftward as a consequence. The likely origin of this asymmetry is the ability of melanopsin activation to outlast the period of illumination by minutes (Emanuel and Do, 2015). Indeed, in leftward-shifting cells, the phototransduction current also tended to be larger during descending irradiance (Figures 6D and S6B). With regard to the rightward shift observed for some IF relations (Figure 4), it is expected from the known characteristics of desensitization in melanopsin phototransduction (Do and Yau, 2013; Wong et al., 2005). The redistribution of tuning curves with light history is likely to reflect cell-to-cell variation in the balance of persistent activation and desensitization in phototransduction.

Axonal Physiology Enables High-Fidelity Signaling during Hyperactivation

Hyperactivation has an apparent disadvantage as a mechanism of response tuning because alterations in channel availability and spike waveform could produce fluctuations in synaptic outflow. Indeed, the somatic spikes of unimodal M1s varied in saturated photocurrent is set below the threshold for overdriving spike generation.

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amplitude and width by >4-fold, and in dV_max by >10-fold, from threshold to silencing (Figures 6B and 6C). Near silencing, somatic spikes were attenuated and often difficult to distinguish from noise (Figures 6C and S5C). However, when we made recordings from the axon, spikes were stereotyped even as their rate fell (Figure 1G).

To identify the basis of axonal spike uniformity, we recorded simultaneously from the axon and soma of single M1s using the loose- and perforated-patch modes, respectively (Figure 7; recording at 23°C for additional stability). As expected, axonal spikes were stereotyped while the corresponding fluctuations in the soma were variable (n = 3 cells; Figures 7A–7D). Propagation efficiency remained high even when sodium channel availability was strongly diminished; events with 2%–10% of the greatest dV_max were still accompanied by axonal spikes (96% of 703 events from 3 cells). At the highest irradiances, somatic spikes were so attenuated that the axon might appear to be firing in isolation (see Figures S7A–S7H for a method of somatic spike detection in such regimes). In actuality, even small fluctuations measured at the soma can correspond to propagating spikes of uniform shape in the axon.

By making axon-only recordings at different distances from the soma, we found that axonal spikes were variable when measured near the soma but uniform when measured farther away (n = 8 cells, Figure 7F; 35°C). To define this relation more precisely, we made paired recordings from the soma and axon using the dual loose-patch mode. We transiently blocked cells with a pulse of light, then monitored the resumption of firing. Somatic spikes were initially distorted and then gradually recovered. Axonal spikes followed the same pattern when measured close to the soma; when measured distally, they were of large and stable amplitude. The correlation between somatic and axonal spike amplitude declined with a length constant of 550 μm, approaching zero at 1,400 μm (n = 8 cells; Figure 7G). Thus, M1 spikes are stereotyped long before arriving at their nearest postsynaptic cells in the brain. Hyperactivation alters the shape and rate of spikes in the soma, but only the rate modulation remains in the distal axon. The result is unimodal tuning to irradiance without an apparent compromise to signaling fidelity.

**DISCUSSION**

M1 ipRGCs have been thought to code irradiance by modulating their firing rates in direct proportion to light intensity. In this view, cells have uniform and broad dynamic ranges. Pooling among them confers accuracy to functions like circadian and pupillary regulation (Laughlin, 1992; Nelson and Takahashi, 1991). On the contrary, we have found that individual M1s fire within restricted bands of irradiance and vary in their sensitivities to collectively span levels from moonlight to full daylight. This

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**Figure 6. Unimodal Irradiance Tuning Arises from Entry into Depolarization Block**

(A) Perforated-patch, somatic recording at 35°C with antagonists of synaptic transmission. An abbreviated irradiance pyramid (top, from I_F to 10⁴-fold I_F) was given to measure membrane voltage (V_m) and spike rate. Dashed horizontal line is ~70 mV, the dark-adapted V_m. Following dark adaptation, a second presentation of the pyramid in voltage clamp (~80 mV) allowed recording of phototransduction current (bottom; gray dashed line represents zero).

(B) The maximum spike upstroke velocity (dV_max), a measure of the availability of voltage-gated sodium channels, for the voltage recording in (A).

(C) Excerpts of V_m from the last 30 s of ascending (a–d) and descending (d–f) irradiance steps, with complete depolarization block evident in epoch d.

(D) Irradiance-response (IR) relations for phototransduction current (top) and subthreshold V_m (middle), as well as the IF relation (bottom). Ascending (solid) and descending (dashed) relations shown.

(E) Steady photocurrent generated at a step irradiance equivalent to the top of the pyramid for unimodal (U) and monotonic cells (M).

(F) Representative monotonic cell exposed continuously to a saturating background light (10⁴-fold I_F) and then given electrical current of different magnitudes, with the largest producing depolarization block. All recording conditions were identical to those in (A).

See also Figures S5 and S6.
population representation is adaptive and includes responses to darkening, which have been considered outside the ipRGC repertoire. Moreover, M1s are tuned by a process of depolarization block that is complemented by axonal spike regularization. These results motivate a reconceptualization of how the mammalian visual system represents stimulus intensity, which we explore here.

**Signaling Irradiance Collectively Rather than Individually**

Formal analyses of unimodal and monotonic tuning curves indicate that the signal/noise ratio dictates where they are most informative (Butts and Goldman, 2006; Yarrow and Series, 2015). This region is on the slope when the ratio is high but switches to the peak when the ratio is low. The ratio appears low for single M1s. Most have a maximum firing rate of <10 Hz (Figure S2F), such that a doubling of irradiance produces only a ~1 Hz increase on the steepest part of the tuning curve. Firing is also irregular, with coefficients of variation being ~3 near threshold and ~1 near peak activation. This slowness and irregularity would suggest that the highest rates are most informative. For unimodal M1s, maximum firing is evoked uniquely by the preferred irradiance; for all, it is the most separable from spontaneous activity. Therefore, the identities of M1s that are firing most vigorously may provide the clearest indication of irradiance. On the other hand, our experiments suggest that activation of the M1 population, albeit unimodal overall, has monotonic intervals. In these intervals, the pooled firing rate may provide a straightforward representation of irradiance. The question is whether it is advantageous to pool from the population of diverse and narrow tunings that we observed, as opposed to the hypothetical case where all tunings are similar and broad. One possible benefit is that diverse tuning allows the many downstream functions of M1s to select signals that are suited to their particular needs (Do and Yau, 2010). Examining how these cells drive their postsynaptic targets would help to clarify the nature of the processing strategies used, including the extent of pooling.

An advantage of dividing irradiance among cells is likely to be efficiency. If all M1s were monotonic, with thresholds similar to those we measured, a large fraction would fire at their saturated rates throughout the day. Saturated firing carries little information and is costly. Indeed, minimizing spike number to decrease energy expenditure has been proposed as a principle of neural function (Laughlin, 2001). Our experiments suggest that M1s
follow this rule. Their spike rates are low. Moreover, as irradiance rises, some unimodal cells fire less as others fire more. Although phototransduction is continuous, it is likely to cost much less than spiking (Okawa et al., 2008; Sengupta et al., 2014) and does not activate the numerous brain pathways downstream of M1s on its own (Morin and Studholme, 2014). Monotonic cells fire steadily, but are relatively insensitive and thus active only when environmental irradiance is highest. Unlike the conventional RGCs that code images, M1s need not uniformly sample each point in space—they are free to disperse across the irradiance axis to achieve dynamic range and efficiency as a population.

Depolarization Block as a Mechanism of Response Tuning

The use of depolarization block is consistent with the principle of efficiency. M1s have a high input resistance, such that the production of picoampere currents by phototransduction can increase firing (Do et al., 2009). We report here that just tens of picoamperes are sufficient to inactivate the voltage gated sodium channels that drive spikes. Hence, relatively little energy is required to maintain the ionic gradients that support M1 excitability.

In principle, altering the level of melanopsin expression would be sufficient for variation in tuning curve position and shape. Melanopsin expression is linked to photon catch and thus sensitivity (Do et al., 2009). If expression were reduced, tuning could convert from unimodal to monotonic as phototransduction became unable to overdrive spiking. The reality is likely to be more complex. For example, our in vivo experiments did not reveal a clear dependence of cellular activation on melanopsin immunoreactivity (Figure 3). In parallel work, we have observed that M1s show extreme variation in their biophysical parameters, including those associated with phototransduction and spiking, with little covariation (A. Emanuel, K. Kapur, and M.T.H.D., unpublished data). A highly individualistic combination of parameter values may underlie each of the tuning curves we measured here, despite the many commonalities in their shapes.

Spike Regularization by Axonal Propagation

The spike distortion that accompanies entry into depolarization block is corrected by properties of the M1 axon. Spikes also become stereotyped during axonal propagation in neurons as different as cerebellar Purkinje cells and CA1 pyramidal cells (Apostolides et al., 2016; Khalil and Raman, 2005). These cells have distinct roles, morphologies, and complements of voltage gated channels (Raman and Bean, 1997), suggesting that there need not be a mechanism of spike regularization that is unique to M1s. In principle, any neuron that employs depolarization block as a tuning mechanism may also display regularization provided that its axon is long enough.

Adaptation of the M1 IpRGC Population

Our data allow certain outcomes of adaptation to be anticipated. At a modest light level, the most sensitive M1s would activate and shift their tuning curves to lower irradiances. These cells would remain active even when irradiance fell below their dark-adapted thresholds and thus support hysteresis. With strong illumination that caused many M1s to silence or approach saturation, the least sensitive cells would also shift their tuning curves, but toward higher irradiances. Saturation would be reduced as a consequence. Subsequent declines in irradiance would cause unimodal cells to unsilence with different delays, providing additional hysteresis. Hence, adaptation of the M1 population supports low-pass filtering and extends dynamic range.

How adaptation manifests should depend on how it is evoked. Persistent activation generally has a shorter lifetime than desensitization in these cells (Emanuel and Do, 2015; Wong et al., 2005). If tuning curves were measured long after a change in irradiance, only desensitization would be evident. Thus, during natural conditions where irradiance changes over multiple timescales—for example, quickly due to motion and slowly due to changes in solar position—the population of M1s is likely to adapt in a complex and dynamic fashion.

Generalization to Other Systems

Cells with unimodal tuning and varied sensitivity have been found at various stages across the sensory modalities of diverse species. Some show evidence of response tuning by depolarization block, though it is frequently unmentioned and must be inferred from indirect measurements. Photoreceptors of the nudibranch, Hermissenda, generate vigorous responses to light that overdrive spike generation (Akon and Fuortes, 1972). The cold-sensing thermoreceptors of vertebrates and invertebrates also show unimodal tuning (Gallio et al., 2011; Spray, 1989), and prominent block has been observed for some (Okazawa et al., 2002). Unimodality and block occur together in the olfactory sensory neurons of many species (Cao et al., 2016; Reisert and Zhao, 2011) and are evident one synapse downstream in the olfactory bulb (Rospars et al., 2013). A synapse farther, in piriform cortex, information about odorant concentration is found in the tuning of responses (Bolding and Franks, 2017). Changes in odorant concentration alter the kinetics of block and thus the spike patterns of the sensory neurons (Ghatpande and Reisert, 2011), raising the possibility that block contributes to the temporal representation of intensity found in cortex. In contrast to these examples, neurons in the auditory system produce unimodal intensity tuning from synaptic integration (Zhou et al., 2012). For these cells, circuit mechanisms are likely preferable to block due to the rapidity of auditory processing. In summary, block may be more relevant to neural function than is generally thought, particularly in systems that operate on intermediate or long timescales. In these cases, block may confer the advantages of flexibility and efficiency that are found in the M1 representation of light intensity.

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
Akon, D.L., and Fuortes, M.G. (1972). Responses of photoreceptors in
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Supplemental Information includes seven figures and can be found with this
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DATA AND SOFTWARE AVAILABILITY
AUTHOR CONTRIBUTIONS
E.S.M. and M.T.H.D. designed experiments, which were conducted by E.S.M.
(with the exception of antibody validations in cell lines). E.S.M. and M.T.H.D.
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**STAR METHODS**

**KEY RESOURCES TABLE**

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**CONTACT FOR REAGENT AND RESOURCE SHARING**

Requests for further information, resources, and reagents should be directed to the Lead Contact, Michael Do (michael.do@childrens.harvard.edu), for fulfillment.

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

In vitro electrophysiological experiments used mice in which ipRGCs express tdTomato from the melanopsin locus of a BAC transgene (> P20; Do et al., 2009). In vivo experiments were performed using mice that were wild-type (C57BL/6J), had one melanopsin allele replaced with the gene for Cre recombinase (Opn4Cre/+, Ecker et al., 2010), or also had the second allele replaced with the gene for β-galactosidase (Opn4Cre/tau-LacZ, Hattar et al., 2002). Opn4Cre/+ mice were also positive for the aforementioned BAC transgene. All mice used for in vivo experiments were > P40 in age to facilitate headpost implantation. For antibody validation, melanopsin-null mice were Opn4Cre/Cre. Mice were of either sex and kept on an alternating light/dark cycle (12 hr each). There was no overt variation in experimental results with age, sex, or circadian time. All procedures were approved by the Institutional Animal Care and Use Committee of Boston Children’s Hospital.
METHOD DETAILS

Tissue Preparation for Electrophysiology
Mice were dark adapted for > 1 hr prior to experimentation and subsequent procedures took place under dim-red or infrared illumination. Animals were anesthetized with Avertin, enucleated, and euthanized. As part of this procedure, a slit was placed in the retina to mark its orientation. The retina was removed in extracellular solution (see below), mechanically freed of vitreous humor, flattened with peripheral cuts, and secured with RGCs facing upward on a glass coverslip coated with poly-L-lysine. For axon-ending recordings, a superficial cut (30-60 μm deep) was placed on the ventronasal quadrant of the retina, 500-700 μm from the optic disc and orthogonal to axon bundles, using a vibratome. The cut was made at room temperature (23 °C) in extracellular solution, and retinas recovered in this condition for 45-60 min before recording.

Cell Imaging and Identification for Electrophysiology
The retina was viewed on an upright microscope through a 60 × water immersion objective, with differential interference contrast optics and infrared transillumination (850-nm center wavelength, 30-nm width at half maximum). Fluorescent M1s were identified with light from a 75-W xenon arc lamp that was conditioned by filters and had a final spectrum that was centered on 545 nm (25-nm width). An EM-CCD camera (Hamamatsu ImageEM) was used to reduce the light intensity required for identification. For somatic recordings, exposure to imaging light was brief (< 1 s, 7.7 log photons μm² s⁻¹). Targeting of axon endings required longer durations and brighter light (20-60 s, ~9.7 log photons μm² s⁻¹), but used a distal, 125-μm diameter spot that minimized illumination of the soma and dendrites (somata were typically 350-1200 μm from the recording site). Cells were allowed to dark adapt for ≥ 10 min before the start of each experiment. M1s identified in both of these fashions exhibited intrinsic responses that were as sensitive as those identified via multiphoton microscopy (Zhao et al., 2014). When recordings were made from both the soma and axon ending, minutes of imaging were required to trace axons between the two locations. Photosensitivity was likely to be reduced as a consequence but these particular recordings did not require complete dark adaptation, since their purpose was to compare somatic and axonal spike waveforms. Only one cell or simultaneous pair was recorded from each retina in order to minimize any effects of cumulative light exposure.

The majority of recordings were made in the ventronasal retina, where M1s have the highest melanopsin expression and are widely distributed (Hughes et al., 2013; Valiente-Soriano et al., 2014). In the reporter line used, the axons of M1s in this region are bright and sparse, which facilitates visualization. During recording, the sensitivity of intrinsic phototransduction, spike width, and spike frequency indicated successful targeting of M1s (Hu et al., 2013; Schmidt and Kofuji, 2009; Zhao et al., 2014). After recording, it was customary to trace the axon to the soma and verify that dendrites extended toward the outermost margin of the inner plexiform layer. Post-recording dye electroporation into the axon often allowed the dendrites to be examined with greater clarity, and M1 stratification was confirmed in all cases (n = 15/15 cells). Both unimodal and monotonic IF relations were represented in this sample and spanned a range of irradiances that was similar to that of the broader sample (~2.3 log units of variation in activation thresholds).

In a subset of experiments, the retinal recording site was confirmed by immunostaining for short-wavelength cone opsin, whose highest expression is in the ventronasal retina (Valiente-Soriano et al., 2014). Most recorded quadrants (19/22 retinas) were well within this region, while a minority (2/22 retinas) straddled the border and one was entirely outside. The latter two cases appeared similar to the rest.

Electrophysiological Solutions
The extracellular solution was bicarbonate-buffered Ames’ medium (Sigma) or “ionic Ames” (in mM, 120 NaCl, 22.6 NaHCO₃, 3.1 KCl, 0.5 KH₂PO₄, 1.2 CaCl₂, 1.2 MgSO₄, and 6 glucose. The solution was equilibrated with 95% O₂/5% CO₂, establishing a pH of 7.4. Unless otherwise noted, synaptic transmission was blocked with 3 mM kynurenic acid (or the combination of 25 μM DNPX and 50 μM D, L-AP5), 100 μM picrotoxin, 10 μM strychnine, and 100 μM D, L-AP4. For perforated-patch recording, the pipette solution was (in mM) 110 K-methanesulfonate, 13 NaCl, 2 MgCl₂, 10 EGTA, 1 CaCl₂, 10 HEPES, 0.1 Lucifer Yellow, and 0.125-0.188 amphotericin B. Amphotericin B was dissolved in DMSO to make 100 μg/mL aliquots, and stored in the dark at −20 °C for up to two weeks. A liquid junction potential of +7 mV has been corrected. The pipette solution for loose-patch recording was HEPES-buffered ionic Ames’ medium (in mM, 140 NaCl, 3.1 KCl, 0.5 KH₂PO₄, 1.2 CaCl₂, 1.2 MgSO₄, 6 glucose, 10 HEPES, and sometimes 0.5 Alexa 488 hydrazide; pH adjusted to 7.4 with KOH). Unless otherwise noted, all recordings were performed at 35 °C, which was established with an in-line heater and continuously monitored by a bath thermistor.

Electrophysiology

Somatic recordings
The glial membrane covering the soma was mechanically removed. For perforated-patch recording, pipettes (3-6 MΩ) were wrapped with parafilm to reduce capacitance. Seal resistances were ≥ 10 GΩ. Series resistances (typically < 60 MΩ) were monitored but not compensated. In current clamp, a steady and slight hyperpolarizing current (< 15 pA) was occasionally applied to produce a stable
baseline rate of firing (comparable to spontaneous firing rates measured in loose-patch recordings). The integrity of the perforated-patch configuration was verified by stability of the intrinsic light response (Do et al., 2009) and by post-recording visualization of Lucifer Yellow, which does not permeate amphotericin B. Loose-patch recordings used wrapped pipettes of 2-5 MΩ resistance and had seals of 10-40 MΩ. Axonal recordings

Pipettes were 5-10 MΩ in resistance and coated with Alexa 594 (conjugated to bovine serum albumin, applied at 10 μM in HEPES-buffered ionic Ames’ medium) to allow simultaneous visualization with tdTomato-labeled axon endings. Seal resistances were typically 10-25 MΩ. To distinguish the recorded cell more clearly from other ipRGCs, Alexa 488-hydrazide was included in the pipette solution and, when experiments were concluded, electroporated using 50-100 square wave pulses (1V, 100-500 ms width; in 28/53 cells, axons were sufficiently bright to trace back to the soma using the Alexa signal; 15 of these 28 also had clear Alexa labeling of the dendritic arbor). On occasion, additional axons with undetectable tdTomato were labeled by electroporation. When synaptic antagonists were included, those recordings showed stimulus-locked spikes from ipRGCs that were typically accompanied by spontaneous spikes from different sources. When antagonists were excluded, varied light responses (e.g., ON, OFF, and ON-OFF) were observed in non-ipRGC axons. In these mixed recordings, spikes were detected and sorted using Wave_Clus for MATLAB (Quiroga et al., 2004). Appropriate thresholds for event detection were typically set at 1.5- or 2-fold greater than the peak-to-peak noise level. If more than one photosensitive unit was detected and spikes were not readily separable by amplitude, the recording was discarded.

Paired soma/axon recording

Axons with intense tdTomato fluorescence were identified and traced to the soma and axon ending (separation of 200-1400 μm). Time-locked spikes at the two sites confirmed that the paired recording was successful. When somatic perforated-patch recording was combined with axonal recording, the experiment was performed at 23° C for additional stability. Paired, loose-patch recordings of the soma and axon were done at 35° C.

All recordings were low-pass filtered at 10 kHz and sampled at 20-50 kHz. Additional filtering and downsampling was conducted in parallel or offline when necessary.

In Vitro Optical Stimulation

Visual stimulus

The standard optical stimulus was generated by a 460-nm LED whose output was band-pass filtered (460-nm center and 10-nm width at half-maximum), collimated, attenuated by neutral density filters (50-ms switching time), and delivered evenly over the entire retina using a diffuser (20°; as epi-illumination) or condenser (as trans-illumination). The light intensity was controlled further using current and pulse-width modulation, which were commanded by an Arduino Uno board and the ArduinoIO package for MATLAB. Filter switching and pulse-width modulation were instantaneous relative to the integration time of M1s (Do et al., 2009; Emanuel and Do, 2015). White light was used in a small subset of experiments (i.e., those illustrated in Figures S7A–S7H). It was generated by a xenon arc lamp and projected through a series of filters to control intensity and deplete ultraviolet as well as infrared wavelengths, then delivered through the diffuser as epi-illumination. The intensity of white light is expressed in terms of equivalent 460-nm irradiance using its spectrum (Emanuel and Do, 2015) and the ipRGC action spectrum (see below). When comparing loose- and perforated-patch recordings (Figures S7I–S7K), it was desirable to limit the persistent activation of ipRGCs that results from short-wavelength or white-light stimulation (Emanuel and Do, 2015). For these experiments, the aforementioned Xe light was band-pass filtered (574-700 nm). Optical stimuli were regularly calibrated using a radiometer, spectrometer, and fast photodiode.

Online estimate of activation threshold

In most experiments, the absolute stimulus intensity was tailored to the estimated activation threshold (Iq) of individual cells. Following the initial period of dark adaptation, Ip was determined by delivering pulses of light (≤30 s, 1.3-2 log photons μm−2 s−1). In the absence of a response, the cell was permitted to dark adapt for 20-30 s before a stimulus of 2- or 3-fold higher intensity was given. This process was repeated until elevated firing was apparent. The irradiance that gave an overt and reproducible increase in firing rate within 10-30 s of stimulus onset was used as Iq. Comparison with 10% thresholds deduced offline from the IF relation (Iq) gave agreement within 0.33 ± 0.23 log units (n = 26 cells recorded with synaptic antagonists). The result without synaptic antagonists was similar (0.35 ± 0.29 log units, n = 27 cells).

Analysis of Irradiance-Response Relations

All irradiance-response relations were constructed from averages taken over the last 30 s of each irradiance step. To analyze the subthreshold membrane voltage, spikes were excised (in windows of 30-60 ms flanking the peak) and the average of the remaining points taken. Phototransduction current was measured in voltage clamp at a holding potential of −80 mV. Tuning curves were compared across cells by interpolation of response values and boxcar smoothing of the result (window size of 0.25 log units). Extracted parameters concerned the irradiances that produced firing at 10, 50, and 95% of maximum (Iq, Iq1/2, and Iq95) as well as those that produced 50 and 90% suppression of firing (Iq1/2, neg and Iq90, neg; specific to unimodal relations). The widths of unimodal tuning curves were measured at 50% of their maximum. For all tuning curves, the dynamic range was taken as Iq90 − Iq.
noted were the spontaneous firing rate, peak firing rate, firing rate at 104-fold Ia, and difference in firing rate in darkness before and after the stimulus (Fdark, Fmax, Fmax and ∆Fdark). Lastly, the change in Isat between the ascending and descending stimulus presentation (∆Isat) was calculated. Isat was used to determine shifts in tuning curve position because many IF relations shifted so far toward lower irradiances with light history that they no longer had a measurable I1/2 (i.e., cells fired at high rates in the dark period that followed the stimulus). Values for these parameters are reported in Figures 2, 4, S1, S2, and S6.

A few unimodal cells (5 of 50) were encountered that remained silent for the entire descent of the irradiance pyramid and only resumed firing in darkness, such that a tuning curve shift could not be measured. This behavior is an extreme case of the more common shift to a lower irradiance (which arises from a large and persistent depolarization that prolongs block; see the cell in Figure S5B, panel ix, for an example). To include these cells in the analysis, they were assigned a descending Isat value equal to Ia (the lowest irradiance presented during the pyramid) and ∆Isat was calculated using that value. This inferred ∆Isat provides a conservative lower bound on the true tuning curve shift.

In Vivo Optical Stimulation
Mice were anesthetized with ketamine/xylazine and implanted with a head post (Cahill and Nathans, 2008). Following 3 days of recovery, mice were dark adapted overnight and then secured such that the left eye was positioned at the aperture of a Ganzfeld diffusing sphere (i.e., coated internally with BaSO4; Do et al., 2009). The sphere was illuminated by a liquid light guide conveying the output of a 460-nm LED that was conditioned by current level and neutral density filters. To maximize intensity, no interference filter was used to narrow the stimulus wavelength; accordingly, photon flux densities were calculated using the measured stimulus spectrum and the ipRGC action spectrum (see below). Both pupils were dilated using topical phenylephrine (3%) and atropine (1%, in saline), with the right eye serving as a dark control. The stimulus had a duration of 90 min in order to promote robust pS6 expression. Immediately upon conclusion of the stimulus, animals were killed by decapitation. Eyes were dissected in ice-cold, HEPES-buffered ionic Ames (above) with Ca2+ substituted by equimolar Mg2+ to suppress further modulation of robust pS6 expression. After the stimulus (Fdark, Fmax, Fmax and ∆Fdark) was calculated. Fmax was used to determine shifts in tuning curve position because many IF relations shifted so far toward lower irradiances with light history that they no longer had a measurable I1/2 (i.e., cells fired at high rates in the dark period that followed the stimulus). Values for these parameters are reported in Figures 2, 4, S1, S2, and S6.

Comparison of Neuronal Activation In Vitro and In Vivo
Single-cell firing rates at 2, 3, 4, 5, 6, and 7 log photons μm−2 s−1 were taken from interpolated curve fits to the IF relations shown in Figure S2. If any of these irradiances were lower than those delivered as part of a cell’s irradiance pyramid, the recorded spontaneous firing rate used was instead. If they were higher than those delivered, the firing rate at the top step of the pyramid was used; this extrapolation is reasonable because the activation level of M1s at the top of the irradiance pyramid was almost always unchanged when tested with even higher irradiances. The median values of firing rate and pS6 fluorescence (across all melanopsin-positive cells tested at a given irradiance) were then plotted together. Using other metrics for analysis, such as means and sums, gave similar results.

Comparison of Reduced Optical Stimuli to Naturalistic Lighting Conditions
The spectrum of each stimulus used (in photons μm−2 s−1 nm−1) was multiplied by the action spectrum of ipRGCs (normalized) and the product integrated to give total irradiance with respect to photon absorption by melanopsin. The exception was monochromatic, 460-nm light; because it activates melanopsin with near-perfect efficiency (≥97%), its measured irradiance was used directly. Environmental spectra were taken from daylight (solar elevation of 90°, 40°, and 10°), twilight (−11°, −7°, −1°, 6°, and 11°), and moonlight (full moon; Johnsen et al., 2006). The ipRGC action spectrum was a nomogram reflecting equal activation of melanopsin from its two silent states (λmax of 471 nm for the cyan state and 453 for the violet state, incorporating both α and β absorption bands), as would be produced by most conditions of natural illumination as well as the white light and short wavelengths used here (Emanuel and Do, 2015). The pupil size at different irradiances was used to calculate retinal irradiance from corneal irradiance (Pennesi et al., 1998; Xue et al., 2011). These calculations indicated that direct sunlight (90°) corresponded to 6.4 log photons μm−2 sec−1 of 460-nm at the retinal surface. Irradiances that saturated the steady-state M1 responses were 3.1–5.8 log photons μm−2 sec−1 (with synaptic antagonists) and 3.9–7.2 log photons μm−2 sec−1 (without). Those that suppressed unimodal M1s to below 50% of their maximum steady firing rate were 3.3–6.2 log photons μm−2 sec−1 (with synaptic antagonists) and 4.5–6.0 log photons μm−2 sec−1 (without).

Immunohistochemistry
Production and validation of a melanopsin antibody
A polyclonal antibody was raised in chicken against a peptide corresponding to the extracellular N terminus of mouse melanopsin, MDS PSG PRV LSS LTO DPS FTT SZC, then affinity purified (Aves Labs). This peptide sequence is practically identical to those used for several, validated IgGs raised against mouse melanopsin and is common to both the short and long splice isoforms (Hattar et al., 2002; Pires et al., 2009; Provencio et al., 2002). As illustrated in Figure S3, the specificity of the novel IgY was verified by heterologous expression of melanopsin in HEK293 cells, the characteristic morphologies of all stained cells in the mouse retina (Ecker et al., 2010),
and the absence of staining in the melanopsin knockout mouse (Lucas et al., 2003). HEK293 cells were obtained from the ATCC. Mouse melanopsin (GenBank: 6693702) was synthesized, subcloned into an expression vector (pCDNA3), and transfected using Lipofectamine 3000.

**Staining and microscopy**

The vehicle for all reagents was phosphate buffered saline (PBS). Retinas and HEK cells were fixed in 4% paraformaldehyde (30 min at room temperature), washed, blocked in 2.5%–10% serum with 1% Triton X-100 (1 hr at room temperature), and exposed to one or more primary antibodies at the specified concentrations in blocking solution: melanopsin (see above), 1:5000; pS6 (Thermo-Fisher at room temperature), washed, blocked in 2.5%–10% serum with 1% Triton X-100 (1 hr at room temperature). These antibodies were donkey a-chicken conjugated to Alexa 488 (Jackson Immunoresearch 703-545-155) or DyLight 405 (Jackson Immunoresearch 703-475-155), donkey a-rabbit conjugated to Alexa 555 (Thermo-Fisher A-31572) or Alexa 488 (Thermo-Fisher A-21206), goat a-chicken conjugated to Alexa 555 (Thermo-Fisher A-21437), and donkey a-goat conjugated to Alexa 647 (Thermo-Fisher A-21447). A final wash was followed by mounting on slides with anti-fade medium containing DAPI (or without DAPI when a 405-nm fluorophore was used). For quantification, retinas were imaged as X-Y-Z confocal montages using a 10 x or 20 x objective. Acquisition parameters were set to maintain the highest pixel values just below saturation.

**Analysis of pS6 immunofluorescence**

For comparison with in vitro experiments, the ventral retina was identified (using staining for S-cone opsin) and RGCs with clear melanopsin expression found. Melanopsin immunofluorescence was measured in the soma (over three frames encompassing 10-14 μm in the Z axis). The region of interest (ROI) for measuring pS6 immunoreactivity encompassed the same depth and was defined in the X-Y plane by the boundary of melanopsin immunolabeling. Average fluorescence in this volume was corrected for background in both channels using a neighboring ROI. To correct for variations in staining intensity, the melanopsin immunofluorescence of each cell was centered and normalized (i.e., by subtracting the average value across all cells within the same retina, then dividing by the standard deviation). The distributions of corrected values were indistinguishable across retinas, suggesting that the same set of M1s was consistently sampled (Figure 3).

The intensity of pS6 immunolabeling was quantified three different ways: raw (i.e., as acquired), normalized by pS6, or normalized by melanopsin. Normalization to pS6 took advantage of scattered, non-ipRGCs in the ganglion cell layer with near-saturated levels of immunofluorescence. Such cells were found in all retinas examined and their fluorescence intensities were uncorrelated with the amount of light given experimentally. Thus, by dividing each cell’s pS6 value over the average of these cells within each retina, variations in staining effectiveness and image acquisition settings were reduced. With regard to melanopsin, its expression level is not expected to change over the course of the experiments described here and its labeling proceeded simultaneously with that of pS6, so its immunofluorescence provides another means of refining the comparison across retinas. Indeed, across retinas—but not across the different irradiances tested—the average immunofluorescence of the several cells most intensely immunolabeled for pS6 (used because their signals are most distinguishable from noise) and melanopsin was correlated (r = 0.5). The relation was described by a line, and that line used as to scale the pS6 values for each cell:

$$pS6 = \frac{pS6_0}{25.8 + 0.7M}$$

where pS6 is the normalized pS6 value, pS6_0 is the raw pS6 value, and M is the melanopsin immunofluorescence. The constants, 25.8 and 0.7, are the intercept and slope of the relation. Raw and normalized pS6 values < 0 were set to 0. The outcomes of these three approaches were comparable (Figures 3 and S4).

To determine which ipRGC types were analyzed in our main dataset, high-magnification (20 x objective), three-dimensional montages were taken of a random sample of melanopsin-immunoreactive cells in the ventral retina (n = 69 cells from 4 retinas). Most (91%) had dendrites that stratified in the outermost margin of the inner plexiform layer, and were therefore of the M1 type. The remaining cells were weakly fluorescent, such that their dendrites were not visible. For cells displaying the top 50% of melanopsin immunoreactivity, 98% were outer-stratifying. This result is consistent with prior studies of type and melanopsin immunoreactivity (Berson et al., 2010; Ecker et al., 2010), and indicates that the majority of cells sampled for our in vivo dataset were M1 ipRGCs.

For experiments concerning melanopsin knockout M1s (Figure S4), Opn4tau-LacZ/Cre mice were used (Ecker et al., 2010; Hattar et al., 2002). Immunostaining the retinas of such mice for β-galactosidase (the tau-lacZ gene product) is known to label exclusively ipRGCs of the M1 type (Bauer et al., 2009). The signal for pS6 in these cells was plotted against that for β-galactosidase, which is expected to be proportional to activity at the melanopsin promoter. With regard to experiments concerning the M4 ipRGCs (also known as sustained ON-α RGCs), these cells could not be identified by conventional immunostaining for melanopsin due to their low expression of this protein (Estevez et al., 2012). Therefore, a viral strategy was used. An adeno-associated virus that drove Cre-dependent expression of tdTomato (AAV2-CAG-FLEX-tdTomato; 2 μl, Vector Core of the University of North Carolina at Chapel Hill) was injected into the vitreous humor of Opn4tau-LacZ mice. Visual stimulation took place > 14 days afterward. M4s were identified by their large size; only tdTomato-positive cells whose somatic areas were equivalent to those of circles that were > 18 μm diameter.
were used (Estevez et al., 2012). Analysis was not confined to the ventronasal retina because the site of viral labeling was variable. Since the level of tdTomato expression in these cells was arbitrary, pS6 intensities were plotted against a random cell index.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Unless otherwise noted, the non-parametric Mann-Whitney U test was used for unpaired data. Otherwise, Student’s t test was employed for unpaired, univariate data and Hotelling’s t-squared test for multivariate data (after validating normality using Kolmogorov-Smirnov tests). To compare pairs of empirical distributions, two-sample Kolmogorov-Smirnov tests were used with both sets of raw data values as inputs. Bonferroni corrections were applied when multiple comparisons were made among the same datasets; otherwise, p ≤ 0.05 was considered significant. Additional details can be found in the figure legends and the Results section. Spearman correlation coefficients are used unless otherwise noted; when Pearson coefficients are used, normality was confirmed using the Kolmogorov-Smirnov test and linearity was verified.

**DATA AND SOFTWARE AVAILABILITY**

Requests for analyses and raw data may be made to the Lead Contact, Michael Do (michael.do@childrens.harvard.edu).
Figure S1. Robustness of IF Relations, Related to Figure 1

(A) Top: To test the stability of IF relations, M1s were exposed to a pair of identical, ascending staircases of irradiance that were separated by a dark period (14 min shown and 14-20 min across the sample) that allowed at least partial recovery from light history. Bottom: Histogram of firing rate from an exemplar, unimodally-tuned M1. The spontaneous firing rate in darkness (blue dotted line) and the zero value (black dashed line) are indicated.

(B) Overlaid relations from the first (black) and second (red) presentation of the staircase for the sample of 8 M1s. The cell depicted in (A) and (B) is included (†). The second IF relation of a pair was sometimes elevated at low irradiances, due to persistent activation from the first staircase (bottom three traces), and sometimes suppressed overall due to residual desensitization (top three traces). One M1 converted from unimodal to monotonic tuning. It was the only case of variant switching in all cells for which IF relations were probed repeatedly, under any stimulus protocol (n = 19 cells). The remaining relations showed suppression at the same irradiance across both stimulus presentations (n = 4/5 cells with steady firing rates reaching < 0.5 Hz).

(C) On average, cells showed slight elevations in $I_q$ and $I_{1/2}$ during the second stimulus presentation (0.4 ± 0.4 log photons µm$^{-2}$ s$^{-1}$, for both parameters) but no detectable change in $I_{sat}$ and $I_{1/2, reg}$ (0.1 ± 0.2 and 0.03 ± 0.3, respectively). Unlike the responses to the ascending and descending limbs of irradiance pyramids, those to repeated ascending staircases showed no correlation between the magnitude in the shift of $I_{sat}$ and the dark-adapted $I_{1/2}$ ($r = 0.1$; see Figure 4G for comparison). Furthermore, the average $\Delta I_{sat}$ between repeated presentations of the ascending staircase was significantly smaller than that between the limbs of irradiance pyramids (0.1 ± 0.1 versus 0.9 ± 0.6, respectively; $p = 0.001$). See Methods for parameter definitions.

(D) Between stimulus presentations, average spontaneous firing rates showed no significant change (0.9 ± 1.5 Hz; $p = 0.13$; measured in the last 30 s of the dark adaptation period). However, average peak firing rates were slightly depressed (−2.1 ± 2.2 Hz; $p = 0.016$).

(E) Schematic of the paired axonal recording configuration.

(F) Firing rate histograms from a pair of M1 axons recorded simultaneously and given the same ascending staircase. One cell was monotonic (blue) and the other unimodal (red). Absolute irradiances are noted in units of log photons µm$^{-2}$ s$^{-1}$, and were chosen to cover the dynamic ranges of all M1s encountered.

(G) IF relations from the two cells in (F). Of 6 simultaneous recordings, unimodal and monotonic relations were paired in 4; the remainder were purely unimodal.

(H) Three parameters of position on the irradiance axis were extracted for each cell ($I_q$, $I_{1/2}$, and $I_{sat}$) and a “separation score” calculated as the average difference between the same parameters within a pair (e.g., a score of 1 indicates that cells of a pair differed in preferred irradiance by a log unit). The score was 0.25 for the pair shown and 0.9 ± 0.4 for all 6 pairs. All error bars are ± SD.
Figure S2. Irradiance Tuning with Synaptic Antagonists Omitted, Related to Figures 1–4

(A) Left: Overlaid IF relations from axonal recordings made without antagonists of synaptic transmission, aligned by $I_{1/2}$ value ($n = 27$ cells; $35^\circ$ C). Right: Averages of the unimodal (red) and monotonic (black) relations from this sample (dashed lines are ± SD).

(B–G) A selection of IF parameters ($I_q$, $I_{sat}$, $D_{Isat}$, $F_{dark}$, $F_{max}$, and $F_{Imax}$; STAR Methods) plotted against $I_{1/2}$ for recordings made with synaptic antagonists excluded (black filled circles) or included (gray open circles). The only significant difference was a higher $F_{max}$, on average, for cells recorded without antagonists ($p = 0.002$). This effect was more pronounced for M1s with lower sensitivity. For $I_{1/2} > 3.4$ log photons μm$^{-2}$ s$^{-1}$ (dashed line), $p = 0.01$ ($n = 17$ and 11 cells without and with antagonists, respectively); for the remainder, $p = 0.29$ ($n = 10$ and 15 cells).

(H) IF relations obtained during ascending irradiances, recorded without synaptic blockers, displayed as heatmaps as in Figure 4. Relations are ordered according to their $I_{sat}$ values.

(I) As in (H) but for descending IF relations, also ordered according to their $I_{sat}$ values. $I_{1/2}$ was correlated with $\Delta I_{sat}$ for recordings both with and without synaptic antagonists ($r = 0.9$ and 0.6, respectively; Pearson correlation coefficients), and these conditions were not significantly different ($p = 0.36$, Hotelling’s t-squared test comparing 27 cells with antagonists and 26 without; see [D]).
Figure S3. Validation of a Custom Melanopsin Antibody, Related to Figure 3

(A) Epifluorescence images of HEK293 cells demonstrating the specificity of melanopsin immunoreactivity. Left: Cells were not transfected with melanopsin, but primary and secondary antibodies were present. Center: Cells were transfected with melanopsin and the secondary antibody was present, but the primary antibody was absent. Including primary but not secondary antibody gave an indistinguishable result. Right: Cells were transfected with melanopsin; both primary and secondary antibodies were present. Blue is nuclear staining with DAPI. Scale bar is 50 μm.

(B) Confocal projections of melanopsin-null (Opn4Cre/Cre; left) and wild-type (center) retinas, immunolabeled for melanopsin. These projections were imaged, processed, and displayed with the same settings. Right: Inset from center panel showing stained ipRGCs at higher magnification. Scale bars are 100 μm (center) and 50 μm (right).
Figure S4. In Vivo Activation of IpRGCs, Related to Figure 3

(A) Left: Cumulative distributions of normalized pS6 immunofluorescence values, from cells pooled across retinas exposed to the same stimulus irradiance. Irradiances were 3.0 (n = 565 cells), 4.1 (621), 5.0 (755), and 6.9 (684) log photons μm^2 s^-1; all stimuli were 90 min in duration. All distributions are significantly different (p < 0.001) except for 4.1 and 6.9 (p = 0.15). Right: quantile-quantile plot comparing selected distributions. The ordinate comprises values from irradiance 6.9 and the abscissa from irradiance 5.0 (orange crosses) and 3.0 (black crosses). Solid line is unity slope.

(B) Raw data from (A). For clarity, equal numbers of cells (565) from each condition are plotted; these cells were randomly selected from 4-5 retinas exposed to the same irradiance. Melanopsin values are centered (by subtracting the mean) and standardized (by dividing over the SD). Values of pS6 are normalized to a calibration standard derived from the average of several highly activated non-ipRGCs (STAR Methods).

(C and D) As in (A) and (B), but pS6 values are normalized by the overall intensity of melanopsin labeling (STAR Methods). All distributions are significantly different (p < 0.001) except for 4.1 and 6.9 (p = 0.22).

(E) Confocal projection of an Opn4tau-LacZ/Cre retina, in which M1s express β-galactosidase (and Cre recombinase, whose activity is inconsequential for this experiment) instead of melanopsin. Arrowheads mark the somata of cells positive for β-galactosidase.

(F) Cumulative distributions of pS6 immunofluorescence values from cells that express β-galactosidase, pooled across retinas exposed to the same stimulus irradiance. Irradiances were 3.0 (n = 399 cells), 5.0 (407), and 6.9 (521) log photons μm^2 s^-1; all stimuli were 90 min in duration. All distributions differed significantly from that of the dark control (n = 400 cells; p < 0.01) but not from one another (p of 0.14 – 0.4).

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(G) Raw data from (F). Immunolabeling intensity of pS6 is plotted against that of β-galactosidase. Equal numbers of cells from each condition are plotted (n = 399 cells from 3 retinas per irradiance, including darkness).

(H) Confocal projection of an Opn4<sup>Cre+/+</sup> retina that was transduced with a virus carrying a Cre-dependent tdTomato construct. Arrowheads mark M4s, which are distinguished by their large soma size.

(I) Cumulative distributions of pS6 immunofluorescence values from M4s pooled across retinas exposed to the same stimulus irradiance. Irradiances were 3.0 (n = 431 cells), 5.0 (305), and 6.9 (366) log photons μm<sup>-2</sup> s<sup>-1</sup>; all stimuli were 90 min in duration. All distributions differed significantly from each other and that of the dark control (n = 380 cells; p < 0.01).

(J) Raw data from (I). Immunolabeling intensity of pS6 is plotted against a random cell index. Equal numbers of randomly selected cells from each condition are plotted (305 cells from 2-3 retinas per irradiance). Values in (A)–(J) are background-subtracted.
Figure S5. Unimodal Tuning through Hyperactivation of Spike Generation by Phototransduction, Related to Figure 6
(A) Irradiance-response relations obtained from somatic, perforated-patch recordings (i-vii; n = 7 cells). Relations obtained from ascending (solid lines) and descending (dashed lines) irradiances are shown for subthreshold membrane voltages (left) and steady firing rates (right). All recordings were made at 35°C. With the exception of rows vi and vii, synaptic antagonists were included.
(B) As in (A), but for cells in which inward photocurrent was measured during a second presentation of the stimulus (as in Figure 6; viii-xi, n = 4 cells). Irradiance-response relations for the steady photocurrent (measured at −80 mV) are shown on the left. Relations from the cell in Figure 6 are shown in row x.
(C) A depolarizing current ramp (bottom) also evoked spike attenuation (top) and a fall in dVmax (middle). Dashed horizontal lines indicate −72 mV (top) and 0 mV/ms (middle). These data are from the cell in (B) (row x) and Figure 6.
(D) Left: Relation of dVmax and the voltage preceding the spike (Vpre) for ascending irradiance (black), descending irradiance (blue), and depolarizing current injection (red). Center: A similar relation, but for maximum downstroke velocity (dVmin) rather than dVmax. Right: For spike amplitude. Depolarization, either through light or through current, affects these parameters similarly.
Figure S6. Adaptation Mediated by Voltage-Gated Channels, Related to Figures 4 and 6

(A) Schematic of the stimulus used to examine the current-firing relations of M1s during increasing and then decreasing excitation, in the absence of evoked phototransduction. Its parameters match those of the abbreviated irradiance pyramid (2 min steps, 5 steps to the apex).

(B) Left column: Current-firing relations measured from M1s using somatic, perforated-patch recording. Right column: As on left, but for subthreshold membrane voltage. Plotted values are averaged over the last 30 s of each step. Relations from ascending steps (solid lines) and descending steps (dashed lines) are shown. Each row is a different cell (i-vi); only cells with $I_{f} > 2.6 \log$ photons $\mu m^{-2} sec^{-1}$ were analyzed. These cells should show unimodal irradiance tuning with leftward adaptive shifts. Recordings were made at 35°C with synaptic antagonists.

(C) Fractional difference in near-maximum (95%) firing rate between ascending and descending irradiance-firing (red) and current-firing (blue) relations. Filled circles are sample means and error bars are SDs. Negative values indicate lower firing rates during decreasing stimulus intensity. For direct comparison, all

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irradiance-driven cells analyzed here had $I_{\text{th}} \leq 2.6 \log \text{photons} \mu m^{-2} \text{sec}^{-1}$ ($n = 11$, with 7 recorded in perforated-patch mode and 4 in loose-patch mode). The same is true of (D) and (E).

(D) As in (C), but for displacement of the tuning curve peak (measured as for $I_{\text{th}}$; i.e., 95% of maximum) on the stimulus intensity axis. In order to compare irradiance to current, displacement is quantified in terms of the tuning curve width (i.e., a value of 1 indicates a shift on the abscissa corresponding to the entire width of the tuning curve, measured at half-maximum). Negative and positive values indicate leftward and rightward shifts due to stimulus history, respectively. Data from the same 11 light-driven cells as in (C) are shown here.

(E) As in (C), but for the subthreshold membrane voltage (with the difference between ascending and descending values averaged across all current steps). Negative and positive values indicate lesser and greater depolarization during descending stimulus intensity, respectively. Data from 7 light-driven cells are shown (i.e., from perforated-patch recordings only).

(F) Left: Relation of $dV_{\text{max}}$ and the voltage preceding the spike ($V_{\text{pre}}$) for ascending (black) and descending (blue) current steps for a representative cell (whose current-firing relation is shown in panel ii of [B]). Center: A similar relation, but for maximum downstroke velocity ($dV_{\text{rep}}$) rather than $dV_{\text{max}}$. Right: Relation of $dV_{\text{rep}}$ and $dV_{\text{max}}$. Light-independent adaptation of M1s appears to have little effect on the voltage-dependence of block.
Figure S7. Technical Considerations for Somatic, Perforated-Patch Recording from M1 IpRGCs, Related to Figure 7

(A) Simultaneous recording from the soma (red) and axon (blue; ~600 μm distal) of an M1. Note the similarity of the first and last somatic fluctuations but the lack of an axonal spike for the latter. The bottom trace (black) is the first derivative of the somatic trace, which tracks axonal spikes. The dashed line shows the spike detection threshold based on this derivative (6-fold $\sigma_{\text{noise}}$, where $\sigma_{\text{noise}}$ was measured in a window lacking obvious spikes. The stimulus was 5.6 log photons μm$^{-2}$ sec$^{-1}$ (2.2 log units above $I_{\text{F}}$). Data from this cell are also shown in (C) and (F).

(B) Error rate for spike detection (i.e., summing false positives and false negatives) as a function of somatic detection threshold. A $\text{dV}_{\text{max}}$-based detection threshold of 6-fold $\sigma_{\text{noise}}$ was effective. The dotted trace indicates data from the cell in (A). Each cell was tested at multiple irradiances, from threshold to block, in order to obtain spikes of different waveforms.

(C–E) Fluctuations in somatic voltage showed continuous variation from unambiguous spikes to noise. The $\text{dV}_{\text{max}}$ values of somatic events that were between 3- and 60-fold $\sigma_{\text{noise}}$ are plotted against event amplitude (i.e., spikes exceeding 30 mV in amplitude and the smallest noise events are omitted, for clarity). Somatic events that were accompanied by axonal spikes are shown in black and others in red. Dashed lines represent the 6-fold $\sigma_{\text{noise}}$ threshold established in (B).

(F–H) Ascending IFs for the cells in B–E, comparing the axonal IF (blue) and somatic IF (red; generated from the 6-fold $\sigma_{\text{noise}}$ threshold). Cells in (A)–(E) were driven with white light, whose intensity is expressed in terms of equivalent 460-nm photons.

(I) Histogram of firing rate measured from the soma during repeated steps of light (5 min) delivered from darkness (2 min; stimulus depicted at top). The measurement was made in the loose-patch configuration before (blue traces) and after (red traces) establishment of perforated-patch recording via a second electrode. Perforation was monitored in voltage clamp (3 min; indicated by the dashed lines) before loose-patch measurement of spiking resumed.

(J) Light-evoked changes in steady firing rates for the sample of 3 cells, with the onset of perforated-patch recording indicated (dashed line). The cell in (I) is distinguished by colored symbols. For all cells, the stimulus was broadband amber light (Xe emission band-passed from 574-700 nm), which facilitated

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comparison among steps by minimizing persistent activation beyond the period of illumination (Emanuel and Do, 2015). The initiation of perforated-patch recording caused a consistent and stable decrease in firing frequency. All data from (A)–(J) were obtained at 23°C with antagonists of synaptic transmission included.

(K) IF relations obtained from the ascending staircases of abbreviated pyramids, using axon-only recordings in the loose-patch mode (black, n = 4 cells) and soma-only recordings in the perforated-patch mode (red, n = 11 cells). Firing rates are displayed in absolute units (left) and normalized to the maximum (right). Relations are aligned by their I_{1/2} values for comparison of shape. The amplitudes but not shapes of tuning curves were altered by perforated-patch recording. Experiments were performed at 35°C with antagonists of synaptic transmission and the standard stimulus (monochromatic 460-nm light).