Biophysical Variation within the M1 Type of Ganglion Cell Photoreceptor

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Summary

Intrinsically photosensitive retinal ganglion cells of the M1 type encode environmental irradiance for functions that include circadian and pupillary regulation. Their distinct role, morphology, and molecular markers indicate that they are stereotyped circuit elements—however, their physiological uniformity has not been investigated in a systematic fashion. We have profiled the biophysical parameters of mouse M1s and found that extreme variation is their hallmark. Most parameters span 1-3 log units, and the full range is evident in M1s that innervate brain regions serving divergent functions. Biophysical profiles differ among cells possessing similar morphology, and between neighboring M1s recorded simultaneously. Variation in each parameter is largely independent of that in others, allowing for flexible individualization. Accordingly, a common stimulus drives heterogeneous spike outputs across cells. By contrast, a population of directionally selective retinal ganglion cells appeared physiologically uniform under similar conditions. Thus, M1s lack biophysical constancy and send diverse signals downstream.

Graphical abstract

Emanuel et al. demonstrate that a type of sensory cell with a defined role and morphology nevertheless exhibits log units of variation in its biophysical parameters. Variation drives functional individualization, even in spike outputs, and is fully available to downstream brain regions serving divergent functions.
Keywords

cell type; melanopsin; photoreceptor; retinal ganglion cell; phototransduction; heterogeneity; membrane excitability; directionally selective retinal ganglion cell; Hb9

Introduction

A precise understanding of the nervous system relies on discernment of its cellular building blocks, with each type defined by its functional role, morphology, molecular composition, and physiological properties (Sanes and Masland, 2015). The definition of type is often operational because these characteristics are incompletely known or give rise to classifications that are incongruent (Parra et al., 1998; Seung and Sümbül, 2014). For instance, some cells share an electrophysiological identity but diverge in their molecular mechanisms, while others have a common morphology yet respond heterogeneously to an identical input (Marder and Goaillard, 2006; Padmanabhan and Urban, 2010; Soltesz, 2006). Indeed, electrical responses and the biophysical parameters that govern them are particularly challenging to define because they are complex, adaptive, and frequently examined using techniques that can introduce variation. An example is provided by cells in brain slices; although they are amenable to deep analysis, they may be partially intact and receive a fraction of their natural inputs (Steriade, 2004). Thus, basic questions remain about the physiological side of cell-type definitions. How much biophysical variation exists among cells that have a common role, morphology, and molecular signature? What are the rules of covariation among parameters? And to what extent is variation related to the requirements of downstream circuits?

A platform for investigation is provided by the intrinsically photosensitive retinal ganglion cells (ipRGCs), which together with the rods and cones account for all mammalian cells that are known to transduce light into electrical signals (Berson et al., 2002; Hattar et al., 2003; Panda et al., 2003). IpRGCs are akin to brain neurons in receiving synaptic input and
communicating with spikes. At the same time, their local circuitry can be kept intact within the ex vivo retina and their natural responses can be evoked with light—ipRGCs initiate phototransduction using a visual pigment called melanopsin (Provencio et al., 2002). These cells are divided into several morphological types (designated M1, M2, etc.; Ecker et al., 2010). The M1s provide an especially informative case for the question of how physiological properties relate to cell-type definitions. One reason is that their identity as an anatomical type is clear, because they form a spatial mosaic and place dendritic arbors of characteristic shape in a discrete sublamina of the retina. The behavioral role of M1s also appears straightforward: To provide a representation of irradiance for functions such as circadian photoregulation and the pupillary light reflex, which are lost when these cells are ablated (Chen et al., 2011; Güler et al., 2008; Hatori et al., 2008). M1s are particularly amenable to precise analysis because, unlike other ipRGCs, their intrinsic light response can drive the full range of spike firing (Ecker et al., 2010; Schmidt and Kofuji, 2009, 2011).

Electrophysiological studies of M1s have uncovered properties of their light responses that are suited to irradiance coding (Do et al., 2009; Do and Yau, 2013; Emanuel and Do, 2015; Wong et al., 2005) and examined how some of these properties differ from those of other ipRGC types (Schmidt and Kofuji, 2009, 2011; Zhao et al., 2014). The degree of physiological variation within an ipRGC type is not known. Although the literature contains hints, it does not address technical sources of variation—differential light history and dialysis caused by whole-cell recording are two of many factors that could give the appearance of diversity (Do and Yau, 2013; Warren et al., 2006; Wong et al., 2005). The question of variation is relevant to M1s because they drive functions that are qualitatively different (reviewed by Do and Yau, 2010). The possibility that the light responses of M1s are diversified to match the requirements of downstream circuits is unexplored.

We have taken a systematic approach to profiling the biophysical parameters that govern the light responses of M1s, primarily using recording methods that provide quantitative information with minimal disturbance to the cell (Do et al., 2009). We have found that these parameters exhibit extreme and continuous variation, whether associated with membrane properties, synaptic input, or phototransduction. In addition, we obtained evidence that the full extent of variation is conveyed to downstream brain regions that have different roles. The biophysical parameters of M1s show little covariation, such that individuals—even those with similar dendritic morphologies—have dissimilar response properties. This diversity is evident at the level of spike output, as a common stimulus evokes distinct firing patterns across cells. Moreover, a population of directionally selective RGCs that we examined as a reference displayed far less biophysical variation than M1s.

Results

Sampling IpRGCs from the General Population and by Projection Target

To estimate the degree of biophysical variation among M1s, we targeted cells in the retinas of BAC-transgenic mice that express the fluorescent protein, tdTomato, under control of the melanopsin gene locus (Do et al., 2009). Random selection (Experimental Procedures) resulted in the inclusion of multiple types of ipRGC, and we discriminated M1s from others using their dendritic morphology or biophysical properties, as described further below.
The defining characteristic of M1s is their placement of dendrites in the outermost lamina of the inner plexiform layer (IPL; Figure S1A; Ecker et al., 2010). To extend this morphological classification to their axonal terminations, we also examined cells that were retrograde-labeled from distinct regions of the brain. Our stereotaxic injections were centered on the suprachiasmatic nucleus (SCN; site of the master circadian clock) or olivary pretectal nucleus (OPN; first brain relay for the pupillary light reflex). We deposited a relatively large amount of tracer, such that neighboring areas were sometimes included, to obtain sufficient numbers of cells for electrophysiological analysis (Figure S1B-E). Injections into either site only resulted in a handful of ipRGCs labeled in each retina (Figures S1F and S1G). These “hypothalamic” and “pretectal” samples are partially independent; murine ipRGCs may project to only one of these regions or split their axons to innervate both (Fernandez et al., 2016). We refer to ipRGCs identified without retrograde labeling as the “general” sample. By comparing hypothalamic and pretectal samples, we test whether any biophysical variation observed in the general sample is partitioned among downstream functions with broadly distinct needs or is a pervasive feature of ipRGC inputs.

Biophysical Profiling of IpRGCs

We analyzed a panel of biophysical parameters that govern the responses of ipRGCs, using the perforated-patch technique to prevent dialysis of phototransduction molecules and thus maintain normal light responses. Due to the breadth of our panel and the extended timescales of ipRGC light responses, we recorded at 23 °C to increase the lifetime and stability of our experiments. In this condition, recordings of high quality were maintained for the required duration (1.5 hrs; Experimental Procedures). Additional experiments indicate that our conclusions hold near body temperature (see below).

Our biophysical profile encompassed aspects of the intrinsic membrane properties, synaptic input, and phototransduction mechanism (Figure 1A). We began each recording by identifying a cell, using brief pulses of imaging light that summed to <2 seconds. This imaging procedure produced little persistent activation of ipRGCs. To minimize desensitization, we imposed ≥20 min of darkness before measuring light responses. We have found that this interval is sufficient to return melanopsin to the dark-adapted state (Emanuel and Do, 2015). During this period, we assessed the baseline membrane voltage (either the resting voltage or the average voltage between spontaneous spikes) and injected current to evaluate passive properties (e.g., input resistance) and aspects of spike generation (e.g., steady rate and regularity; Figure 1B). These elements of electrical excitability translate the conductances evoked by synaptic input and phototransduction into the particular output of the cell.

Subsequently, we voltage-clamped the cell and delivered weak flashes. The response to each flash often was biphasic (Figure 1C; Wong et al., 2007). In order to compare across cells, we calibrated our stimulus such that the slow phase, which appeared to be entirely intrinsic in origin, had a peak of ~5 pA or less (within the linear range of melanopsin phototransduction; Do et al., 2009). We measured the fast transient at this flash intensity and then blocked it by applying pharmacological antagonists of synaptic transmission. In some cells, the antagonists also eliminated a component of standing inward current (“synaptic...
Once block was complete, we analyzed the intrinsic light response driven by melanopsin phototransduction. Our approach was grounded in a conceptual framework that was established for the rods and cones, then augmented to encompass properties that M1s share with the rhabdomeric photoreceptors commonly found in invertebrates. This framework allows the biophysical parameters of phototransduction to be rigorously compared between M1s and other photoreceptors, and used to predict the responses of individual cells to various illumination conditions (Do et al., 2009; Do and Yau, 2013; Emanuel and Do, 2015).

We examined four fundamental components of the intrinsic light response and the transitions between them. First is the single-photon response, which originates from activation of one melanopsin molecule and is the basis of the macroscopic response. This response is exceptionally prolonged in ipRGCs and therefore promotes temporal integration (Do et al., 2009). It is challenging to visualize due to its small size but can be deduced from responses evoked in the linear range of the intensity-response relation. Such “dim-flash” responses (Figure 1E) are the superposition of single-photon responses. As such, dim-flash and single-photon responses have the same waveform. Following convention, we summarized its kinetics using a fit to the convolution of two exponentials. We also quantified its duration as the integration time, defined as $t_i = \int \frac{f(t)}{f_p} dt$ where $f(t)$ is the waveform and $f_p$ its peak amplitude. By measuring the trial-by-trial variation in the amplitude of the dim-flash response, we estimated the magnitude of the single-photon response (Figure 1E; Do et al., 2009). Moreover, dividing the amplitude of the dim-flash response by the stimulus intensity provided an absolute measure of sensitivity (i.e., the amount of current generated per photon in a unit area).

Next is the saturated response evoked by an intense flash, which arises from the activation of many melanopsin molecules and represents the maximum, instantaneous capacity for phototransduction. We extracted parameters of activation, magnitude, and termination (Figure 1F). Following the saturated response is a long-lived increase in inward current and noise, which we measured with respect to baseline (Figure 1G). This persistent response can last for minutes, extends the integration time of the cell over this interval, and reflects the thermal stability of melanopsin’s signaling conformation (Emanuel and Do, 2015).

After acutely decreasing the persistent response with 560-nm light (Figure 1G), our protocol finished with five steps of light, each brighter by approximately one log unit (Figure 1H). The responses to these steps reflect the balance of activation with a mechanism of adaptation that helps maintain phototransduction within the dynamic range of spike generation (Do and Yau, 2013; Wong et al., 2005). We measured the kinetics of adaptation onset, the extent of adaptation, the interval needed to reach steady state, and the rate of deactivation following the cessation of illumination. Taken together, these responses to flashes and steps of light provide a quantitative and comprehensive view of ipRGC light responses.

### Discrimination of M1 IpRGCs

Our data set comprises 97 ipRGCs, of which 46 were of the general population while 24 and 27 others were retrograde-labeled from the hypothalamus or pretectum, respectively. After profiling each cell, we converted from the perforated-patch to whole-cell mode in order to
dialyze neurobiotin into the cytosol. Subsequent fixation, staining, and confocal analysis allowed us to define dendritic stratification and thus morphological type. Of 29 cells that were successfully recovered using this procedure, 18 were M1s, 9 were M2s, and 1 was an M3 (Figures 2A and S2A). The paucity of M3s was anticipated by prior studies (Berson et al., 2010). We expected the remaining types to be undetectable in our reporter line due to their low expression of melanopsin, and thus presumably of fluorophore (Ecker et al., 2010). We encountered no cells with the large somata and dendritic fields that characterize M4s, though one had a dendritic field resembling that described for M5s (Figure S2B). The large fraction of M1s in our sample likely reflects the disproportionate contribution of this type to innervation of the SCN. Examining only the general population, we find that its M1 fraction (10/16 cells) is similar to that previously observed in the mouse retina by systematic, anatomical reconstruction (Berson et al., 2010).

As expected, the somatic diameters of identified M1s were significantly smaller than those of M2s (mean ± SD of 12.7 ± 1.9 vs 15.5 ± 1.7 μm; p = 0.004, Wilcoxon rank test; Berson et al., 2010). These types also showed biophysical differences, expected from previous observations, in sensitivity, saturated photocurrent amplitude, input resistance, instantaneous firing rate, maximum firing rate, current for maximum firing rate, and amplitude of the synaptic response (all p values ≤ 0.001, Wilcoxon rank test; Figure 2B; see Schmidt and Kofuji, 2009; Zhao et al., 2014). Our profile revealed 18 additional parameters that differ significantly between M1s and M2s, of the 37 assessed in total (Figure S2C).

Divergence of the established, biophysical parameters allowed us to predict whether cells with unverified morphologies were of the M1 type. We developed a binary logistic regression model that used the most informative subset of these parameters to separate verified M1s from verified non-M1s (Experimental Procedures and Figures 2C and S3). The model gave no false negatives (i.e., all predicted M1s had verified M1 morphology) and one false positive (one predicted non-M1 had verified M1 morphology; Figure 2C). Applying it to cells with unverified morphology produced the sample of predicted M1s that will support the remainder of our analyses.

**Biophysical Diversity of M1 IpRGCs**

Examining the biophysical parameters of all M1s in our sample, an overt characteristic is their extreme degree of variation. Of the 37 parameters examined, 7 spanned at least two log units and 22 others spanned at least one (Figures 3A and S4; detailed further in Table S1). Such variation was found in parameters concerning synaptic input, intrinsic membrane properties, and phototransduction. Variation is likely to be impactful even for parameters that showed relatively little dispersion. An example is the ~2-fold range in baseline membrane voltage, which is salient given that membrane voltage is constrained to a narrow physiological band and subtle changes in its value can have large effects due to the nonlinearities of spike generation. Thus, the biophysical parameters of M1s are not stereotyped across individuals.

The biophysical diversity of M1s may be expected to segregate across their downstream brain targets, which can diverge markedly in their use of visual information (e.g., Laughlin, 1992; Keenan et al. 2016; Nelson and Takahashi, 1991). We examined the profiles of M1s
that were retrograde-labeled from the hypothalamus (locus of the master circadian clock) and pretectum (a primary control center for the pupillary light reflex), and found that they were almost completely overlapping. All 37 parameters lacked differences between groups (Table S1). Notably, these profiles are similar to those of the general sample (Figures 3A and S4). Cells of the hypothalamic (n = 17), pretectal (n = 21), and general (n = 32) groups are also intermingled when visualized in principal component space (Figure 3B). Hence, the full range of variation present among M1s appears to be reflected in their inputs to two distinct brain regions.

**Controls for Variation of Technical Origin**

We considered that part of this variation may be due to technical matters. Access resistance is of particular concern, as it may differentially filter responses across cells and result in a lack of voltage control, but it was not correlated with the first principal component of the biophysical parameters (p = 0.54; Experimental Procedures). With regard to individual parameters, inadequate access should have a disproportionate effect on events that are fast and large. We found cases of the opposite: Some cells had saturated responses that were among the fastest and largest in the sample, together with dim-flash responses that were among the slowest and smallest (see below). High access resistance distorts events with more rapid kinetics; thus, although we compare parameters concerning spike pattern across cells, we do not compare those relating to spike waveform. Additional concerns regarding technical sources of variation are light history, circadian time, and cell damage. However, when we recorded from multiple cells in a retina, we ensured that their dendritic fields were well-separated, and found no relationship between the order in which a cell was recorded and its sensitivity or other biophysical parameters. In addition, the cells we filled after recording had intact dendritic arbors, and those with similar arbors nevertheless showed diverse biophysical parameters (see below). Statistical prediction of M1 identity could also introduce variation. However, the variation we observed among predicted and verified M1s was similar (Table S1; also compare Figures 2 and 3); for example, dim-flash sensitivities spanned a range of more than 2 log units in both. Therefore, variation is likely biological in origin.

To provide additional confidence that variation was authentic, we compared biophysical parameters between ipRGCs recorded simultaneously as pairs (Figure 4A). We targeted M1s by their small somata and high reporter expression, then used dye-filling to verify their dendritic stratification. To reliably fill cells and better control access resistance, we used whole-cell rather than perforated-patch recording. We reduced the impact of dialysis by testing an abbreviated set of biophysical parameters, using fine electrodes, and initiating recordings at the same time for cells in a pair. We found that the largest variation within pairs of M1s was dim-flash sensitivity, which differed by as much as 690% (or as little as 10%; n = 8 pairs). The integration time and single-photon response magnitude spanned ranges that were as broad as those in the larger sample of M1s recorded singly, with maximum differences of 250% and 60%, respectively (n = 6 pairs for which these measurements could be made for both cells; Figure 4B). Other parameters, including those concerning synaptic and intrinsic properties, were likewise distinct across simultaneously recorded M1s (Figures 4C, D). These experiments also controlled for variation due to...
regional differences across the retina, because members of a pair were close together. Moreover, they provided direct evidence that variation in our sample was not driven by differences in light history, circadian time, global neuromodulatory state, retinas, or animals. Rather, biophysical variation appears to be an inherent quality of M1s.

An additional concern is that the variation we observed at 23 °C may not reflect that at body temperature. Although recording lifetime is too brief at high temperature for extensive biophysical profiling, it is sufficient for analysis of select parameters; we chose dim-flash sensitivity and integration time because they are among the most and least variable, respectively. Perforated-patch recording at 35 °C (Figures 4E and 4F) revealed dim-flash sensitivities between $3.8 \times 10^{-6}$ and $4.4 \times 10^{-4}$ pA photons$^{-1} \mu m^2$ ($n = 17$ cells; mean ± SD of $1.5 \pm 1.4 \times 10^{-4}$ pA photons$^{-1} \mu m^2$). As expected from prior work (Do et al., 2009), these sensitivities were higher than those measured at 23 °C (by 3.3-fold, $p = 0.0002$, Wilcoxon rank test). They spanned more than 2 log units at both temperatures. Integration times also covered similar ranges at 35 and 23 °C (6.4- and 5.3-fold, respectively; $n = 13$ cells at 35 °C for which waveforms were well-resolved); comparing population means yielded a temperature dependence of 2.2-fold (9.9 ± 3.4 and 21.5 ± 5.9 s at 35 and 23 °C, respectively; $p = 2 \times 10^{-9}$, Wilcoxon rank test). These experiments also allowed the time constants of the dim-flash response to be extracted, and these spanned broad ranges at 35 °C (3.8-fold for $\tau_1$ and 9.9-fold for $\tau_2$; Figure 4F). Thus, the biophysical parameters of M1s appear to show extreme variation near body temperature.

To contextualize the biophysical heterogeneity of M1s while providing an additional control for technical sources of variation, we studied another RGC type. We selected the directionally selective RGCs that are fluorescently labeled in the Hb9:EGFP mouse line; these cells show morphological and functional homogeneity (Sabbah et al., 2017; Trenholm et al., 2011). We examined biophysical parameters that could be compared between these cells and M1s. We targeted Hb9s in various retinal locations, used whole-cell recording to obtain reliable dye-filling for morphological verification (Figure 4G), and verified that they had the expected ON-OFF light response (Figure 4H). Many biophysical parameters of Hb9s showed significantly less variation than those of M1s, and none showed significantly more (Figure 4I and Table S2).

**Flexibility of Biophysical Diversification**

To what extent are biophysical parameters coordinated within individual M1s? For example, if a cell has a large number of pigment molecules, it should capture photons effectively and thus have the capacity to respond with high sensitivity. Because the maximum number of photons it can capture is high, it is also poised to have a large saturated response. In other words, one might predict that sensitivity and the saturated response are correlated. Another prediction may be that prolonged single-photon responses are correlated with large persistent responses and mild adaptation, because these are all reflections of a phototransduction cascade that is biased against rapid termination. One might also hypothesize that some parameters are either correlated or anti-correlated. For instance, a small single-photon response could be paired with low input resistance and little synaptic input to produce low sensitivity; alternatively, a small single-photon response could be
compensated by high input resistance or large synaptic input. We investigated the relationships among biophysical parameters to infer the rules that govern the expression and function of molecules that mediate phototransduction, membrane excitability, and synaptic transmission.

We examined all pairwise relations between measured biophysical parameters and calculated their correlation coefficients. Approximately one third of the correlations were statistically significant (227 of 666, or 34%, where \( p \leq 0.05 \)). The matrix of these significant correlations is shown in Figure 5A (see Figures S5 and S6 for a full correlation matrix and coefficient of determination matrix, respectively). Of these significant correlations, 6% generally would be considered strong (\( |r| \geq 0.7 \)), 18% moderate (\( 0.7 < |r| \leq 0.5 \)), 50% mild (\( 0.5 < |r| \leq 0.3 \)), and 26% weak (\( |r| < 0.3 \); Figure 5B). Strong correlations appeared trivial, as they were only found among aspects of the same property. For example, saturated responses of large amplitude also had large slopes of activation (Figure 5C). Many of the moderate and mild correlations were of a similar kind, while the remainder were otherwise unsurprising (e.g., the larger the saturated response, the larger the persistent response that followed). Overall, we detected remarkably few correlations of note. None of the expectations articulated above were strongly supported (Figure 5D): There was only mild correlation between dim-flash sensitivity and the magnitude of the saturated response; no correlation between the duration of the single-photon response and the magnitude of the persistent response; and no correlations between the size of the single-photon response and input resistance or the magnitude of synaptic input. These observations indicate that an M1 that sets one of its biophysical properties retains a high degree of flexibility for setting the others.

A complementary perspective on variation is provided by examination of individual biophysical profiles. Shown in Figure 6A are compact visualizations of these profiles. Each is composed of vectors representing the measured parameters; their lengths are proportional to the parameter values, with the unit length representing the maximum observed and half that value representing the sample mean. Inspection of these visualizations shows that no single M1 bears a strong resemblance to the average M1 or any other M1. The biophysical profiles of M1s appear to be highly individualistic.

### Relationship between Morphology and Physiology

To compare biophysical and morphological parameters, we reconstructed M1s in our sample and extracted parameters such as soma diameter, dendritic length, and number of primary dendrites. These parameters showed relatively broad and continuous variation (Figure S7A). Furthermore, cells with similar morphologies diverged in their biophysical profiles (Figure 6B). Although the first principal component of the biophysical parameters was correlated with that of the morphological parameters (Figure 6C), anticipated correlations between individual biophysical and morphological parameters were not found. For example, although larger cells may be expected to have a lower input resistance and a higher capacity for phototransduction, dendritic length was neither correlated with input resistance (\( p = 0.19 \)) nor the amplitude of the saturated photocurrent (\( p = 0.08 \); Figures S7B and S7C). Thus, morphology does not place an overt constraint on the biophysical variation of M1s.
Biophysical Variation is Reflected in Spike Outputs

Some neurons have stereotyped outputs despite variation in their underlying mechanisms of excitability (Marder and Goaillard, 2006). We do not expect this to hold for M1s because the biophysical properties we measured are largely uncorrelated and thus unlikely to counterbalance one another in any obvious manner. In addition, some evidence can be found in previous work for variation in spiking across ipRGCs, whether they were recorded simultaneously using multielectrode arrays (Mawad and Van Gelder, 2008) or sequentially via patch-clamp electrophysiology (Do and Yau, 2013). These recordings were conducted at body temperature, which suggests that biophysical variation is reflected in spike outputs under physiological conditions.

To ask whether M1 outputs are also diverse under the experimental conditions that are required for biophysical profiling, we used loose-patch recording to monitor spikes at 23 °C. We targeted M1s based on their soma size and high reporter expression, and delivered two steps of white light that differed in intensity by 100-fold (Figure 7A). Based on our biophysical profiles, these steps should drive the intrinsic responses of individual cells at different points in their dynamic ranges to evoke spike outputs with varied time courses and patterns. The outcome is summarized in Figure 7B-D. Diversity in spike outputs was apparent even in darkness, with all cells exhibiting spontaneous firing of various rates (between 0.01 and 2.1 Hz) and patterns (coefficients of variation, CVs, of interspike intervals between 0.7 and 2.1; n = 11 cells). When exposed to light of the lower intensity, cells increased firing with divergent patterns (CVs of interspike intervals between 0.6 and 3.3; n = 11 cells). Upon cessation of illumination, a fraction of cells returned to baseline with little delay while others exhibited long-lasting, persistent responses. When presented with light of the higher intensity, an additional component of variation became apparent: While some cells maintained an elevated rate during illumination, many only fired a transient burst at its onset and had no detectable firing afterward. Varied responses also followed the return to darkness, with cells remaining active or silent for different intervals before relaxing toward baseline. These variations in spiking at high intensity, like those at low intensity, are consistent with the diversity of biophysical profiles we have measured.

Discussion

M1s exemplify the idea of a cell type in their distinctive role, complement of molecular markers, and morphology. They provide the essential conduit for retinal signals to influence functions like circadian photoregulation and pupillary constriction: furthermore, melanopsin phototransduction is required for such functions to exhibit their normal dynamic range and sustained nature (reviewed by Do and Yau, 2010). M1s are distinguished from conventional RGCs by molecular markers that include melanopsin and pituitary adenylate cyclase-activating polypeptide (Hannibal et al., 2002; Provencio et al., 2002). In addition, they have a characteristic anatomy, with dendrites that branch within a distinct sublamina of the retina and axons that innervate a circumscribed set of brain regions (Ecker et al., 2010). This view is established for rodents and is likely to hold across species (e.g. Hannibal et al., 2017; Hannibal et al., 2014; Nasir-Ahmad et al., 2017). It is against this background of stereotyped features that we have found extreme, continuous, and flexible variation in the biophysical
parameters of M1s. Variation is a robust trait of these cells, spanning a similar range in the general sample and in those retrograde-labeled from hypothalamus or pretectum. Furthermore, M1s with similar morphologies nevertheless diverge in their biophysical parameters.

**Generalization to Other IpRGC Types**

Our sample of M2s shows comparable dispersions in parameters concerning synaptic input, intrinsic membrane properties, and phototransduction (Figure S2). These data are consistent with select aspects of M2s measured previously (Hu et al., 2013; Schmidt and Kofuji, 2009). Studies concerning the remaining types of ipRGCs also contain hints of variation (Estevez et al., 2012; Hu et al., 2013; Schmidt and Kofuji, 2011). Because the goal of these studies was to explore differences among types rather than within them, technical origins of variation were not excluded. Nevertheless, M1s are unlikely to be alone among ipRGCs in their physiological heterogeneity.

**Comparison with the Classical Photoreceptors**

Natural counterpoints to M1s are provided by rods and cones, which together with ipRGCs are the only mammalian cells known to transduce light into electrical signals. Of the parameters that can be compared between these cell types, and focusing on those of the mouse, a few appear to have a similar degree of variation. For example, the integration time has a CV of ~0.2-0.3 for rods, cones, and M1s (Cao et al., 2014; Mendez et al., 2001). This degree of invariance may indicate the particular importance of integration time, as it sets the balance between sensitivity and temporal resolution. Indeed, it is regulated at multiple stages in the phototransduction cascade (Burns, 2010). Overall, however, parameters showed greater variation in M1s. Sensitivity is a prominent case, exhibiting a CV that is ~2- and ~4-fold higher in M1s than in rods and cones, respectively; its range in the M1 population is comparable to the separation between the classical photoreceptors. Likewise, the CV of the saturated response is ~2-fold greater in M1s than in rods or cones (Azevedo and Rieke, 2011; Cao et al., 2014). The degree of correlation among parameters is especially salient. With normalization for the mild variation in sensitivity and the saturated response, the intensity-response relations of individual rods and cones are superimposable (Baylor et al., 1979; Schnapf et al., 1990; note that these studies concern toad rods and primate peripheral cones, respectively). By contrast, we have found that these and other parameters of M1s are largely uncorrelated. Thus, M1s are exceptional among photoreceptors of a type in the breadth and individual flexibility of their biophysical variation.

**Biophysical Variation in IpRGCs and Conventional RGCs**

M1s may also be compared to conventional RGCs (cRGCs), which divide into dozens of variants that extract different features from the visual scene (Sanes and Masland, 2015). Examining the biophysical variation within ten cRGC types examined previously (in the cat retina; O'Brien et al., 2002), we find only one case in which the CV is as large as that of M1s, which is the steady firing rate of the delta RGC. Even for this parameter, the CV of M1s exceeds the average CV of the ten types by 1.7-fold and the minimum CV by 6.1-fold. The heterogeneity of M1s is prominent when comparing the other parameters that are common to both data sets: baseline membrane voltage (the CV of M1s exceeds the
maximum CV of the ten types by 2.2-fold, the average by 5.6-fold, and the minimum by 14-fold), input resistance (1.8-, 3.1-, and 6.5-fold), and peak firing frequency (1.9-, 2.7-, and 10.2-fold). This divergence is unlikely due to species differences, because we have found that a population of direction-selective cRGCs in the mouse retina also exhibits biophysical constancy. Thus, the electrophysiological properties of M1s are exceptionally heterogeneous.

A Broader Survey of Biophysical Variation

An analogy to M1s is provided by olfactory sensory neurons (OSNs), which are also both receptor and projection cells. The activity of an OSN is governed by the identity of its odorant receptor. Like M1s, individuals with the same receptor can have parameter values that span log units. Moreover, a few of these parameters have been examined for correlations, with none found (Connelly et al., 2013; Grosmaitre et al., 2006). Moving to the brain, one finds a substantial amount of biophysical variation in several neuronal types (Soltesz, 2006), even considering technical factors (e.g., measured synaptic connectivity can rise steeply with modest increases in the thickness of brain slices; Steriade, 2004).

Biophysical variation may recur throughout the nervous system.

Implications of Biophysical Variation

Physiological diversity signifies that the mechanism of the M1 light response lacks a strict template across cells. The components of phototransduction and membrane excitability are variable, with differences potentially arising in the identities, expression levels, and posttranslational modifications of signaling molecules. Candidates for diversification among M1s have been found, including distinct developmental lineages and splice isoforms of melanopsin, though their relations to cellular function remain to be established (Chen et al., 2011; Pires et al., 2009). The continuous and independent variation we observed indicates that these molecular differences may be subtle, perhaps more so than the sum of weak differences that separate rods and cones (Ingram et al., 2016). Therefore, making precise connections between molecular composition and physiology requires the study of single cells. Analyses of pools would not yield accurate views of individuals. Likewise, a simulated M1 built from average parameters would be a poor representation of any real M1.

Because the biophysical parameters of M1s are varied, the influence of these cells on the organism is not readily predictable. For example, the threshold of a particular function may reflect activation of the most sensitive M1, that preferring a particular intensity, or progressive recruitment of several over a span of intensities. The absolute sensitivities of phototransduction that we measured cover a >100-fold range. We estimate that this dispersion of sensitivities corresponds to spiking thresholds ranging from roughly 30 to 3,000 photons μm^-2 s^-1 at body temperature (Supplemental Information). In parallel work performed under physiological conditions, we have directly observed this range of spike thresholds and examined its consequences for coding irradiance (Milner and Do, in press).

From the experiments presented here, it is apparent that a given light intensity will activate M1s that have overlapping sensitivities but exhibit broad dispersion in other biophysical parameters. Heterogeneity and decorrelation of spiking is understood to improve stimulus encoding in several systems (Padmanabhan and Urban, 2010; Pitkow and Meister, 2012) and...
future work will determine whether they confer advantages to the M1 representation of irradiance.

**Experimental Procedures**

Complete methods are provided in Supplemental Information.

**Animals**

All animal procedures were approved by the Institutional Animal Care and Use Committee of Boston Children’s Hospital. Mice were wild-types, BAC-transgenics in which ipRGCs express tdTomato (Do et al., 2009), or transgenics in which directionally selective RGCs express EGFP (Wichterle et al., 2002). They were adults (P23 – P113) of either sex. Biophysical parameters did not vary according to age, sex, or circadian time.

**Retrograde Labeling**

Hypothalamic injections were centered on the suprachiasmatic nucleus and kept dorsal to the optic chiasm. Pretectal injections were centered on the olivary pretectal nucleus, avoiding the optic tract and superior colliculus.

**Electrophysiology**

Following dark adaptation, mice were anesthetized and retinas collected. IpRGCs and Hb9s were identified with <2 s of imaging light. When 470-nm light was used on ipRGCs (for green-emitting fluorophores), 545-nm light followed to minimize persistent activation (Emanuel and Do, 2015). Additional dark adaptation (≥20 minutes) preceded the measurement of light responses in ipRGCs. Because the aim was to assess the degree of biophysical variation, no criteria were used to exclude cells from analysis apart from detectable fluorescence, an accessible soma, lack of visible damage, achieving a satisfactory seal resistance, and maintaining a stable access resistance. Exclusion criteria derived from prior studies were also tested for ipRGCs (Schmidt and Kofuji, 2009) but did not change the distributions of the biophysical parameters. Therefore, all cells were analyzed.

**Type Prediction**

To predict morphological type using biophysical parameters, a binary logistic regression model was developed using ipRGCs with known morphology and the 7 parameters that have previously been shown to differ between M1 and other types (Hu et al., 2013; Schmidt and Kofuji, 2009). To select a subset of parameters that would minimize prediction error, a Lasso penalty was implemented (Taylor and Tibshirani, 2015). To further assess the reliability of this model, two independent methods to classify ipRGCs based on the same subset of biophysical parameters were used. Unsupervised cluster analysis and linear discriminant analysis each produced two groups that were nearly identical to those generated by the regression model (i.e., 5% and 6% of cells were classified differently, respectively; Figure S3). The regression model is preferred because it uses the minimum number of meaningful parameters and employs cross-validation, which both reduce the susceptibility to overfitting. Furthermore, it does not require assumptions about the distributions of parameters (e.g., normality and linear separability).
**Statistical Methods**

Parametric and non-parametric statistical tests were applied as appropriate, with post-hoc tests used when multiple comparisons were made.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Biophysical variation is extreme within a well-defined type of retinal neuron.
- Key parameters span log units and differ more within than between related types.
- Parameter variations are largely independent, allowing flexible diversification.
- Biophysical variation is associated with population diversity in spike patterns.
Figure 1. Profiling the Biophysical Parameters of IpRGCs
A. Sequence of recording and parameter extraction, designed to minimize effects of light history. See Figure S1 for identification of ipRGCs by retrograde labeling.
B. Response of an ipRGC to hyperpolarizing and depolarizing current injection.
C. Response of an ipRGC to a flash of light (480 nm, 50 ms, $4.4 \times 10^5$ photons $\mu m^{-2}$) without (black) and then with (gray) antagonists of synaptic transmission.
D. Example ipRGC that exhibited a decrease in the holding current and noise in synaptic antagonists. A periodic, 10-mV hyperpolarizing pulse (bottom) was used to monitor input resistance and recording parameters.
E. Left, top: Four dim-flash responses of an ipRGC (480 nm, 50 ms, $5.5 \times 10^4$ photons $\mu m^{-2}$). Left, bottom: Amplitude of all dim-flash responses over time. Right, top: Mean response (black) fit with a convolution of two exponentials (gray, $\tau_1 = 1.42$ s, $\tau_2 = 19.8$ s; complete decay to baseline not shown). Right, middle: Trial-by-trial fluctuation in the dim-flash response. Right, bottom: Comparison of variance and mean$^2$ waveforms (normalized by their peak amplitudes) indicates that most variation arises from the number and not shape of the underlying, single-photon responses. Dividing the variance by the mean gives the amplitude of the single-photon response.
F. Response of an ipRGC to saturating light (xenon, 200 ms, $2.0 \times 10^{-2}$ μW μm$^{-2}$, equivalent to $3.8 \times 10^{6}$ lux). Inset: Expanded time scale for resolution of activation kinetics. A subset of extracted parameters is illustrated.

G. Persistent response of an M1 and measurement periods for its mean and standard deviation (140 s after cessation of the stimulus, which is the same stimulus as in F). 560-nm light (70 s, $2 \times 10^{9}$ photons μm$^{-2}$ s$^{-1}$; preceded by a 50-ms flash of $1 \times 10^{8}$ photons μm$^{-2}$ to monitor the transient response) acutely reduced the response.

H. Responses of an ipRGC to 60-s steps of 480-nm light (intensities relative to dimmest stimulus, $5.6 \times 10^{4}$ photons μm$^{-2}$ s$^{-1}$, on right). Steps were delivered from dimmest to brightest. Parameters related to the transient peak were measured from the step with the largest transient, and parameters related to the steady response were measured from the step with the largest steady response. The maximum difference between the peak and steady responses was also extracted. All recordings were at 23 °C. Antagonists of synaptic transmission were included for E-H.
**Figure 2. Biophysical Discrimination of M1 from Non-M1 IpRGCs**

A. Confocal projections of filled M1s and M2s (red) counterstained with DAPI (blue) to show retinal layers. Orthogonal views (below) taken from boxed regions.

B. Distributions of seven parameters from morphologically-verified M1s and M2s, previously established to differ between these types. Asterisks indicate p ≤ 0.05, Wilcoxon rank test.

C. All ipRGCs, filled and not filled, visualized using the first two principal components of the parameters in B. Binary logistic regression was used to predict whether individuals were M1 (magenta) or not (cyan). See also Figures S2 and S3.
Figure 3. Variation of Biophysical Parameters among M1 ipRGCs
A. Values of selected parameters displayed as cumulative distributions. No differences were detected between hypothalamic (red; n = 17), pretectal (blue; n = 21), or general (black; n = 32) samples (Kruskal-Wallis ANOVA). See also Figure S4 and Table S1 for distributions and statistics of all parameters.
B. Hypothalamic, pretectal, and general M1s plotted on the first three principal components of all 37 biophysical parameters.
Figure 4. Controls for Technical Variation
A. Schematic of paired, whole-cell recordings from M1 ipRGCs. All cells were filled with Alexa 488-hydrazide and verified to have M1 morphology.
B. Dim-flash responses (470 nm, 50 ms, $3.4 \times 10^5$ photons μm$^{-2}$) recorded from a pair of cells (average of 5 trials for each cell). Synaptic antagonists were included.
C. Membrane voltages of the same cells during injections of current. The left cell was hyperpolarized (-2 pA) to match the resting potential of the right cell.
D. Summary plots for all cells, with pairs connected.
E. Example of a dim-flash response (average of 4 trials; 480 nm, 50 ms, $5.0 \times 10^4$ photons μm$^{-2}$) fit with a convolution of two exponentials ($\tau_1 = 0.7$ s; $\tau_2 = 5.5$ s). 35 °C. Synaptic antagonists were included.
F. Range (left) and CV (right) of dim-flash sensitivity, integration time, $\tau_1$, and $\tau_2$ at 23 and 35 °C (white and black bars, respectively).
G. Confocal projection (left) and morphological tracing (right) of an EGFP-expressing (green) Hb9 RGC filled with Alexa 555 hydrazide (red) during whole-cell recording. Counterstain is DAPI (blue). Scale bar: 40 μm.
H. Example responses to a step of light (top; 480 nm, 2 s, $1.4 \times 10^3$ photons μm$^{-2}$ s$^{-1}$) and to steps of current injection (bottom; 2 s) of the Hb9 depicted in G.
I. Range (left) and CV (right) of 12 biophysical parameters measured from Hb9s (red markers, n = 12) and 50 representative sets of 12 randomly sampled M1s (black markers). Asterisks denote parameters for which the value for M1s was lower than that for Hb9s in <0.5% of 100,000 subsamples (i.e., p < 0.005). The range of the spontaneous spiking rate is not shown, being undefined due to division by 0.
Figure 5. Correlations among Biophysical Parameters of M1 IpRGCs
A. Heatmap showing Pearson coefficients for all significant pairwise correlations between biophysical parameters. Pairs with insignificant correlations are in gray. Colors along the ordinate indicate categories of biophysical parameters: red, synaptic; black, dim-flash response; green, saturating flash response; magenta, persistent response; cyan, step response; gray, passive membrane properties; and blue, active membrane properties. See also Figures S4 and S5.
B. Histogram of all Pearson correlation coefficients (magenta and gray denote significant and insignificant values, respectively).
C. Scatter plots of the four parameter pairs with the strongest, significant correlations (i.e., with the highest $|r|$; parameters standardized and centered). Axis labels are colored according to parameter categories as defined in A.
D. Scatter plots of parameters as in C, providing examples ranging from weak but significant correlations (left) to insignificant correlations (right).
Figure 6. Biophysical Profiles of Individual M1 IpRGCs
A. Visualizations of all biophysical parameters measured for all individual cells with full profiles. Each vector within a plot represents the relative value of a parameter measured for the cell (with 0.5 and 1 representing the population mean and maximum). Visualizations are ordered by Euclidean distance to the mean in the first two principal components, from closest (top left) to furthest (bottom right). The gray letter at the bottom right of each plot indicates the group (h, hypothalamus-projecting; p, pretectum-projecting; or g, general). The two cells shown in 6B are specified by i and ii.
B. Tracings and parameter visualizations of two cells with similar morphologies (e.g., dendritic lengths of the left and right cells are 1,450 μm and 1,620 μm).
C. Scatter plot of the first principal component of the morphological parameters versus the first principal component of the biophysical parameters for filled M1 ipRGCs (n = 18). See also Figure S7.
Figure 7. Diverse Spike Outputs of M1 IpRGCs

A. Loose-patch recording of a cell (top) with spike histograms (1-s bins, gray, and 5-s bins, black). The stimulus was a step of white light (xenon, 60 s, $2.3 \times 10^{-8} \mu W \mu m^{-2}$, equivalent to 7 lux) followed by darkness (220 s) and then a ~100-fold brighter step (60 s, $2.4 \times 10^{-6} \mu W \mu m^{-2}$, equivalent to 720 lux).

B. Spike histograms of exemplar cells (n = 6 of 11, excluding the one depicted in A; 5-s bins) in response to the dimmer (left) and brighter (right) step. All responded with a ≥10% change in firing rate to the dimmer step. Stimulus timing is shown by the bottom trace and the vertical, dashed lines. The latter are expanded to accommodate histogram bins. These cells are represented by the symbols on the right in C and D.

C. Scatter plots for all cells in the sample, showing the firing rate during each step of light (CV of 0.4 for dim and 2.0 for bright) versus the firing rate preceding each step (CVs of 0.8 and 1.0). The dashed line represents no change in firing rate.

D. Maximum instantaneous spike rate for each cell during designated epochs of the recording (CVs of 1.5, 0.8, 1.8, 0.7, and 1.7 for each respective epoch).