Retinal output changes qualitatively with every change in ambient illuminance

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The collective activity pattern of retinal ganglion cells, the retinal code, underlies higher visual processing. How does the ambient illuminance of the visual scene influence this retinal output? We recorded from isolated mouse and pig retina and from mouse dorsal lateral geniculate nucleus in vivo at up to seven ambient light levels covering the scotopic to photopic regimes. Across each luminance transition, most ganglion cells exhibited qualitative response changes, whereas they maintained stable responses within each luminance. We commonly observed the appearance and disappearance of ON responses in OFF cells and vice versa. Such qualitative response changes occurred for a variety of stimuli, including full-field and localized contrast steps and naturalistic movies. Our results suggest that the retinal code is not fixed but varies with every change of ambient illuminance. This finding raises questions about signal processing within the retina and has implications for visual processing in higher brain areas.

The mammalian visual system functions over a wide range of light intensities, spanning roughly a dozen orders of brightness magnitude. Specialized photoreceptors, the rods and cones, are active at low and high light intensities, respectively. At low light intensities, only rods are active (scotopic vision). With increasing luminance, cones become active (mesopic vision), while at high luminance, rods saturate but cones remain active (photopic vision). In the outer retina, signals from the photoreceptors are both combined within and distributed across more than ten different bipolar cell types. In the inner retina, the bipolar cell terminals interact with amacrine cell interneurons to bring about sophisticated responses in the output neurons of the retina, the ganglion cells. The diversity of ganglion cells is characterized by physiological parameters1, as well as by functional specifications such as directional selectivities, approach sensitivity, object motion sensitivity and many more2. On a simpler level, all ganglion cells can be classified by their response polarity to step-like changes in brightness: ON cells increase spiking activity to light increments, OFF cells to light decrements, and ON-OFF cells to both. This property is often called “polarity” and is one of the most basic features for further classification of ganglion cells in the vertebrate retina.

It is not well understood how the properties of ganglion cell responses (that is, the retinal output) vary with changes in ambient luminance. On one hand, it is conceivable that adaptation in retinal circuitry counteracts the changes in ambient luminance to maintain a stable representation of the incoming visual scene. On the other hand, several reports suggest that the retinal output changes with changing ambient luminance. Some of these changes are linked to the switch from scotopic to mesopic vision; that is, from purely rod-mediated to mixed rod- and cone-mediated signaling. Examples include color vision3, changing responses due to surround activation4–6, changes in temporal and spatial frequency processing7,8, 2-amino-4-phosphorobutanoic acid (APB)- and strychnine-resistant OFF responses appearing in response to dim high-contrast stimuli9, or luminance-dependent inhibitory modulation of rod signals10.

In addition, the coexistence of several parallel rod pathways11 might allow different retinal processing within the scotopic range as well: for example, the primary rod pathway shifts from encoding of single photons to encoding of contrast modulations12. Furthermore, light adaptation switching from circuit-based to photoreceptor-based mechanisms has been found within both scotopic13 and photopic regimes14. Finally, melanopsin-driven changes in retinal responses have been described within the photopic range15. Most of these reports concentrate on individual building blocks of the retinal circuit, and each describes luminance-dependent changes over a limited range of light intensities. What is missing is a systematic description of the retinal output and its modulation across a wide range of light intensities, from scotopic to photopic light levels.

We asked whether luminance-dependent changes of the responses of ganglion cells are a widespread phenomenon or whether they are restricted to few cell types or specific luminance transitions. Using multielectrode array (MEA) recordings from isolated mouse retina, we systematically surveyed ganglion cell responses across many orders of ambient luminance, in discrete steps separated by 1 log unit. We found that the output of the retina was qualitatively different at each tested light level. For example, we found OFF cells gaining or losing ON responses, and vice versa. Such response changes occurred

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to both simple stimuli and complex natural movies. Sometimes, but not always, these changes depended on modifications of the center-surround receptive field structure or on GABA-mediated inhibition. Consequently, diverse mechanisms seem to underlie the response changes in different ganglion cell types. In addition, we show that such alterations of the retinal output are not restricted to the isolated mouse retina but can also be observed in vivo, where the changing output of the retina is reflected by changing activity of dorsal lateral geniculate nucleus (DLGN) neurons, and in the retina of another species, the pig. It thus appears that luminance-dependent changes of retinal output are a phenomenon that is preserved across species and that higher visual centers are exposed to these changes.

RESULTS
Experimental procedure
We presented our visual stimuli, grayscale images, to isolated mouse retinas using a digital projector (Supplementary Fig. 1). The ambient light level was set by placing neutral density (ND) filters into the light-path, such that the intensity of the stimulus could be attenuated without changing the computer-controlled images presented by the projector. Consequently, the contrast of the stimuli remained constant during the experiment (Fig. 1a), independent of the ambient light level. The actual physical intensity of the stimuli associated with each ND-filter is shown in Figure 1b. We estimate (see Online Methods) that ND8 and ND7 correspond to scotopic conditions, ND6 weakly activates cones, ND5 is fully mesopic, and ND4 is photopic. Unless otherwise noted, we started our experiments from low intensity (ND8) and increased it in the course of the experiment (that is, from ND8 to ND4 in 1-log-unit steps). The retina was kept at each ambient luminance for 20 to 70 min, and we showed the same set of stimuli at each light level.

With this experimental procedure, we recorded from ganglion cells using MEAs and compared their responses across different ambient light levels, initially using spatially homogeneous contrast steps (‘full-field steps’) of positive and negative contrast (‘white step’ and ‘black step’, ±66% Weber contrast; Fig. 1a). We will refer to increases of a cell’s spike rate to light increments (both after the white step onset and black step termination) as ON responses and to increases of a cell’s spike rate to light decrements (both after the black step onset and white step termination) as OFF responses.

Luminance-dependent changes of retinal output
To our surprise, most ganglion cells changed their response type (ON, OFF or ON-OFF) at different ambient luminance. The example cell in Figure 2a had OFF responses to all light decrements, but its ON responses were not consistent across light levels. First, they were absent at ND8 but present at ND7 to ND4. Second, when present, they occurred with either short or long latency (‘early’ or ‘delayed’, respectively), measured as time to peak of the firing rate. Third, at any given light level, the ON responses to the two stimuli (white and black steps) were either the same—that is they were absent (ND8) or had the same latency (ND4)—or they had different latencies (ND7, ND6 and ND5). We will refer to the latter as ‘asymmetry’ of the response at a given luminance. In summary, the OFF responses of this cell at the different light levels (ND8 to ND4) differed from each other only quantitatively (amplitude, duration and moderate latency changes), whereas the ON responses were affected qualitatively.

We take a ‘qualitative change’ of a response across light levels to mean not only its presence versus absence, but also alternations between early and delayed responses. Indeed, early and delayed responses, as seen in Figure 2a, seem to be two distinct response categories, and not merely separate realizations of a continuous latency distribution. The distributions of the response latencies (Fig. 2b), measured separately in ON cells and OFF cells and separately for ON and OFF responses, was unimodal for the preferred contrast—that is, for ON responses in ON cells and for OFF responses in OFF cells—with a median time to peak between 130 and 140 ms. In contrast, the distributions of latencies for responses to the anti-preferred contrast had an additional mode peaking between 600 and 800 ms, in both ON cells and OFF cells. In other words, delayed ON responses occurred only in OFF cells, whereas delayed OFF responses occurred only in ON cells. The bimodality of the distribution indicated two categories of responses and let us treat early and delayed responses as qualitatively different.

In our analysis below, we concentrate only on the qualitative response changes. Quantitative aspects were not considered.

The response patterns of ganglion cells usually remained stable while probed at the same luminance level, tested up to 70 min (luminance levels with unreliable responses were excluded from the analysis; see Online Methods). When the response pattern of a cell changed at luminance transitions, the new pattern was observed from the very first stimulus presentation. The earliest time point we tested was 10 s after the luminance transition because a luminance increase by 1 log unit itself evoked a strong response in all cells.

The cell in Figure 2a could be classified as OFF at some light levels and as ON-OFF at other light levels on the basis of its full-field step responses. Since such luminance-dependent response changes were common in many ganglion cells, we used an ON/OFF classification based on properties of the cell’s linear filters. We calculated the linear filters from responses to Gaussian white noise full-field flicker (see Online Methods). Cells with a downward deflected linear filter were marked as OFF and cells with an upward deflected filter as ON. In contrast to full-field step responses, almost all cells had consistent linear filter polarities over all luminance levels. The cell in Figure 2a fell into the OFF category at each light level, despite its changing ON responses. Note that with such a classification scheme, ON-OFF cells will not be categorized as such, but would fall into either the ON or OFF category, depending on which input was predominant; similarly, cells with an exceptionally strong surround might be mistaken for a cell of opposite polarity. Furthermore, if ON and OFF inputs were very well balanced, the cell would have a noisy linear filter. However, such cases were rare, and we excluded from the analysis all cells with noisy or changing linear filters across light levels (34 out of 517 recorded units were excluded).

We obtained 219 OFF and 264 ON cells (as based on their linear filter properties) from 15 wild-type retinas. The validity of this ON/OFF classification approach was supported by the observations that >97.5% of ganglion cells from the ON group consistently responded to light increments (that is, their preferred stimulus) at all light levels and >97.4% of cells from the OFF group consistently
responded to light decrements. It follows that luminance-dependent changes mostly occurred in response to the anti-preferred contrast. In the following analysis, we concentrated on the responses to anti-preferred contrast steps (Fig. 3), and we describe the ON responses in OFF cells first.

**ON responses in OFF ganglion cells**

Across all light levels tested, only 9% of our OFF cells never had an ON response. The number of cells displaying early or delayed ON responses changed at different ambient light levels (Fig. 3a). Almost 100% of OFF cells had no ON responses at ND8, whereas at ND5, this number fell below 20%. Notably, the early and delayed responses could also occur together (most often at ND5). They were still easily separable in most cases because of the considerable difference in their latencies (for examples, see Fig. 4 and Supplementary Fig. 2).

At every transition of ambient luminance, the ON responses of a considerable fraction of OFF cells changed (Fig. 3b), ranging from 38% at the ND8–ND7 transition (within the scotopic regime) to 83% at the ND6–ND5 and ND5–ND4 transitions. Overall, 89% of the OFF cells changed their responses at least once between ND8 and ND4. The response changes were diverse. At any given light level, some cells would lose a certain response type, others would gain it, and some cells would not change. Furthermore, the responses to white steps and black steps changed asymmetrically (Fig. 3a). For example, at ND6 there was a predominance of delayed responses to the white step and early responses to the black step, whereas at ND5 the ratio was opposite.

In summary, the presence of ON responses and their variability across light levels were two prominent features in OFF cells: we found that early and delayed ON responses in OFF cells could appear or disappear with changing ambient light levels, that they could occur independently or together during a response and that they could differ for white and black contrast steps. These findings suggest that these early and delayed ON responses in OFF cells may have independent origins and be heterogeneously affected in different OFF cell types by the immediate stimulus history (that is, white or black step) and by ambient luminance.

**OFF responses in ON ganglion cells**

Occurrences of OFF responses in ON cells (Fig. 3c,d for summary, Fig. 4a,b for examples) were less common than occurrences of ON responses in OFF cells. In fact, most ON cells were strongly suppressed by light decrements, such that their spiking activity fell below their spontaneous firing rates, often to zero. Black steps often suppressed spiking for the entire stimulus duration (2 s); white step termination, for about 500 ms (Fig. 4a). Strong pre- or postsynaptic inhibition may have counteracted excitation and decreased the occurrence of the OFF responses. Indeed, there were almost no OFF responses to black steps (Fig. 3c), with the exception of the photopic ND4 light level, at which 11% of ON cells had early OFF responses. Delayed OFF responses were observed quite frequently after white step termination, especially in scotopic and mesopic light levels (ND7 to ND5).

In our experiments, the luminance-dependent qualitative change of response patterns was such a surprising and yet prominent feature of most ganglion cells that this raises concerns about how trustable and stable these observations are. We tested the following: (1) How strongly are the different response types bound to a particular ambient luminance? (2) Do these response changes occur in morphologically identified ON and OFF cells? (3) Is this finding restricted to *in vitro* conditions?

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**Figure 2** Early and delayed anti-preferred responses. (a) Responses (firing rate) of a single OFF ganglion cell to white and black full-field contrast steps (average firing rate to 45 repetitions at each of five different light levels, ND8 to ND4). (b) Histogram of response latencies (time to peak) in OFF cells (left column) and ON cells (right column), measured from responses of all units at all light levels, to both black and white full-field steps.

**Figure 3** Summary of luminance-dependent response types. (a) Fraction of OFF cells displaying no, early and delayed ON responses at each luminance level to white and black full-field step stimuli. (b) Fraction of units with stable and changing responses. Cells were defined as stable if they had the same response type (no, early, delayed or both) at all compared light levels, both to the black step and to the white step. All other cells were defined as changing. (c,d) Same statistics as in a and b for OFF responses in ON cells. Numbers indicate units included in the analysis, selected on the basis of their reliable responses at these light levels (see Online Methods).
conditions, or may it also be observed in vivo?
(4) How much of the responses variability is due to the unnatural stimulus properties of full-field contrast steps? Furthermore, we investigated the contribution of center-surrond receptive field interactions, GABAergic inhibition and rod-cone interactions to the mechanism of qualitative luminance-dependent response changes.

Response patterns are bound to individual light levels
As described above, the response patterns of ganglion cells were stable at each individual light level but could change after a luminance increase. We next tested whether ganglion cell responses would revert when the luminance returns to the previous level (Fig. 5a). Indeed, in the ND8 to ND5 luminance ranges, all recorded cells that changed their responses at a luminance transition (n = 16 from 2 retinas) immediately reverted to the previous pattern after an intermittent exposure to either lower or higher luminance levels (Fig. 5b,c). However, once exposed to ND4 (photopic level), cells did not immediately return to the response they had at ND5 earlier. This may be due to stronger bleaching caused by this light level (∼10^4 R* rod^−1 s^−1) or to some light adaptation triggered by this light level that reverses only slowly.

In further experiments discussed below (Supplementary Fig. 2), 2 of the 15 cells studied did not revert to their previous response pattern at the ND7 light level after they had a different pattern during an interleaved exposure to ND6, while 13 of 15 cells did revert to their previous response pattern. Taken together, these results suggest that specific response patterns of ganglion cells are strongly associated with distinct luminance levels rather than with the history of luminance or with a luminance-independent drift.

Confirmation using single-cell recordings
Most cells in our data set had ON-OFF responses at least at one light level. Our cell type classification based on linear filter polarity cannot identify ‘classical’ ON-OFF cells (that is, cells stratifying in both ON and OFF sublaminae of the inner plexiform layer and having short-latency responses to both light increments and decrements) and distinguish them from ‘real’ ON cells or OFF cells (that is, cells with dendrites stratifying exclusively in the ON or OFF sublamina). To confirm that the latter can indeed have responses to anti-preferred contrast steps at some light level(s), we recorded action potentials from individual ganglion cells using patch electrodes. Most cells were filled with neurobiotin and imaged with confocal microscopy to assess whether they had typical ON or OFF morphology (Fig. 6a–c).

We recorded from three PV-5 ganglion cells, the well-studied mouse homolog of the transient OFF-alpha cell (monostratified in the OFF sublamina of the inner plexiform layer; n = 2 of 3 cells confirmed with the neurobiotin marker). All three cells had delayed ON responses up to ND5 that disappeared at the photopic light level ND4. For one cell, we repeatedly switched between ND4 and ND5, and the responses reliably reverted (Fig. 6d). Consistent with the related MEA experiments (Fig. 5), switching from ND4 back to ND5 did not lead to an immediate reappearance of the delayed ON responses; here they reemerged about 1 min after the luminance switch. Four out of 5 more cells of unknown types, stratifying exclusively in the OFF (n = 3) or ON (n = 2) sublamina (Fig. 6e), had luminance-dependent response changes, confirming our findings based on MEA recordings.

Luminance-dependent response changes in vivo
One caveat of the results described so far is that they have been recorded from the isolated retina, and that these experiments can last several hours. Do luminance-dependent response changes also happen in vivo? To test this, we recorded from the dLGN of anesthetized mice (Fig. 7a) and projected step stimuli into their eyes that were comparable in absolute intensity and contrast to the stimuli we used for the in vitro recordings (Fig. 7b). Consistent with our findings in the in vitro retina preparation, in the dLGN 18 out of 28 units (n = 5 mice) changed their responses qualitatively with changing ambient luminance (Fig. 7c). We could also test higher light levels (ND3 and ND2) in vivo than in vitro (see also Discussion). More than one-third of the recorded neurons changed their responses within the photopic regime as well (ND4–ND3 and ND3–ND2), including the example shown in Figure 7d. These observations suggest that luminance-dependent qualitative changes of retinal ganglion cell responses also occur in vivo and that these changes are reflected in the thalamus. This confirms scattered reports of this phenomenon in the literature.3

Luminance-dependent changes to naturalistic movies
Full-field contrast steps are easy to analyze and interpret. However, they are not a natural stimulus for the retina and visual system in general. The retina might employ specific mechanisms to stabilize the output to a more natural stimulus when it is presented under varying luminance conditions. We tested this by stimulating the retina with a naturalistic movie repeatedly shown at different light levels.

Ganglion cells (n = 172 units from 8 retinas) responded to the natural movie with interleaved sequences of spike bursts (events) and silence, as described previously.18 Such bursting events presumably correspond to features in the movie that are relevant to this ganglion
If a cell had a robust bursting event at some light levels but not at others, we classified this as a qualitative response change (see Online Methods for details).

We observed such qualitative changes in 57% of the units (n = 98 of 172). For each of these units, some features (scenes of the movie) evoked a response at all light levels tested, and other features evoked a response only at certain light levels (Supplementary Fig. 3a, b). Some units (n = 55) were also tested with our full-field step stimulus. Response changes to the movie stimulus and to the full-field step stimulus could occur independently from each other (Supplementary Fig. 3c). This suggests that ambient luminance can alter different receptive field properties of ganglion cells, some of which are triggered by a homogeneous full-field step and some by a stimulus with more complex temporal and spatial properties.

Cells’ peripheries involved in only some response changes
Most ganglion cells’ receptive fields consist of a spatially distinct center and periphery. Stimulation of the center and periphery can evoke responses of opposite polarities in some ganglion cells19. Furthermore, it is known that the receptive field structure of some cells changes during light adaptation5. Thus, the changing response patterns that we observed in our experiments might have been caused by luminance-dependent changes in the balance of the receptive field center and periphery. To test this, we stimulated the retina with disks of 150 μm diameter with identical contrast properties to the full-field steps (n = 107 units in 4 retinas).

We observed the same variety of response types to the localized disk stimulus as for the full-field stimulation. 80% of the units changed the response type to the disk stimulus at least at one luminance transition, while 20% had stable responses at all light levels (Fig. 8a). At any individual luminance transition, between 44% and 61% of the units changed their responses. We also mapped the receptive fields of all units using a binary noise checkerboard flicker stimulus and measured how much of the disk stimulus lay within the receptive field center (Fig. 8b). For more than half the units, both with changing or stable responses, 80% or more of the disk stimulus was contained.
ChAT staining was not successful. Most cells had luminance-dependent response changes.

The structure of the central receptive field alone. Taken together, our results suggested that it influenced some but not all luminance-dependent qualitative response changes.

Nevertheless, the receptive field periphery did influence the responses of many units: the responses to the local disk and full-field stimuli differed from each other at least at one light level in 67 of the 107 units. Distinct responses to localized and full-field stimulation could be observed at all light levels, from ND8 (scotopic) to ND4 (photopic), suggesting that at least some ganglion cells possess a receptive field surround in scotopic conditions.

Notably, we observed several units that stably maintained their response type to disks with changing luminance but that qualitatively changed their responses to full-field steps (Fig. 8d). In these units, it is likely that a reorganization of the overall receptive field structure (for example, of center-surround interactions) is responsible for the changes of the responses, and not a reorganization of the central receptive field alone. Taken together, our results suggest that most units can change their responses to local stimulation but that a dynamic reorganization of the overall receptive field structure can be responsible for some qualitative luminance-dependent response changes as well.

GABAergic inhibition involved in some response changes
GABA-mediated inhibition can mask responses of ganglion cells20,21; release from GABAergic inhibition at some light levels might therefore be a valid mechanism for luminance-dependent response changes. To test this, we compared the responses of ganglion cells to full-field contrast steps at ND7 and ND6 with and without blockade of ionotropic GABA receptors (5 µM SR-95531 and 100 µM picrotoxin; Supplementary Fig. 2a). From two retinas, we extracted 37 units with stable responses during the two repeats of ND7 in control conditions.

The drugs had diverse effects on the ganglion cell responses (Supplementary Fig. 2b–e): in some cells, GABA blockers prevented luminance-dependent response changes, whereas in other cells they enabled such changes. In yet other cells, responses were not influenced by GABA blockade. In summary, we found that the mechanism of GABAergic response regulation was highly diverse and that it influenced some but not all luminance-dependent qualitative response changes.

Response changes do not require rod-cone interactions
Many ganglion cells changed their response pattern at transitions within the scotopic regime (ND8–ND7). This suggests that rod-cone circuit interactions are not required for all response changes.
Figure 7 Luminance-dependent qualitative response changes in the dLGN. (a) Recording locations in the dLGN, outlined on the left. Middle, reconstructed positions (colored dots) of recording sites in three rostrocaudal positions relative to the bregma. Reconstruction was based on DiI labeling of electrode shanks (right); blue line, maximum depth of recording electrode. Brain schematics based on Paxinos and Franklin. (b) Absolute stimulus intensities used for the in vivo experiments (black) in comparison to the intensities used during in vitro experiments (gray; see Fig. 1b). Note that the stimulus range is extended to higher intensities. Scot., scotopic; mes., mesopic. (c) Fraction of light-responsive units in the dLGN with changing or stable responses. Conventions as in Figure 3b. (d) A single ON unit from the dLGN that has both changing and asymmetric OFF responses at different ambient light levels.

To further explore how much of the response variability is brought about by the rod pathways, we used three different mouse models with nonfunctional cone photoreceptors (‘rod-only retinas’): Gnat2<sup>−/−</sup>, Pde6c<sup>−/−</sup> (Cpfl1) and Cnga3<sup>−/−</sup> mice, which carry mutations in cone-specific members of the phototransduction cascade: a transducin, phosphodiesterase and cyclic nucleotide–gated channel, respectively.

In retinas from all three cone-deficient mouse lines, we found a similar prevalence of luminance-dependent response changes as in wild-type retinas (Supplementary Fig. 4). Together, these results confirm that not all luminance-dependent response changes rely on rod-cone interactions, as such changes can be observed in retinas with nonfunctional cones. Instead, some response changes might reflect more subtle changes in processing due to engaging different rod-mediated pathways<sup>11</sup> at low and high scotopic light levels.

Generalization to other species
To exclude the possibility that luminance-dependent response changes are a feature restricted to the mouse retina, we recorded from the isolated pig retina, using the same procedure as for the mouse retina. Luminance-dependent response changes were also commonly observed in pig ganglion cells (n = 98 cells, three retinal pieces from two different animals; Supplementary Fig. 5). While the pig and mouse data differed in some details (for example, hardly any delayed ON responses in pig OFF cells), the phenomenon of luminance-dependent qualitative response changes was observed in both species with comparable frequencies.

DISCUSSION
We studied the responses of retinal ganglion cells to full-field contrast steps over 5 log units of background light intensities. We classified ganglion cells into ON and OFF groups based on their linear filter and found that most OFF ganglion cells and a large fraction of ON cells behave as ON-OFF at least at some luminance levels. In both groups, the responses to the anti-preferred stimulus contrast could have short latency (early responses) or long latency (delayed responses). Early and delayed responses, which may occur together in many cells (Fig. 3a,c), appeared to be distinct response categories (Fig. 2b) that can be regulated independently (Supplementary Fig. 2). Most intriguingly, over 80% of cells displayed different response types to the anti-preferred contrast at different background luminance (Fig. 3b,d). It is noteworthy that the linear filter polarity, obtained as spike-triggered average to full-field Gaussian white noise flicker, was stable at all light intensities despite changing responses to step stimuli.

Despite such a high degree of variability in the responses of ganglion cells, we found them to be reliably bound to the specific luminance: most cells would always respond in a similar way at a particular light intensity.

Figure 8 Luminance-dependent response changes to small localized disk stimuli. (a) Percentage of units with stable or changing responses across different luminance levels. Conventions as in Figure 3b, but combining both ON and OFF cells. (b) Histogram showing how much of the disk stimulus was contained within the receptive field center, as determined by a binary checkerboard flicker stimulus. (c) Example unit changing its responses to localized stimulation of the receptive field center. Right, overlap of the disk stimulus (red) with the receptive field (blue ellipse shows 2.5 σ of Gaussian fit). (d) Example unit that had stable responses to the disk stimulus but changing responses to the full-field step at the ND6–ND5 luminance transition.
level, even if such trials were interleaved with exposure to higher or lower light levels (Fig. 5). Moreover, luminance-dependent qualitative changes of the responses were also demonstrated in recordings from dLGN neurons in vivo (Fig. 7) and to spatially heterogeneous stimuli, such as small disks (Fig. 8) and a naturalistic movie, which is a more ecologically relevant visual stimulus for the retina and the visual system in general. In several single-cell recordings from ganglion cells identified to be morphologically ON or OFF, we observed similar light-dependent response changes (Fig. 6), further corroborating the conclusions drawn from the MEF recordings. Finally, we found that luminance-dependent response changes were not restricted to the mouse retina but existed in pig retina as well (Supplementary Fig. 5).

In the isolated retina, stimulation at light levels higher than ND4 (corresponding to 10^4 R* rod^1 s^-1) led to subtle changes in response properties that are likely associated with excessive bleaching of photopigment (data not shown). While the retina continued to respond well to visual stimulation, the results obtained at those high intensities probably do not reflect normal retinal processing as it would happen in the intact eye (data not shown), and hence we excluded these higher light levels from our analysis. The recordings from the dLGN therefore not only confirm that luminance-dependent response variability occurs in vivo, but they also expand the range of light intensities at which that phenomenon was observed. Overall, we found luminance-dependent response changes over all intensity ranges and at each luminance transition we tested, from scotopic to photopic light levels.

The collective activity (firing pattern) of all retinal ganglion cells in response to a visual stimulus is sometimes referred to as the retinal code, which is, simply put, "what the eye tells the brain" about the visual world. The recent advances in retinal prosthetic technology, including electrical retinal implants37–39 and optogenetic approaches40–43, have raised the bar on the stated goals in vision restoration: the goal is no longer to simply confer light perception on the blind patient, but to try to fully restore normal function. Ideally, an implant would encode the light stimulus such that the induced retinal output would be as natural as possible. Our work suggests that the ‗natural‘ retinal output is a moving target. This may, in fact, be advantageous for prosthetics that lack cellular specificity, such as electrical retinal implants. They have always suffered from the problem of not being able to specifically stimulate ON or OFF cells (but see ref. 44). According to our results, ON responses are a common feature in OFF cells. Nonspecific electrical stimulation at light onset might therefore not confuse the brain as much as has been feared. Whether or not this really is the case, however, depends on how the retinal output is decoded.

The second topic, decoding of the retinal output, views the retina as a black box and asks questions about how the output of the retina is treated by receiving neurons. Is the exact spike timing important45,46, or is the firing rate the relevant unit47,48? How is the correlation structure of multineuron firing patterns taken into account49? When we started this research project, we expected to see only a moderate influence of illuminance on the retinal output, maybe with more pronounced effects at certain brightness thresholds (namely, cone activation threshold and rod saturation threshold). Overall, however, we assumed that adaptation in the retina largely would compensate for illuminance differences, so that the retinal black box delivers a rather stable input to the visual brain. Since this does not seem to be the case, there is a whole new dimension that is added to the already existing questions on decoding. How does the brain deal with the changes of the retinal output? Are they successfully filtered out and discarded, or do they indeed carry important information, maybe even used to identify viewing conditions?
The data we present are probably insufficient to even start tackling these questions. Furthermore, in the current work we have only focused on qualitative response changes. In addition, there are widespread quantitative changes in response to both preferred and anti-preferred contrast steps (for example, response amplitude, transparency), as can be seen in many of the example responses depicted in our figures. Various aspects of quantitative luminance-induced changes have also been described by others. In the future, it will be desirable to monitor the luminance-dependent changes of the retinal output on a better spatial scale. In particular, it will be important to test whether the information transmitted to the brain by a population of ganglion cells is, in aggregate, luminance independent despite the luminance-dependence of single cells. It is also possible that the phenomenon of changing output described in this paper allows the retina to encode the visual stimulus more efficiently in the ever-changing and dynamic luminance conditions of natural viewing.

METHODS

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

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

A.-T.H., K.R. and T.A.M. designed the study. MEA recordings and spike sorting were performed by A.-T.H., K.R., H.S. and A.H., and analyzed by A.-T.H., K.R. and T.A.M. Patch-clamp experiments and immunohistochemistry were conducted and analyzed by H.S. and T.A.M. In vivo experiments were designed by C.A.P., A.E.A. and R.J.L., performed by C.A.P. and A.E.A., and analyzed by C.A.P., A.E.A. and K.R. Pig eyes were provided by M.S. The manuscript was prepared by A.-T.H., K.R. and T.A.M. with the help of H.S., C.A.P., A.E.A. and R.J.L.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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Animals. As wild-type animals, we used PV-Cre × Thy-S-Y mice17 (B6;129P2-Pvdh/2McIra/j x C57BL/6-tg(ThystopFYP)) and C57BL/6j mice. For cone-deficient mice, we used Cnga3−/− (ref. 51, kindly provided by M. Biel, LMU Munich), Cpf1 (B6.CXB1-Ptd6cqa/1), Jackson strain 3678), kindly provided by B. Chang (The Jackson Laboratory, Bar Harbor, ME), and Gnat2cpfl3 mice (B6.Cg-Gnat2cpfl3/J, Boc, Jackson strain 6795). Wild-type animals were 5 weeks to 6 months old at the time of the experiments, Cnga3−/− animals 4.5–6 weeks old, Cpf1 animals 11–13 weeks old and Gnat2cpfl3 animals 12 months old. We used both male and female mice for all experiments. Mice were kept in groups of one to five animals. Animal use was in accordance with German, UK and European regulations and approved by the Regierungsräsidium Tübingen (in vitro experiments).

Pig retinas were obtained from two female domestic pigs sacrificed during independent scientific studies at the Department of Experimental Surgery, University of Tübingen. Pigs were sedated and anesthetized by injection of atropine, azaperone, benzodiazepine (midazolam), and ketamine, and sacrificed with embutramide (T61). Before administration of embutramide, heparin was injected. During sedation and anesthesia, the pigs were dark-adapted for 15–20 min. After death, the eyes were enucleated immediately under dim red light conditions, the cornea, lens and vitreous removed, and the eyecup kept in CO2-independent culture medium (Gibco) and protected from light. After transportation to the laboratory, pieces ~4 × 4 mm2 were cut from the mid-peripheral retina. Recordings were performed identically to those in experiments with mouse retina. In vitro MEA recordings. Mice were kept on a 12/12 h light/dark cycle, dark-adapted for 4–16 h before the experiment, and sacrificed under dim red light by cervical dislocation. The eyecups were removed, put in Ringer solution (in mM: NMDA, 1% bovine serum albumin (BSA), 0.5% Triton X-100, diluted in 0.5% Triton X-100 in PBS). Retinas were washed in PBS, incubated with DAPI (2.5 µg/ml in PBS) for 20 min, washed again and mounted in Vectashield (Vector Laboratories).

All steps were carried out at room temperature. Confocal image stacks of the filled ganglion cells were taken on a Zeiss LSM710, using a 40X NA1.3 oil immersion objective. xy image and z-stack size were chosen such that they covered the complete ganglion cell, including its entire dendritic arbor, and encompassed the full thickness of the inner plexiform layer. Dendritic stratification depths relative to the ChAT bands and DAPI-stained nuclei of inner nuclear layer and ganglion cell layer were determined on several dendritic locations of each cell using a custom-written Mathematica script.

Light stimuli during in vitro experiments. Intensities. Light stimulation was performed with a digital light processing (DLP) projector (PG-F212X-L, Sharp) and focused onto the photoreceptors through the condenser of the microscope (Supplementary Fig. 1). The light path contained a shutter and two motorized filter wheels with a set of neutral density (ND) filters (Thorlabs NE10B-A to NE50B-A), having optical densities from 1 (‘ND1’) to 5 (‘ND5’). To achieve light attenuation stronger than 5 log units, we serially combined an ND5 filter in one filter wheel with another ND filter in the second filter wheel. We refer to the filter settings as ND4 (brightest setting used, 104-fold light attenuation) to ND8 (darkest setting used, 106-fold light attenuation). While changing the ND filters during the experiment, we closed the shutter to prevent intermittent exposure to bright light. We usually started the experiments at ND8, and step by step increased the ambient stimulation lumiance by changing the ND filters by 1 unit. Unless otherwise noted, we presented the same set of visual stimuli at each ND level during an experiment.

The stimulus projector output spanned 3 log units of light intensities (that is, a 1,000-fold difference between black (0) and white (255) pixels). We linearized the projector output, and limited our visual stimuli to the range of 0 to 60, with the background set to 30 (Fig. 1b). As a consequence, the brightest pixels at any given ND-filter setting were fivefold dimmer than the background illumination at the next brighter ND-setting (Fig. 1b).

Light intensity measurements. We measured the spectral intensity profile (in µW cm−2 nm−1) of our light stimuli with a calibrated USB2000+ spectrophotometer (Ocean Optics). We transformed the stimulus intensity into equivalents of photostimulations per rod and per second, assuming dark-adapted rods42. Briefly, the spectrum was converted to photons cm−2 s−1 nm−1, convolved with the normalized spectrum of rod sensitivity5, and multiplied with the effective collection area of rods (0.5 mm2)35. The results for a stimulus intensity of 30 ranged from 1 R+1 s−1 per rod (ND8) to 104 R−1 s−1 per rod (ND4) (Fig. 1b). These calculations, as well as recordings from mice lacking functional rods and functional cones (data not shown), suggest that ND8 and ND7 correspond to scotopic conditions, ND6 weakly activates cones, ND5 is fully mesopic and ND4 is photopic. Note that our characterization of ND7 as scotopic may partly be owed to our use of low-contrast stimuli. We cannot exclude the possibility that stimuli with stronger contrast might activate cones even at ND7 (see, for example, refs. 5,56).

Light stimuli. All stimuli were grayscale images with pixel values between 0 (black) and 60 (white). The background was kept at 30 (gray), and the stimuli were balanced to keep the mean intensity over time at 30.

Our stimulus set for MEA recordings contained the following: (1) Full-field steps (Fig. 1a,b). ON step: stepping to an intensity of 50 for 2 s from the background of 30 (66% Weber contrast); OFF step: stepping to 10 for 2 s (~66%). (2) Full-field Gaussian flicker, 30 s or 1 min. Screen brightness was updated every frame (60 Hz) or every other frame (30 Hz) and was drawn from a Gaussian distribution with mean 30 and s.d. 9. This stimulus was used to calculate the linear filters of ganglion cells57. (3) Disk stimulus. Disks (diameter, 150 µm on the retina) were presented on a gray (30) background for 2 s and had the same contrast as the full-field stimulus (10 for black disks, 50 for white disks). They were centered over the recording electrodes. The sequence of disk locations was chosen such that the next disk was always at least 600 µm away from the previous disk, and at least 7 white and 7 black disks were presented at each location at
each ND level. (4) Binary checkerboard flicker, 15 min. The screen was divided into a 40 × 40 checkerboard pattern; each checker covered 60 × 60 μm² on the retina. The intensity of each checker was updated independently from the other checkers and randomly switched between 10 and 50. This stimulus was used to calculate the spatial receptive field of ganglion cells. (5) Natural movie, 22 s. It consisted of sequences taken from the music video “Rip It Up” by Bill Haley (https://www.youtube.com/watch?v=HdIlZ4213zM). The contrast of the moving image was compressed so that it spanned brightness values between 0 and 60.

We used different combinations or subsets of these stimuli in different experiments, repeated several times at each ND filter. The complete experimental stimulus set lasted at least 20 min at each ND. See results for details.

Our stimulus set for single cell recordings contained the following: (1) Full-field steps (see above). (2) Full-field Gaussian flicker (see above). (3) Disk stimulus (see above). Disks were centered over the patched cell’s soma. (4) Annulus stimulus. Full-field contrast steps (see above) with an inner hole (diameter, 500 μm on the retina) staying at gray (30) background, centered on the patched cell’s soma. The same set of stimuli was presented at each ND from ND8 to ND4, taking a total of 35 min. Only one cell was recorded from each retina.

Data analysis. Spike sorting. Data were high-pass filtered (500 Hz, tenth-order Butterworth filter), and spike waveforms and spike times were extracted from the raw data using Matlab (The MathWorks Inc., MA, USA). Spike sorting (assignment of spikes to individual units, presumably ganglion cells) was performed semimanually with custom written software (Matlab). The quality of each unit was individually and manually assessed by inter-spike interval and spike shape variation. Data analysis was based on the spiking responses of individual units.

Calculation of cell polarities and receptive fields. We calculated linear filters in response to full-field Gaussian flicker and to binary checkerboard flicker by summing the 500-ms stimulus history before each spike. Linear filters calculated in response to the full-field were used to determine cell polarity. Latency and amplitude of the first peak of the filter were determined. If the peak was positively deflected, the cell was categorized as an ON cell. If negatively deflected, the cell was categorized as an OFF cell. Linear filters calculated in response to the full-field checkerboard flicker were used to determine the spatial receptive field. For each checker, we determined the s.d. along the 500-ms temporal kernel. From the resulting 40 × 40 matrix entries, we calculated the mean and s.d., set all checkers deflected, the cell was categorized as an ON cell. If negatively deflected, the cell was categorized as an OFF cell. Linear filters calculated in response to the binary checkerboard flicker were used to determine the spatial receptive field. For each checker, we determined the s.d. along the 500-ms temporal kernel. From the resulting 40 × 40 matrix entries, we calculated the mean and s.d., set all checkers to zero, fit a two-dimensional Gaussian, and took the 2.5-σ ellipse as a representation for the receptive field (Fig. 8c,d).

Firing rate calculation. We estimated the instantaneous firing rate by convolving the spike train (time series of 0s and 1s) with a Gaussian with σ = 40 ms and amplitude = 0.25 σ² e⁻¹²σ² (≈10 Hz for σ = 40 ms), unless otherwise noted.

Algorithm to detect and classify early and delayed responses. For the step-stimuli (full-field and disk), we applied an algorithm to automatically detect ON responses in OFF cells or OFF responses in ON cells and to classify them as early or delayed (see Results for definitions). Responses were rejected as unreliable for specific light levels if less than 50% of them were strongly correlated with each other (‘strong correlation’ was defined here as pairwise Pearson correlation coefficient of at least 0.4; 0.2 for experiments where automated classification was only taken as a suggestion and manually corrected). Then we applied an automatic algorithm to detect and classify early and delayed responses at each reliable light level. Briefly, we compared the maximal firing rates during spontaneous activity on the one hand and the relevant time windows for early (50–350 ms after the stimulus) and delayed (350–1,000 ms) responses on the other hand. If the peak firing rate in the response windows was higher than during spontaneous activity and also more correlated from trial to trial, we categorized the response as present, regardless of its absolute amplitude (that is, binary classification ‘absent/present’). Additional checks were implemented to distinguish these responses from ‘tails’ of sustained responses to the preferred contrast and to distinguish a delayed response from a slowly declining early response (in both cases, we checked for ‘valleys’, or firing rate decreases, before the response peak). Mostly, the specific parameters used by the algorithm were based on heuristics and we made extensive checks to confirm that the automatic classification was valid. The responses to the small disk and in Gnat2 retinas had smaller signal-to-noise ratio; for those responses we treated the result of the automated algorithm only as a suggestion and confirmed each individual response by hand. Responses during GABA blocker application had different shapes in some cells (sharp peaks, thus slightly different latency distribution). Responses obtained during these experiments were checked manually and corrected where necessary. Responses of the LGN neurons were classified by hand.

We next compared the responses across light levels. Overall, a cell was classified as stable if, at all light levels being compared, it always had the same response type to the black step (that is, no response, early response, delayed response, or both early and delayed response) and always the same response type to the white step. Otherwise the unit was classified as changing. If a cell had unreliable responses at some light level (see above), this light level was not considered for the analysis. For example, if a cell had unreliable responses at ND6, we did not compare this cell’s responses for the ND7/6 or the ND6/5 transition, but we still compared its responses between all other light levels, for example, between ND7 and ND5. This is the reason for the different numbers of cells for each luminance transition in the plots showing the fraction of changing and stable units (for example, Fig. 3b,d). As a consequence, a cell may be classified as stable even if it had unreliable responses at one or more light levels. The fraction of changing cells can therefore be viewed as a conservative estimate.

Analysis of movie responses. Responses to the movie typically consisted of interleaved sequences of spike bursts (‘events’) and silence. To test whether the response to the movie would change across light levels, we analyzed whether a cell would have an event during some light level(s), but not other(s). This analysis proceeded in several steps: (1) Alignment. We calculated the average spike rate for each light level (see above) with a σ of 10 ms, and calculated the pairwise cross-correlation to estimate the relative temporal shift of the spike trains (spiking always gets faster at higher intensities). We then aligned the spike trains across the different light levels. (2) Event detection. (a) From the aligned spikes, we calculated the average firing rate across the whole experiment with a σ of 30 ms. Events were preliminarily defined as periods where the spike rate exceeded the mean firing rate of the 2 s before movie onset + 3 STD. (b) If spike bursts occur close to each other, they are fused into 1 event because the calculated firing rate does not drop below the threshold between the bursts. We therefore identified local minima in the spike rate and split events at those minima. (c) Of the resulting events we discarded those that were shorter than 20 ms and those that had a peak firing rate smaller than 5% of the second-largest event. (3) Response strength. We counted the spikes in each event at each light level, and converted that count into an average spike rate (number of spikes/s per movie presentation). We refer to this as the activity of the cell during an event and at each light level. (4) Light levels with very low activity. Events are inherently defined by high activity. To look for qualitative response changes across light levels, we therefore identified light levels during which there was low activity during an event. We applied 2 criteria to identify such ‘silent’ light levels: (a) Comparison across light levels within an event: the activity during a silent light level had to be lower than 10% of the maximal activity during this event. (b) Comparison across events within a light level: The activity during a silent event had to be lower than 10% of the mean activity across all events at that light level. For analysis we counted only such events as silent that fulfilled both criteria (dark gray in Supplementary Fig. 3).

Statistical analysis. No statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those generally employed in the field. No statistical tests were required for analysis of the data presented.

In vivo recordings. Five adult female C57 wild-type mice (6–8 weeks, housed in a 12-h light-dark cycle with 6 animals per cage) were used for experiments between 8 a.m. and 6 p.m. Mice were anaesthetized by i.p. injection of 30% (w/v) urethane (1.5 g/kg; Sigma, UK) and placed in a stereotaxic apparatus (SR-15 M; Narishige International Ltd., UK). Additional top up doses of anesthetic (0.2 g/kg) were applied as required and body temperature maintained at 37 °C with a homeothermic blanket (Harvard Apparatus, Kent, UK).

An incision to expose the skull surface was made and a small hole (~1 mm diameter) drilled 2.5 mm posterior and 2.3 mm lateral to the bregma, targeting the dorsal LGN. The pupil, contralateral to the craniotomy, was dilated with topical 1% (w/v) atropine sulfate (Sigma) and the cornea kept moist with mineral oil. A recording probe (A48x-5 mm-500-200–413; Neuronexus, MI, USA) consisting of four shanks (spaced 200 μm apart), each with eight recordings sites (spaced 50 μm apart) was then positioned centrally on the exposed surface in the coronal plane, and lowered to a depth of 2.5–3.3 mm using a fluid-filled micromanipulator (MO-10; Narishige).

Once the recording probe was in position and light responses confirmed, mice were dark adapted for 1 h, which also allowed neuronal activity to stabilize after
probe insertion. Neural signals were acquired using a Recorder64 system (Plexon, TX, USA). Signals were amplified ×3,000, high-pass filtered at 300 Hz and digitized at 40 kHz. Multiunit activity (spikes with amplitudes >50 µV) were saved as time-stamped waveforms and analyzed offline (see below).

Light stimuli ($\lambda_{\text{max}}$, 460 nm; half peak width, ± 10 nm) were generated by a custom-built LED-based light source (Cairn Research Ltd.), passed through a filter wheel with various ND filters and focused onto a 5-mm-diameter piece of opal diffusing glass (Edmund Optics Inc., York, UK) positioned 3 mm from the eye contralateral to the recording probe. LED intensity and filter wheel position were controlled by a PC running LabView 8.6 (National instruments). At each intensity, starting from the lowest ($6.1 \times 10^{-1}$ R* rod$^{-1}$ s$^{-1}$), a 2-s light increment from background (+66% contrast) was followed by a 5-s inter-stimulus interval of background light, after which a 2-s light decrement (−66% contrast) was presented. This was repeated 120 times at each background level before being increased by a factor of ten, spanning a 6-log-unit range in total. Mice were otherwise kept in complete darkness.

At the end of the experiment mice were transcardially perfused with 0.1 M PBS followed by 4% PFA. The brain was removed, postfixed overnight, cryoprotected with 30% sucrose and sectioned at 50 µm on a freezing sledge microtome. Sections were mounted with DPX (Sigma), coverslipped and electrode placement in the dLGN confirmed by visualization of a fluorescence dye (Cell Tracker CM-DiI; Invitrogen Ltd. Paisley, UK) applied to the probe before recording. Multichannel, multiunit recordings were analyzed in Offline Sorter (Plexon). Following removal of cross-channel artifacts, principal component–based sorting was used to discriminate single units, identifiable as a distinct cluster of spikes in principal component space with a clear refractory period in their inter-spike interval distribution. Following spike sorting, data were exported to Neuroexplorer (Nex technologies, MA, USA) and Matlab R2013a for construction of peristimulus histograms and further analysis. Light-responsive units were identified as those for which the peristimulus average showed a clear peak (or trough) that exceeded the 99% confidence limits estimated from a Poisson distribution derived from the prestimulus spike counts.

Corneal irradiance was measured using a calibrated spectroradiometer (Bentham Instruments, Reading, UK; Ocean Optics, FL, USA). Retinal irradiance was calculated by multiplying these values by pupil area/retinal area, based on calculations by Lyubarsky et al.58, where a pupil size of 3.2 mm² and retinal area of 17.8 mm² were used to generate a correction factor of 0.18. Effective photon flux was calculated by multiplying retinal irradiance by spectral transmission through the mouse lens59. Photoisomerizations were calculated as described for MEA recordings. All procedures conformed to requirements of the UK Animals (Scientific Procedures) Act, 1986.

A Supplementary Methods checklist is available.

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