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Visual Experience before Eye-Opening and the Development of the Retinogeniculate Pathway

Colin J. Akerman,[1,3] Darragh Smyth,[1] and Ian D. Thompson[1,2]
[1]University Laboratory of Physiology
Oxford University
Oxford OX1 3PT
United Kingdom

Summary

Visual experience before eye-opening is not usually thought to have any developmental significance. Here we show that naturalistic visual stimuli presented through unopened eyelids robustly activate neurons in the ferret dorsal lateral geniculate nucleus. Further, dark-rearing prior to natural eye-opening has striking effects upon geniculate physiology. Receptive field maps after dark-rearing show increased convergence of On- and Off-center responses, and neurons frequently respond to *both* bright and dark phases of drifting gratings. There is also increased selectivity for the orientation of the gratings. These abnormalities of On-Off segregation can be explained by the finding that the responses of immature On and Off cells to naturalistic stimuli are strongly anticorrelated.

Introduction

Activity-dependent changes in neural connectivity are seen in the mammalian visual system before the eyes open. Not surprisingly, these changes have been thought to reflect a role for spontaneously generated neuronal activity (Shatz and Stryker, 1988, Katz and Shatz, 1996; Wong, 1999). For example, the segregation of retinal afferents into eye-specific territories in the dorsal lateral geniculate nucleus (dLGN) occurs before the retina is light responsive and is affected by manipulations of neural activity (Shatz and Stryker, 1988; Penn et al., 1998; Stellwagen and Shatz, 2002). In the case of the ferret, the segregation of retinal afferents into eye-specific laminae is complete by postnatal day 9 (P9). This segregation process is proposed to rely upon the patterns of activity in the two eyes and upon how these binocular interactions are interpreted by the postsynaptic cells in the dLGN (Wong, 1999; Stellwagen and Shatz, 2002). Importantly, the patterns of retinal ganglion cell (RGC) activity during this period have many of the appropriate spatio-temporal features that could drive ocular segregation (Wong, 1999; Eglen, 1999). The activity of cells within the same eye are likely to be correlated, while cells from the two eyes are more likely to be anticorrelated. Assuming that the postsynaptic cells respond "preferentially" to coactive inputs, a Hebbian-type mechanism would favor the convergence of coactive inputs and the segregation of inputs that are not coactive (Wong, 1999).

Later in development, ferret retinogeniculate fibers segregate according to their receptive field (RF) types. The terminals of RGCs that respond to increases in light ("On") and those that respond to decreases in light ("Off") are initially intermixed in the ferret dLGN and segregate during development. This segregation process begins at around P14 and is essentially complete before eye-opening at P32 (Linden et al., 1981; Hahm et al., 1991, 1999). At the level of individual afferents, the anatomical segregation emerges as a result of growth of terminal arbors in the correct regions of the dLGN and the elimination of extraneous branches in incorrect regions (Hahm et al., 1999). Normal On-Off segregation is disrupted by blocking action potentials in the eye (Cramer and Sur, 1997) or by blocking NMDA receptors in the geniculate (Hahm et al., 1991, 1999). Since this activity-dependent process also occurs before the time of eye-opening, it too has been thought to rely only on spontaneous activity.

It has been argued that observed differences in the spontaneous activity of On and Off cells could account for the segregation of the two pathways. In vitro recordings from retinae have found Off RGCs to be three times more active than On cells during the period of segregation (Wong and Oakley, 1996; Myhr et al., 2001). And although On cells burst synchronously with Off cells, the difference in relative amount of activity could drive segregation (Wong, 1999). Meanwhile, in vivo recordings from the postsynaptic cells indicate no difference in the total amount of activity in On and Off dLGN cells (Weliky and Katz, 1999). Spontaneous activity recorded in vivo from dLGN cells of the same type (e.g., On and On) was found to be the most correlated, but the activity in cells of different type (i.e., On and Off) were also positively correlated. It is therefore still not clear exactly which patterns of activity in the retinogeniculate pathway might instruct the segregation of On and Off pathways during development.

The observation that neurons in the visual system of the ferret are responsive well before eye-opening (Chapman and Stryker, 1993), and that they show orientation selectivity for stimuli presented through the naturally closed lids (Krug et al., 2001), raises another possibility: responses through unopened eyelids could pattern neural activity in the visual system and thus provide developmentally relevant information which is used in the refinement of connections. However, it could be argued that the high-contrast stimuli that have been used to demonstrate closed eyelid responses represent an artificial pattern of visual stimulation. Therefore, in this paper we address whether closed eyelid experience is a likely source of activity in the normal rearing environment of neonatal ferrets. We then go on to address the developmental significance of this source of activity for the ferret retinogeniculate pathway.

[2]Correspondence: ian.thompson@physiology.ox.ac.uk
[3]Present address: Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724.

Results

Responses to Natural Visual Stimuli through Unopened Eyelids

Ferrets open their eyelids around P32. Yet neonatal ferrets start to leave the nest before the end of the third postnatal week (Porter and Brown, 1985; our unpublished data). At this age, the kits make fairly stereotyped head movements, which we have characterized from video recordings (6 kits; P18–P24). The typical head movement covered 100° of visual space, lasted 1.1 s, and was interleaved with periods lasting 2.2 s when the head was still. These parameters were used, together with digital photographs of the cage environment, to construct movies that mimicked the spatio-temporal properties of natural visual stimuli that might reach the neonatal ferret's closed eyelids. Three different movies were constructed, which differed in spatial but not temporal structure. The movies were presented to the *naturally closed* eyelids of paralyzed, anaesthetized ferret kits (P20–P26, n = 6 animals) prepared for single-unit recording.

Figure 1 shows the stimulus sequence and the responses of three different dLGN neurons. The digitized photographs were preceded by a blank frame of a luminance equal to the mean of the digital images. The first frame of the visual stimulus was presented for 2.2 s (head still), and then there was a rapid sequence of frames (0.99 s in length, mimicking head movement), and the last frame of the movie was presented for 2.2 s (head still again). The whole sequence was repeated 50 times, and the neuronal responses were shown as raster plots.

The responses in Figure 1B clearly show that the naturalistic stimuli provide a potent visual stimulus for geniculate neurons, even when presented through closed lids. The three rasters illustrate a variety of neuronal responses to the stimulus protocol. The first cell, from a P20 animal, responded only during the movie, and the response was reliable; it happened on most trials and also occurred at the same time in each presentation. Responses in slightly older animals were more robust (P23 is still 9 days before eye-opening) and often showed more complex patterns of responses. For instance, the cell in the middle panel had relatively high levels of spontaneous activity (indicated by the spikes in the first gray box). During the movie (the second gray box), this cell initially responded at a high rate, and then midway through its activity was suppressed for approximately 500 ms, and toward the end of the movie, the cell fired again in a stimulus-locked fashion. The burst of firing at the beginning of the blank resulted from the transition between the last frame and the blank. The final cell in Figure 1B showed three time-locked responses. Two of these occurred during the movie period, and the other occurred at the beginning of the blank and reflected the transition from the last frame.

For analysis purposes, each sequence was divided into two separate periods: a "spontaneous period" recorded during the blank and a "movie period" that encompassed the simulated head movement (see Figure 1). A cell was classed as movie responsive if the neuron showed statistically significant variation in spike firing during the movie period, but not during the spontaneous period ($p < 0.05$, Kruskal Wallis). By this criterion, 47 out of a total of 56 dLGN neurons (84%) recorded between P20 and P26 were statistically responsive to the movies. Across all 56 cells and all movies, the mean firing rate during the movie period was 0.90 spikes per second (±0.007), whereas the mean firing rate during

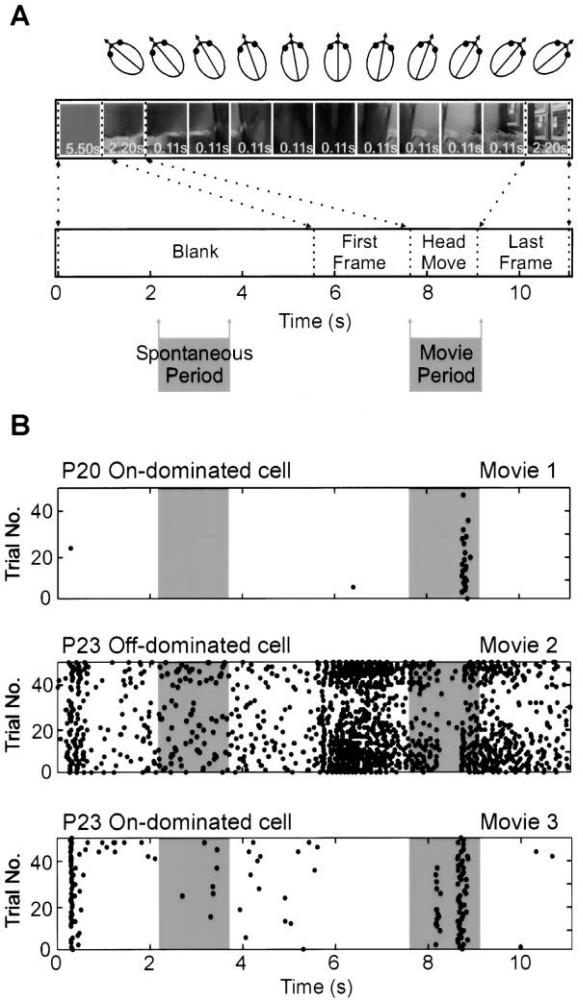

Figure 1. Ferret dLGN Neurons Respond Robustly to Natural Visual Stimuli Presented to the Unopened Eyelids

(A) Short movies were constructed which mimicked the spatio-temporal stimulation induced by a typical neonatal ferret head movement, measured as described in the text. The digital images were taken inside the home cage, and each represented a rotation of 10° of visual space from the previous image. For analysis, each sequence was divided into two parts: the "spontaneous period" and the "movie period" (indicated by gray boxes). The movie period began at the end of the first frame of the movie, included the nine frames of the head move (each 0.11 s), and ended 0.44 s into the last frame. The spontaneous period matched the movie period in length, but was taken from the central part of the blank.

(B) Examples of dLGN responses when movie stimuli were presented to the closed eyelids in animals 9–12 days before eye-opening. The raster plots show robust and time-locked responses during presentation of the natural scenes. Phase-locked spikes seen at the start of the blank are flash responses to the transition from the last frame of one sequence to the blank of the next sequence. Stated above each raster is the movie code, animal age, and cell type (see Experimental Procedures).

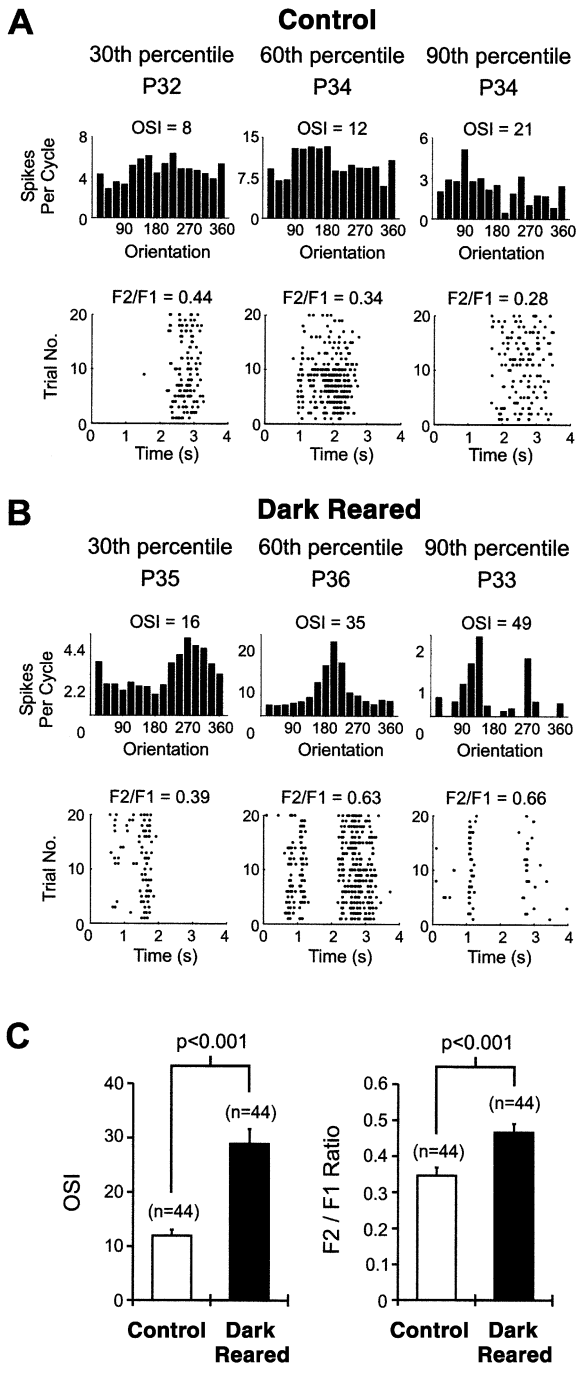

Figure 2. Dark-Rearing before Eye-Opening Affects dLGN Responses to Oriented Grating Stimuli

(A and B) Orientation tuning curves and raster plots of the cells at the 30th, 60th, and 90th percentiles are shown for control (A) and dark-reared (B) conditions. Cells were ranked by OSI. The tuning curves reveal that while the control cells responded approximately equally to all orientations, the dark-reared cells showed bias for different grating orientations. The raster plots show that while control cells responded at only one phase of the stimulus, the dark-reared cells often responded at two phases of the grating cycle. Raster plots were constructed from the responses recorded during 20 presentations of the optimum grating (temporal frequency of 0.25 Hz). The OSI and F2/F1 ratio are stated above the tuning curve and raster plot, respectively. The age of each recording is stated above the tuning curve.

(C) Mean OSI (left) and mean F2/F1 ratio (right) in control and dark-reared dLGN neurons. Both the OSI and F2/F1 ratio were significantly greater in the dark-reared neurons ($p < 0.001$, Mann-Whitney). Error bars represent the standard error of the mean. Stimulus-evoked firing rates of control and dark-reared cells were not significantly different ($p > 0.05$, Mann-Whitney). In control animals, the mean firing rate to the optimal stimulus was 11.0 ($\pm$1.4) spikes per cycle, and for the dark-reared cells the equivalent mean was 12.9 ($\pm$4.0) spikes per cycle. Mean spontaneous activity (recorded during presentation of a blank screen at mean luminance and expressed as spikes per cycle) was slightly higher in the dark-reared (2.2 $\pm$ 0.6) than in the controls (1.0 $\pm$ 0.2), although not significantly different ($p > 0.05$, Mann-Whitney).

the spontaneous period was 0.41 spikes per second ($\pm$0.007). But does the ability of geniculate neurons to respond to naturalistic stimuli presented through the closed lids have any developmental importance?

Dark-Rearing Prior to Eye-Opening Affects Response Properties in the dLGN

To address directly the question of developmental significance, we dark-reared ferrets starting at P16 (the earliest visual responses we have obtained are at P19; our unpublished data) to the time of normal eye-opening (P29–P35). Recordings were compared between four dark-reared ferrets (from two litters) and three age-matched control ferrets that were raised under normal lighting conditions (from three litters). The responses of dLGN neurons in these animals were investigated through *open* eyelids. In order to describe the geniculate physiology of these animals, we used three different types of conventional, well-controlled visual stimuli: (1) drifting gratings, (2) whole-field flashes, and (3) flashed square stimuli. Results from the different stimuli were consistent, and all indicated a dark-rearing effect upon the excitatory feedforward of On and Off inputs to dLGN cells. Data from each type of stimulus will be described below.

Drifting square-wave grating stimuli allowed us to test two response parameters: first, the extent of orientation bias, and second, the modulation of the response. Orientation tuning curves were obtained for 44 units from dark-reared animals and 44 units from control animals, and the orientation selectivity index (OSI) was calculated (Worgotter and Eysel, 1987, Chapman and Stryker, 1993; see Experimental Procedures). Neurons from control animals and from dark-reared animals were separately ranked according to their OSI, and the 30th, 60th, and 90th percentiles are shown in Figure 2. The histograms illustrate how the response varied with the orientation of the drifting grating. The rasters reveal the timing of individual action potentials during 20 repeats of the grating at the optimum orientation. While the responses of geniculate neurons in normally reared animals were not surprising, those from dark-reared animals were unusual in two respects.

Most dramatic was the finding that the responses of neurons in the dark-reared animals could vary considerably with the orientation of the drifting grating. This is apparent when the histograms in Figures 2A and 2B are compared. In control animals, the histograms were essentially flat—there is little variation in response with

orientation. Consequently, 93% (41/44) of the neurons in control animals had OSIs of 25 or less. In contrast, many units in the dark-reared animals displayed selectivity for orientation—the responses to some orientations of grating were consistently greater than to others. While not all dark-reared cells had high OSIs (for instance the 30th percentile had an OSI of only 16), 48% (21/44) of the cells had an OSI above 25, the value that has been used to indicate orientation selectivity in ferret visual cortex (Chapman and Stryker, 1993). Thus, the 60th percentile cell in Figure 2B responded best to a narrow range of orientations and was also directional. The most selective dark-reared cells, such as the 90th percentile, not only responded best to a restricted number of orientations but also failed to respond at orthogonal orientations.

The raster plots revealed a second difference between the responses of neurons in control and dark-reared animals. As the examples in Figure 2A show, neurons in control ferrets displayed the anticipated pattern of spike times and responded during a single phase of the grating—either the bright or the dark phase. This observation is consistent with normal dLGN neurons being either On- or Off-centered. While this was the case for some neurons from dark-reared animals, such as the 30th percentile example in Figure 2B, many dark-reared neurons responded *twice* during the stimulus cycle. Thus, in both the 60th and 90th percentile raster plots shown in Figure 2B, there are two bursts of spikes separated by roughly half a cycle. Such a double modulation to a drifting grating could occur if the cells had *both* On- and Off-center inputs. F2/F1 ratios were calculated in order to quantify the extent to which the responses of dark-reared and control cells where either singly or doubly modulated (see Experimental Procedures). Cells that responded at a single phase of the stimulus cycle have an F2/F1 less than 0.5, while those that responded at two parts of the stimulus cycle have F2/F1 ratios greater than 0.5. This is apparent in Figures 2A and 2B, where the 60th and 90th percentile dark-reared cells are the only cells out of the six shown with F2/F1 values greater than 0.5. Indeed, whereas 43% (19/44) of the dark-reared cells had an F2/F1 greater than 0.5, only 7% (3/44) of the control cells fell into this category. This observation is consistent with many more dark-reared cells receiving both On and Off inputs.

The population data (Figure 2C) revealed a highly significant effect of dark-rearing on both the orientation selectivity and modulation of responses. Mean OSI for neurons in dark-reared animals was 28.9 ($\pm$2.7; $n = 44$), which was significantly higher ($p < 0.001$, Mann-Whitney) than that in controls, where the mean was 12.0 ($\pm$1.1; $n = 44$). The difference in action potential modulation patterns was also significantly different between the two populations. The mean dark-reared F2/F1 ratio was 0.47 ($\pm$0.02), which was significantly greater than that of the control cells at 0.35 ($\pm$0.02; $p < 0.001$, Mann-Whitney). Neither the level of spontaneous activity nor the mean firing rate to optimal stimuli was significantly different (see Figure 2).

Changes in dLGN Receptive Field Structure Underlie the Dark-Rearing Effects

The examples in Figure 2B suggest that neurons with high OSIs tended also to show double modulation. This was confirmed in the population data by the fact that there was a positive and significant correlation between a neuron's OSI and its F2/F1 ratio ($p < 0.0001$, Spearman $r = 0.41$). Such a correlation implies that the same mechanism might underlie both effects. Indeed, a parsimonious explanation for these observations is that the balance of On and Off inputs was altered by dark-rearing and that this generated both the bias for orientation and the double modulation. This hypothesis was investigated by using flashed stimuli to describe the dLGN RF structure in control and dark-reared animals.

First, however, we tested On and Off responses in control and dark-reared dLGN cells with whole-field flash stimulation. An On-Off ratio was calculated from a cell's mean spike discharges in response to bright and dark whole-field flashes (see Experimental Procedures). In this measure, higher values indicate cells that were dominated by either On *or* Off, while lower values indicate cells that were more equally responsive to both On *and* Off. Consistent with the grating experiments, the flashed stimuli generated a significantly lower On-Off ratio in the dark-reared cells ($p < 0.05$, Mann-Whitney). The mean On-Off ratio in controls was 0.79 ($\pm$0.07; $n = 20$), whereas the dark-reared mean was 0.55 ($\pm$0.08; $n = 16$).

We then proceeded to explore the spatial structure of the cells' RF with flashed square stimuli (bright or dark squares presented in different locations in visual space; see Experimental Procedures). Figure 3 shows the RFs of six neurons (three from control and three from dark-reared animals) that were analyzed with flashed squares. Five panels are shown for each neuron. The first two panels show the post stimulus time histograms (PSTHs) of the mean number of spikes recorded in response to white ("On") and black ("Off") flashed squares, averaged across all stimuli. The next two panels display the response-weighted spatial map of effective stimuli, again separately for bright and dark. The last panel is a smoothed representation of the RF, combining responses to stimuli of both polarities.

The control cells in Figure 3A were clearly dominated by a single type of input. The PSTHs show a clear time-locked response to either the bright or dark stimuli, but not both. Furthermore, their RFs tended to be symmetrical, with a continuous single polarity "hot-spot" confined to one part of visual space. In agreement with previous work (Tavazoie and Reid, 2000), some control cells at this age (such as in Figure $3A_3$) showed evidence of a surrounding "halo" of inputs as well as the central hot-spot. The RF profiles of the three dark-reared cells were very different. As the PSTHs illustrate, the dark-reared cells showed clear, time-locked responses to *both* bright and dark stimuli. Furthermore, the corresponding On and Off subregions of the RF were often discontinuous and usually occupied separate parts of visual space. This meant that when the two spatial maps were combined to generate the RF, dark-reared cells had some regions that were dominated by On and others that were dominated by Off. Of the 19 neurons in which RF maps were generated in control animals, 17 (89%) showed RFs that were dominated by a single type of input that was localized in space. Of the 19 dark-reared cells whose RFs were described, 13 (68%) showed asymmetric fields with a mixture of On and Off subregions. The mean OSI and F2/F1 for this type of dark-reared cell

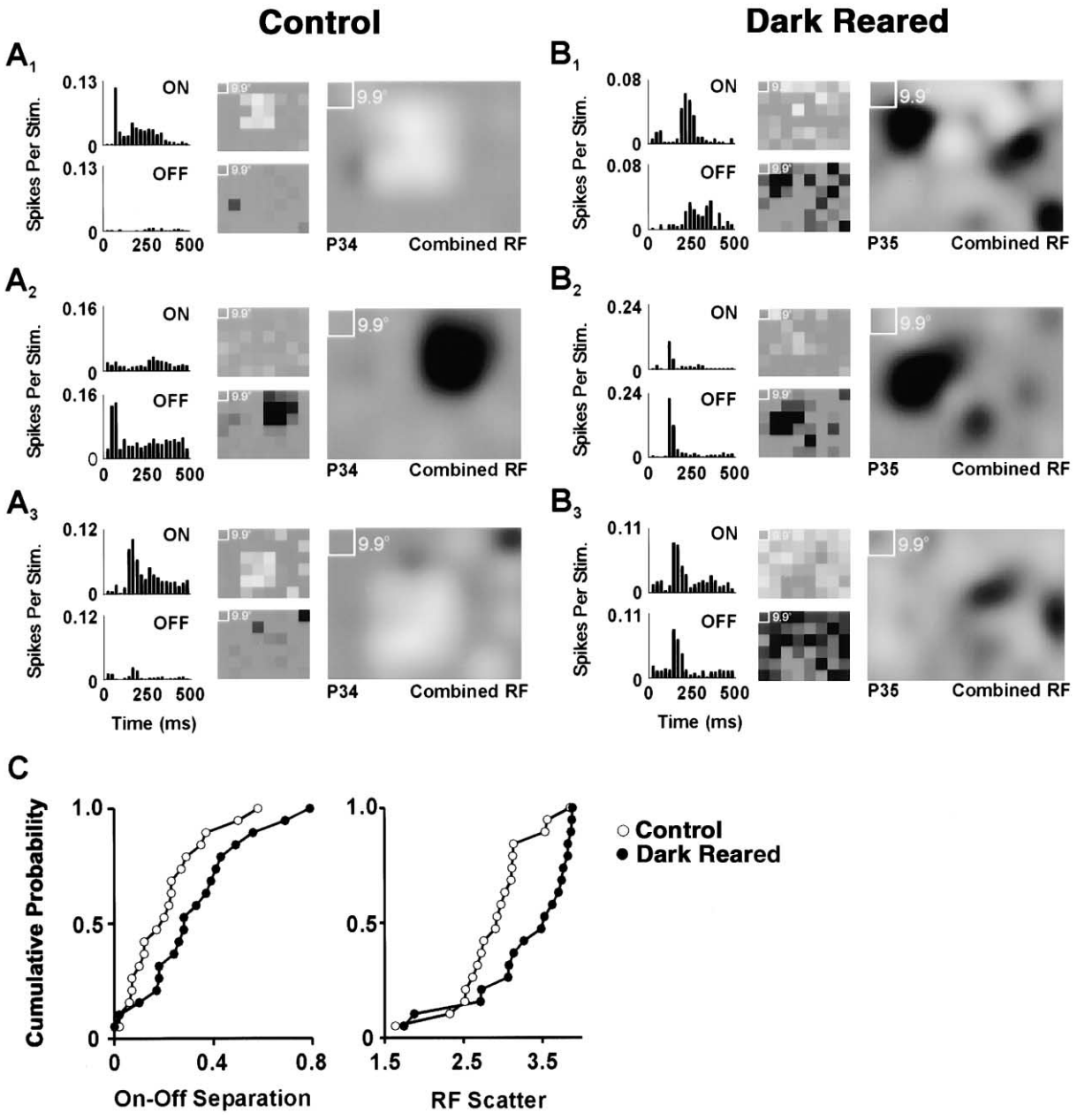

Figure 3. Dark-Rearing before Eye-Opening Affects the Receptive Field Structure of dLGN Neurons

For both control and dark-reared cells, response histograms and response-weighted RF profiles were constructed from the responses to sequences of 48 white and 48 black flashed square stimuli presented at different locations on a gray background. Each stimulus was presented for 500 ms, and all responses recorded during this time were included in the analyses. For each cell, a series of plots is presented: PSTHs of the mean number of spikes recorded in response to white ("ON") and black ("OFF") flashed squares, averaged across all stimuli (left); the raw RF profiles that corresponded to these PSTHs (middle); and the combined RF map when the ON and OFF channels were brought together (right). The age is stated below the combined RF, and the square outlines in the RFs represent 9.9° of visual space.

(A_1–A_3) RF structures of three dLGN neurons recorded in control ferrets. These cells were clearly dominated by a single type of input. Their RFs tended to be symmetrical with a continuous single polarity hot-spot located at one point in space. Grating responses in these control cells generated OSI (and F2/F1) values of 14 (0.44), 5 (0.17), and 11 (0.46), respectively.

(B_1–B_3) RF structures of three dLGN neurons recorded in dark-reared ferrets. In contrast to the control cells, the dark-reared cells showed robust responses to both white and black stimuli. The corresponding ON and OFF subregions of the RF were often discontinuous and occupied separate parts of visual space. Grating responses in these dark-reared cells generated OSI (and F2/F1) values of 10 (0.57), 42 (0.87), and 53 (0.50), respectively.

(C) Cumulative probability plots of On-Off separation (left) and RF scatter (right) in control and dark-reared dLGN. Values from both measures were significantly greater in dark-reared neurons ($p < 0.05$, Mann-Whitney).

were 46.3 (±6.2) and 0.56 (±0.06), respectively. The other dark-reared cells were more reminiscent of the cells in the control animals in that they responded to stimuli of only one polarity at a single coherent location in space. The mean OSI and F2/F1 for this type of dark-reared cell were 7.8 (±3.2) and 0.43 (±0.05), respectively.

In order to quantify the effect of dark-rearing on dLGN RFs, we developed two measures of spatial organization (see Experimental Procuedures). The first measure (On-Off separation) tested whether the On and Off responses came from different parts of the RF. In control dLGN RFs, the two responses should be approximately co-centered (low values). The first cumulative probability plot in Figure 3C shows that dark-rearing significantly increased the spatial separation of the On and Off responses ($p < 0.05$, Mann-Whitney). The second measure (RF scatter) addressed whether responsive RF locations within On and Off maps were focused into a single hot-spot (low values) or were scattered across visual space (high values). The second plot in Figure 3C shows that RF scatter was also increased in dark-reared cells ($p < 0.05$, Mann-Whitney), suggesting that early visual experience could also be involved in the topographic coherence seen in control dLGN RFs at the time of eye-opening.

Simple cells in striate cortex show spatially separated On and Off input that predicts their orientation tuning. Could this be the case for the orientation selectivity found in dark-reared dLGN cells? To investigate this possibility, we further analyzed those cells that showed substantial bias for grating orientation (with OSI values greater than 35, to ensure that the estimate of optimal orientation was reliable) and that had RF maps ($n = 6$). The RFs were Fourier analyzed to extract the predicted best orientation (see Experimental Procedures), and this was compared with the experimentally measured preferred orientation. On average, the predicted and measured orientation optima differed by 26.3° (±9.0°), and all measured optima were within two orientation stimuli (45°) of the predicted optima. For such a small population, the probability of this happening by chance is 0.06 (see Experimental Procedures), which suggests that some component of a dark-reared cell's orientation bias can be predicted from the spatial structure of the RF.

Finally, as with the grating stimuli, the responsivity of the dark-reared cells to the square stimuli was not different from controls ($p > 0.05$, Mann-Whitney). When spikes were averaged across all bright and dark stimuli, the mean output of the dark-reared cells was 0.50 (±0.11) spikes per stimulus, and the corresponding value in the controls was 0.49 (±0.11). This is noteworthy for two reasons. First, it argues that the different response properties observed between the two conditions are not the result of a nonspecific effect on responsivity. Second, it suggests that the absolute number of inputs is unaltered in dark-reared dLGN cells and that the different response properties of these cells reflect changes in the *balance* of inputs.

The fact that dark-rearing before eye-opening does alter dLGN responses shows that visual experience through closed eyelids is developmentally important. More specifically, our analyses argue that this early experience contributes to the development of On and Off pathways. We now return to our studies of normal ferrets before eye-opening (P20–P26, Figure 1) to investigate whether response patterning by naturalistic stimuli through closed eyelids could explain this effect of dark-rearing.

Response Patterns to Natural Visual Stimuli Contain Developmentally Relevant Correlations

As was seen in Figure 1, naturalistic stimuli can strongly modulate the firing of neurons in the developing thalamus of normal ferrets, even through the closed lids. What is the relevance of this to the development of On and Off pathways? The data presented in Figure 4 compare responses to the movie sequences of On-dominated and Off-dominated cells in normal ferrets whose eyelids were still closed (aged P20–P26). Figure 4A shows the pattern of responses of four geniculate neurons (two On-dominated and two Off-dominated) to three different movies. The movies differed only in their spatial content, and all four neurons were recorded on a single electrode penetration in a normal P23 ferret.

The histograms show that for any given neuron, the temporal structure of responses is different for each of the three movies. More importantly, the *type* of cell (whether it was On-dominated or Off-dominated) was found to be a powerful predictor of how a neuron responded to a particular movie; cells of the same type tended to be active at the same time, while cells of different type were active at opposite times. Considering movie 1, the pair of On-dominated cells (upper two cells) fired at the same point in the movie, and the pair of Off-dominated cells (lower two cells) fired at the same point in the movie, but there is almost no overlap in the firing between the two pairs. Similar behavior was elicited by movies 2 and 3.

This stimulus-dependent dissociation in the activity patterns of cells of different type during natural visual stimulation was very robust and remained when data from all the recorded neurons were pooled for each animal. Note that the recordings were made from single neurons at different times, and the contribution of RF location was minimized by always attempting to center the cell's RF on the display screen. Figure 4B plots the mean correlation coefficients calculated for all possible pairs of neurons of "same type" (e.g., Off-dominated versus Off-dominated) and for all pairs of neurons of "different type" (i.e., On-dominated versus Off-dominated) for each of the movies. For each of the three movies, the mean correlations for same type cell pairs were positive and significantly different from zero (means = 0.44, 0.55, 0.57; $p < 0.001$ in each case, one sample t test). In contrast, the correlations between different type cell pairs were negative but also significantly different from zero (means = −0.34, −0.29, −0.51; $p < 0.001$ in each case, one sample t test).

In summary, dark-rearing ferrets in the period before eye-opening has striking effects upon the normal segregation of On and Off inputs in the retinogeniculate pathway. Consistent with this, the correlations contained in neuronal responses to natural stimuli presented to unopened eyelids are ideally suited for driving and/or refining the convergence of same type inputs and the segregation of opposite type inputs.

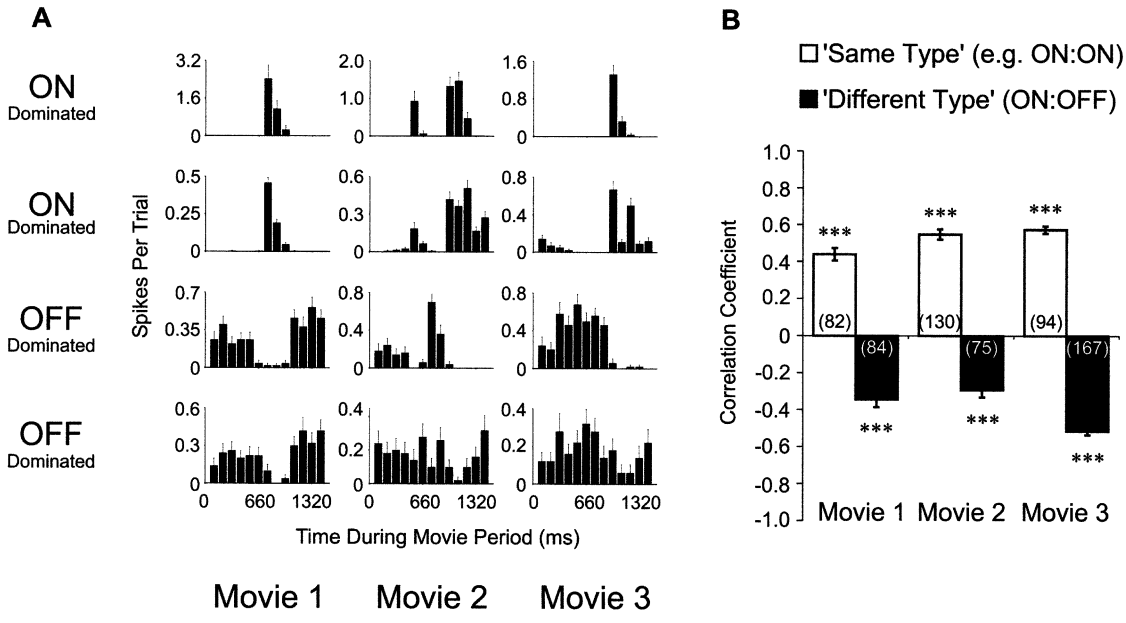

Figure 4. Response Patterns to Natural Visual Stimuli Contain Developmentally Relevant Correlations

(A) Histograms show the responses of four example dLGN cells to three different movies. Each histogram was constructed from responses recorded during the movie period (bin width 110 ms; see Figure 1) across all presentations of a particular movie. All four cells were recorded within a distance of 1000 μm on the same electrode penetration in a P23 ferret. The two On-dominated cells (upper) and two Off-dominated cells (lower) demonstrate that for each movie, cells of the same type (ON versus ON and OFF versus OFF) tended to be active at the same time, while cells of different type (ON versus OFF) were active at opposite times.

(B) Mean correlation coefficients (Spearman r value) calculated for pairs of cells of the same type and for pairs of cells of different type. For each movie, the activity of same type cell pairs was positively and strongly correlated, whereas the activity of different type cell pairs was negatively and strongly correlated. Statistical comparisons were performed for each pair type and each movie, against a hypothetical mean of zero (***$p < 0.001$, one sample t test). Number of cell pairs is indicated in parentheses. Error bars represent the standard error of the mean.

Discussion

Previous studies had shown that visual responses can be elicited through the closed eyelids (Krug et al., 2001; Huttenlocher, 1967). This study demonstrates that the visual system can respond to natural stimuli through unopened eyelids and that this activity contributes to the development of functional connections. Prior to the time of eye-opening, On and Off pathways from retina to dLGN are known to be segregating in an activity-dependent manner (Cramer and Sur, 1997; Hahm et al., 1991; Bodnarenko and Chalupa, 1993). We have shown that dark-rearing during this period results in dLGN cells that receive converging On and Off responses. Furthermore, the patterning of neuronal activity induced by naturalistic stimuli should promote On-Off segregation.

The Source of the Dark-Rearing Effect

Normally at the time of eye-opening, most neurons in the dLGN of ferrets and cats are clearly dominated by inputs from either On- or Off-center RGCs (see Results; Tavazoie and Reid, 2000; Stark and Dubin, 1986; but see Daniels et al., 1978). This was found not to be the case in ferrets that were dark-reared prior to eye-opening. Dark-reared dLGN neurons frequently responded at two phases of drifting gratings, and their RF structures had regions that responded to increases in luminance *and* regions that responded to decreases in luminance. The simplest explanation for these results is that they reflect a change in the feedforward excitatory drive for the dLGN cells.

During the period in which the ferrets' eyelids are closed, the functional connectivity of On and Off pathways within the retina, and from retina to dLGN, has been shown to be refining (Bodnarenko et al., 1999; Wang et al., 2001; Hahm et al., 1991, 1999; Linden et al., 1981). Furthermore, experiments in which neurotransmission has been manipulated have demonstrated that the segregation of On and Off is "plastic." For instance, the gross anatomical segregation of On and Off RGC afferents in the dLGN has been shown to require retinal activity prior to eye-opening (Cramer and Sur, 1997; Dubin et al., 1986) and can be blocked by infusion into the dLGN of NMDA receptor blockers (Hahm et al., 1991). In normal ferrets, anatomical reconstructions of single RGCs show that at P14/15, RGC axon terminals span an entire eye-specific lamina of the dLGN. By P19/21, the axon terminals show evidence of being segregated into On and Off sublaminae (Hahm et al., 1999). Since P19 is the earliest age at which visual responses have been recorded, it may be that visual experience is less involved in the initial segregation of these afferents but plays an important role in subsequent refinement and/or consolidation of afferent segregation.

It may also be relevant that whereas previous studies have mainly relied upon anatomical methods to demonstrate the segregation of On and Off pathways, we have used quantitative single-unit physiology in vivo in order to observe developmental events. These electrophysio-

logical methods may offer a more sensitive resolution at which to detect developmental changes in the functional connectivity. Indeed, a recent study using similar methods has found that even after eye-opening, there are changes in the convergence of ferret RGC axons onto dLGN neurons that underlie changes in the RF properties (Tavazoie and Reid, 2000).

Previous studies have not explicitly investigated the role of visual experience prior to eye-opening on visual system development. In the mammalian retina, neither dark-rearing nor intraocular tetrodotoxin affects the lamination of dendritic arbors (Leventhal and Hirsch, 1983; Dubin et al., 1986; Lau et al., 1990; Wong et al., 1991), but dark-rearing in turtle does affect arbor size (Sernagor and Gryzwacz, 1996). In the cat, prolonged dark-rearing from birth has been reported to have no effect on orientational and directional properties of dLGN neurons (Zhou et al., 1995), while in cat and ferret cortex, dark-rearing eventually causes loss of orientation selectivity, even though development initially proceeds normally (Blakemore and Van Sluyters, 1975; Zhou et al., 1995; White et al., 2001). In this regard, it will be interesting to determine the effect of early dark-rearing on cortical RFs in the ferret.

Comparing Patterns of Activity

It is widely believed that precise connections between pre- and postsynaptic neurons are governed by "learning" mechanisms that are able to interpret patterns of neural activity in order to strengthen or weaken connections (Katz and Shatz, 1996; Crair, 1999). Under such a scheme, the segregation of On and Off pathways would be achieved via a Hebbian-type learning algorithm that "favors" connections that are coactive and "discourages" connections that are not coactive. When considering the significance of a particular source of activity, it is therefore important to consider what correlations exist within/between populations of cells and the frequency and the temporal fidelity of the activity. One strength in the literature is that close attention has been paid to the correlational structure of spontaneously patterned activity (Meister et al., 1991; Feller et al., 1997; Weliky and Katz, 1999). In contrast, direct investigation of the correlated activity under visual stimulation during development has been limited.

In vitro recordings from ferret retinae have found that before On and Off pathways segregate, putative On and Off RGCs exhibit similar patterns of spontaneous activity (Wong and Oakley, 1996; Myhr et al., 2001). Then, for the last two weeks before eye-opening, putative Off-center RGCs become three times more active than On RGCs. This relative difference in amount of activity could drive On-Off segregation in the dLGN (Wong and Oakley, 1996). However, the fact that On RGCs continue to burst synchronously with Off RGCs has been recognized as not necessarily the strongest patterning to drive segregation (Wong, 1999). More explicitly, in the case of a strictly Hebbian process, these patterns would not be the most efficient way to retract Off RGC axons from the On sublamina of the dLGN. In vivo multiunit recordings from the postsynaptic cells have also revealed that, although dLGN cells of different type (i.e., On and Off) do not show different levels of spontaneous activity, they are still positively correlated (Weliky and Katz, 1999).

The responses to natural visual stimuli through closed eyelids offer both an elevated level and a robustly patterned source of activity. Either, or both, of these features could underlie the dark-rearing effects reported here. First, the mean level of activity was higher during periods when natural visual stimuli were presented than during periods without stimulation. Such elevated levels of activity during visual stimulation could act permissively to allow molecular plasiticity mechanisms to function (Crair, 1999). The second feature of early visual stimulation was the temporal reliability of responses—across multiple presentations, the spiking of dLGN neurons was phase-locked to features of the visual stimuli. Such fidelity defines the temporal boundaries for correlated activity within, and across, populations of neurons. Indeed, under visual stimulation, strong correlations were observed even at the relatively short timescales investigated (bin width, 110 ms); comparing spiking patterns under natural visual stimulation for On-dominated and Off-dominated dLGN neurons revealed positive correlations for cells of the *same* type but anticorrelated activity for cells of *different* type. Such patterns are well suited to a Hebbian learning rule by which they would strengthen connections between cells of the same type, while also punishing the "aberrant" connections between cells of different type.

As well as stimulus-dependent correlations, correlated activity in the developing thalamus is likely to occur as a direct consequence of anatomical connections, both within the retina and through converging inputs to the dLGN. The stimulus-dependent correlations reported here were evident even though responses were sampled at different times, at different locations in the dLGN, and across different animals. Therefore, this form of correlation might reflect a locally robust pattern that could dominate correlations that are not stimulus dependent. Our recordings from individual neurons mean that cell-cell correlated activity under visual stimulation is inferred. Subsequent experiments in which the activity of multiple isolated neurons is recorded simultaneously will be required to further define the strengths, sources, and time courses of correlations during development. The present study has also used activity in the dLGN to make inferences about activity throughout the retinogeniculate pathway. This approach seems reasonable given that the activity in the postsynaptic cells reflects, at some level, the activity of the input. However, to further understand how activity in input cells might govern their connections with target cells, it would be informative to record from presynaptic cells and ultimately from pre- and postsynaptic cells simultaneously, as has been possible in the adult retinogeniculate pathway (Usrey et al., 1998). Equally, it will be important to determine the exact time scale over which presynaptic correlations lead to changes in connections in the developing mammalian visual system.

As yet, we do not know the full range of factors that underlie the patterning of responses to naturalistic stimuli or whether these have other developmental implications. The fact that cortical neurons show orientation selectivity for gratings presented through the lids (Krug et al., 2001) argues that some spatial structure of the stimuli is preserved. We also have preliminary evidence that response patterns are different when the individual

movie frames are replaced with blanks of matched mean luminance (M. Grubb et al., 2001, Soc. Neurosci., abstract). Nevertheless, given the extent of RFs at eye-opening (this study; Tavazoie and Reid, 2000) together with the optical blurring by the lids, it would seem probable that responses to naturalistic stimuli are correlated over a considerable spatial scale. Thus, early vision might actually *limit* the extent of activity-dependent topographic refinement. If true, this could explain the polyneuronal convergence seen in the retinogeniculate projection at eye-opening (Tavazoie and Reid, 2000), and in this regard, it is interesting that some of the dark-reared RFs (Figure 3) resembled the fragmented fields observed during the course of polyneuronal elimination in ferret dLGN.

More generally, the onset of vision represents a significant stage. First, neural activity in the animal's visual pathways may be associated with important modulatory influences, such as the animal's state of arousal. Second, vision can combine features of the stimulus within the same neural pattern. For instance, at the same time cells relay information about the contrast or luminance of a stimulus, they can also relay spatial information about that stimulus. We suggest that similar methods to those adopted here could be used to study the correlated activity during natural stimulation in order to address exactly how these contribute to experience-dependent changes during development.

Experimental Procedures

Rearing

Pigmented ferret kits were studied between P18 and P36 (P0 is day of birth). Control animals were housed under a light/dark cycle of 16 hr light and 8 hr dark. Local luminance values in the light ranged as follows: within the cage, 2–48 cd m^{-2}; from the front of the cage, 4–89 cd m^{-2} (below the horizontal) and 89–342 cd m^{-2} (above the horizontal); within the nest boxes, 0.4–24 cd m^{-2} (lid closed). Dark-reared ferrets were transferred to a dark-rear facility at P16. The facility had a three-stage dark-proofing system, and infrared goggles were used to monitor and feed the animals. Dark-reared litters had normal body weights. Visual experience was minimized during experimental set-up by covering the eyes with lightproof material.

Electrophysiology

Electrophysiological recordings were performed as described previously (Krug et al., 2001). The exception was that, in the experiments investigating the consequences of dark-rearing, responses were recorded after opening the eyelids and protecting eyes with plano-contact lenses. An ophthalmoscope (maximum luminance ~500 cd m^{-2}) was used to identify visually responsive neurons and to classify cells as responding more to an increase in luminance (On-dominated) or a decrease in luminance (Off-dominated). Only 5% of dLGN cells (3/56) recorded through closed lids could not easily be classified as either On- or Off-dominated. For quantitative studies, stimuli were presented on a CRT monitor controlled by a Cambridge Research Systems VSG 2/4 graphics card. At viewing distance of 28.5 cm, the display subtended 70° × 55° and comprised 800 × 600 pixels.

Electrolytic lesions (3–4 μA for 3–4 s) were used to reconstruct electrode tracks. Reconstruction showed that, for control animals, 39 of the cells were recorded in the A layers and 5 in the C layers of the dLGN and that, for dark-reared animals, 39 were recorded in the A layers, 3 in the C layers, and 2 in MIN. All cells were included in the analyses, and identical conclusions were reached when only cells from the A layers were analyzed. All statistical tests were two tailed and values are reported as mean (± standard error of the mean).

Movie Stimuli

Awake, behaving ferret kits were videoed (40 Hz frame rate) in their home cage environments. Frame-by-frame analysis provided values for the angle covered by each head movement and the temporal duration and frequency of each head movement. Based upon these measurements, three short movies were constructed from digital photographs taken inside the animals' home cage. Each digital photograph represented a rotation of 10° from the previous image and was scaled such that its maximum luminance did not exceed the reflected luminance of the walls of the cages (48 cd m^{-2}).

Grating Stimuli

Drifting square wave gratings (approaching 100% Michelson contrast, spatial frequency 0.01 cycles per degree, temporal frequency 0.25 Hz) were presented in eight different orientations and two directions. Only cells that showed a significant increase in firing to the optimal stimulus, compared to a blank of matched mean luminance, were analyzed further. Orientation tuning was assessed with the orientation selectivity index (OSI) of Worgotter and Eysel (1987) and Chapman and Stryker (1993). Temporal modulation of the response was assessed by Fourier analysis of peristimulus time histograms (binwidth 100 ms) constructed from responses to the optimal stimulus. The amplitude of the first (F1) and second (F2) harmonics were extracted and used to calculate the F2/F1 ratio:

$$F2/F1 = (F2 - F1)/(F1 + F2)$$

Cells for which the F2/F1 ratio was greater than 0.5 are consistent with the cell responding at two phases of the cycle, separated by half a stimulus cycle.

Flashed Stimuli

The On-Off ratio was calculated from responses to bright (On) and dark (Off) whole-field flashed stimuli. To measure the On responses, bright whole-field flashes (99 cd m^{-2}; between 500 and 1000 ms duration) were presented after an interstimulus period (4 s) during which the screen was dark (2 cd m^{-2}). Off responses were measured in an identical fashion except that the polarity of the stimuli was reversed. Responses were averaged over at least 30 repeats, and spontaneous activity was subtracted. The On-Off ratio was defined as

$$\text{On-Off ratio} = |(On_{flash} - Off_{flash})/(On_{flash} + Off_{flash})|$$

The RF structure of dLGN neurons was investigated with randomized sequences of flashed white and black square stimuli (Jones and Palmer, 1987). Each square (measuring 29° × 29° of visual space) was presented for 500 ms in 48 different locations on the screen. With the screen size used (see above), the stimuli overlapped and thus enabled receptive fields to be described with a resolution of 9.9° × 9.9° of visual space. Separate On and Off RF profiles were constructed by counting the total number of spikes elicited when a stimulus, of a particular polarity (white squares for On and black squares for Off) and centered at a particular point on the screen, was presented. The response at all points was normalized by dividing by the response to the optimal stimulus, regardless of position or polarity. The On and Off profiles were then overlaid to construct the "combined" profile which was smoothed with a Gaussian filter, chosen such that within the distance between two adjacent points of the original profile, the Gaussian decayed to less than 10%.

We used two measures to quantify and compare the RF maps from light- and dark-reared cells. A measure of On-Off separation was used to quantify the spatial segregation of the On and Off subfields:

$$\text{On-Off Separation} = 1 - \frac{|\sum_i (ON_i * OFF_i)|}{||ON|| * ||OFF||}$$

where ON_i and OFF_i denote the sensitivity to bright and dark stimuli, respectively, at spatial position i of the RF map, and

$$||ON|| = \sqrt{\sum_i ON_i^2}$$

denotes the norm magnitude of the vector of ON spatial sensitivities. More specifically, the On-Off Separation function is one less the normalized dot product of the On and Off sensitivity maps. It is

maximized when the maps are segregated to different spatial locations and minimized when the maps occupy the same positions.

While the measure above quantifies the separation of subfields of different polarities, there can also be considerable separation of multiple subfields of the same polarity. This was quantified by a measure of RF scatter:

$$\text{RF scatter} = \langle dist(i, j)\rangle_{ij}^{ON>T} + \langle dist(i, j)\rangle_{ij}^{OFF>T}$$

where $dist(i, j)$ denotes the distance between positions i and j, and $\langle dist(i, j)\rangle_{ij}^{ON>T}$ denotes the mean distance between all unique pairs of positions i and j whose ON sensitivities exceed a threshold T. The units of distance were in stimulus spacings since all stimuli were the same size across the cell population. The threshold was 10% of the strongest sensitivity from across both ON and OFF maps for that cell, although the results were robust across a range of threshold values (2%–20%). The use of a threshold instead of a sensitivity-weighted sum of distances ensures a nonparametric measure that makes no assumptions about the relative strengths across the sensitivity profile. RF scatter is maximized when the ON and OFF subfields are individually scattered across visual space and minimized when they are each localized into single subfields.

Fourier analysis of RF maps was used to predict the orientation preference of dLGN cells. This analysis transforms a spatial map of luminance sensitivity to a polar map of orientation and spatial frequency contrast sensitivity. The peak of the Fourier amplitude spectrum corresponds to the optimal stimulus for that RF map. Before transforming, each RF map was inserted into a much larger empty matrix to increase the resolution in the Fourier domain. Following transformation of this large matrix, the peak was extracted and fitted with a Gaussian function to give the optimal orientation at the preferred spatial frequency. The probability that, by chance, the optimal orientation measured with gratings was within two stimuli (45°) of the predicted optima is 0.625 for one cell and 0.06 ($= 0.625^6$) for the total of six cells investigated.

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