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An Instructive Role for Retinal Waves in the Development of Retinogeniculate Connectivity

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Summary

A central hypothesis of neural development is that patterned activity drives the refinement of initially imprecise connections. We have examined this hypothesis directly by altering the frequency of spontaneous waves of activity that sweep across the mammalian retina prior to vision. Activity levels were increased in vivo using agents that elevate cAMP. When one eye is made more active, its layer within the LGN is larger despite the other eye having normal levels of activity. Remarkably, when the frequency of retinal waves is increased in both eyes, normally sized layers form. Because *relative,* rather than absolute, levels of activity between the eyes regulate the amount of LGN territory devoted to each eye, we conclude that activity acts instructively to guide binocular segregation during development.

Introduction

In mammalian neural development, precise patterns of connectivity are refined from initially diffuse projections, a process known to require neural activity (Katz and Shatz, 1996; Feller, 1999; O'Donovan, 1999). For example, retinal ganglion cells in each eye subserving the same regions of visual space project to the same visual thalamic relay, the lateral geniculate nucleus (LGN). The axonal projections from the two eyes are initially intermingled, but segregate into eye-specific layers during early development (Linden et al., 1981; Rakic, 1976; Shatz, 1983; Sretavan and Shatz, 1986). The axons segregate before vision is possible, but the process still requires neuronal activity in the form of spontaneous retinal waves (Feller et al., 1996; Meister et al., 1991; Wong et al., 1993). Blockade of neural activity, either directly in the retina (Penn et al., 1998) or in the LGN (Shatz and Stryker, 1988; Sretavan et al., 1988), prevents the axons from the two eyes from segregating into eye-specific layers. Neural activity has been theorized to drive a competition between the two eyes for LGN territory (Shatz, 1996), and indeed blocking activity in one eye results in the loss of much of the territory that eye's projection normally occupies in the LGN to that of the active eye (Penn et al., 1998).

Previous experiments could not distinguish between a requirement for some threshold level of neural activity, versus a genuine activity-based competition, because they have relied upon *removal* of neural activity. It remains possible that activity is merely permissive—that is, activity may only be required for axons to read hypothetical molecular cues that specify eye-specific layers. However, this "permissive" model is difficult to reconcile with the three-eyed frog experiments of Constantine-Paton and colleagues, where the optic tectum would certainly not be expected to harbor eye-specific molecular cues arrayed in stripes, which desegregate when NMDA receptors are blocked (Constantine-Paton et al., 1990; Cline, 1991). Unfortunately, this experimental manipulation again relies on activity blockades, which cannot reveal a requirement for specific spatiotemporal patterns of activity, as opposed to activity per se. To make such distinctions requires modulating either the level or pattern (or both) of spontaneous activity, rather than removing all activity.

An experiment involving the retinotectal system of cold blooded vertebrates has perhaps come closest to achieving this goal: strobe-rearing, which correlates the activity of all retinal ganglion cells, prevents the refinement of the retinotopic map in goldfish (Eisele and Schmidt, 1988; Schmidt and Buzzard, 1993). In mammals, electrical stimulation of one optic nerve can prevent the emergence of highly orientation-tuned receptive fields in primary visual cortex while leaving the overall orientation map intact (Weliky and Katz, 1997). These experiments, in which the activity of *all* retinal ganglion cells has been synchronized, indicated that temporal information is critical in driving the refinement process. However, these manipulations disrupt development without addressing how activity might be used during normal development. Specifically, the experiments have not addressed the question of whether an activity-dependent competition is involved because the activity of *all* ganglion cell inputs has been synchronized. To address this question, it is necessary to alter activity patterns between two sets of inputs that are hypothesized to compete. Here we have done this by altering the spatiotemporal properties of the retinal waves in either one or both eyes during development of the ferret retinogeniculate projection, and allowing competition to proceed.

Waves of correlated retinal activity occur spontaneously during early development (Wong et al., 1993), throughout the period of eye-specific layer and subsequent on-off sublayer segregation in the ferret LGN (Hahm et al., 1999; Linden et al., 1981). Although the retina lacks mature photoreceptors, the waves propagate through a synaptic network of amacrine and ganglion cells (Feller et al., 1996; Wong et al., 1995; Zhou, 1998). Propagation during the period of layer segregation requires cholinergic synaptic transmission (Feller et al., 1996; Penn et al., 1998). Further, waves have distinct spatiotemporal properties, occurring approximately every 1–2 min, and having limited domain sizes and propagation velocities (Feller et al., 1996, 1997). These spatiotemporal parameters of waves are regulated by the levels of cyclic-AMP in the retina, controlled

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at least in part by adenosine acting through A2 adenosine receptors (Stellwagen et al., 1999). Increasing pharmacologically the level of cAMP in the retina results in larger, faster, and more frequent waves. Using this fact, we can now elevate levels of activity in one or both eyes without perturbing drastically the overall spatiotemporal dynamics of wave activity, and examine the consequences on the patterning of the retinogeniculate projection.

Results

Pharmacological agents that elevate intracellular levels of cAMP increase wave activity within the retina (Stellwagen et al., 1999). To produce an increase in wave activity in vivo, intraocular injections were made of forskolin or cholera toxin, each known to increase production of cAMP (Seamon and Daly, 1986; Tsai et al., 1987). Alternatively, 8-[(4-Chlorophenyl)-thio]-cyclic adenosine monophosphate (CPT-cAMP), a nonhydrolyzable membrane-permeable analog of cAMP shown to be effective in elevating cAMP intraocularly (Shen et al., 1999), was employed. Previous quantitative studies of wave dynamics following such treatments in vitro indicate that waves occur more frequently, travel much faster, and propagate over much larger areas than normal (Stellwagen et al., 1999). More frequent waves lead to more frequent bursts of action potentials by individual retinal ganglion cells in vitro, which participate in each wave as seen by microelectrode recordings combined with fluorescence imaging of calcium indicators (Figure 1A).

Although the duration of action for these agents is difficult to assess in vivo, we can take advantage of the long-lasting action of cholera toxin, which generates prolonged elevations in cAMP levels through ADP-ribosylation of stimulatory G proteins (Tsai et al., 1987). A single intraocular injection of cholera toxin can produce sustained alterations in wave parameters in vivo, for at least 24 hr. Ferret kits received a monocular injection of cholera toxin; 24 hr later, the retinas were removed, loaded with calcium indicators, and the resulting fluorescence monitored in vitro to assess the level of retinal activity (Figure 1B). Wave frequency was 0.79 $\pm$ .07 waves per minute for treated retinas 24 hr following an intraocular injection of cholera toxin, significantly higher than the 0.58 $\pm$ .01 waves/min observed in the uninjected retinas from the same animals ($p < 0.05$; $n = 3$ pairs of retinas). While the increase in frequency is more modest than that seen immediately following an acute application of cholera toxin, it demonstrates that wave frequency can be increased chronically in vivo by at least 35%.

Monocular Elevation of cAMP Increases Size of LGN Layers

To examine whether an increase in wave activity confers a competitive advantage to the projection from the more active eye, monocular intraocular injections were made, leaving the other eye with normal levels of activity. Forskolin, CPT-cAMP, or cholera toxin was injected monocularly in ferrets kits every 48 hr between postnatal day 1 (P1) and P9, when retinal axons have normally segregated into the eye-specific layers in the LGN (Hahm et al., 1999; Linden et al., 1981; Penn et al., 1998). Injections did not affect the overall development of the ferrets, as injected kits gained weight and size at the same rates as their uninjected littermates. The status of the retinal projections to the LGN was then examined at P9 by injecting the treated eye with an anterograde tracer (Figure 2A; see Experimental Procedures). The percentage area within the LGN occupied by the ipsilateral projection from the treated retina was measured to obtain a quantitative assessment of the consequences of the treatment (Figure 2B).

Nine days of treatment with CPT-cAMP produced a dramatic increase in the percentage of the LGN occupied by the ipsilateral projection from the treated eye (Figure 2). Normally, the ipsilateral projection occupies 13.5% $\pm$ 1.2% of the LGN area (measured objectively from the four sections containing the largest projection; $n = 6$ untreated control littermates), but with CPT-cAMP, the ipsilateral projection occupancy increased to 22.7% $\pm$ 1.0% ($n = 4$). Injections of CPT-cAMP every 24 hr, instead of every 48 hr, yielded no significant further increase in occupancy by the ipsilateral projection: 25.2% $\pm$ 1.8% of the LGN ($n = 3$) versus 22.7% $\pm$ 1.0%. This observation suggests that a near maximal effect on the ipsilateral projection can be achieved even with injections every other day. Similar increases in the area of the ipsilateral projection occurred with monocular injections of forskolin (22.5% $\pm$ 2.8% of the LGN; $n = 3$), or cholera toxin (23.6% $\pm$ 3.3%; $n = 3$). All treatment groups were significantly increased compared to untreated controls ($p < 0.011$; one way ANOVA followed by Bonferroni's t test). Thus, agents known to increase retinal waves by elevating cAMP also increase the amount of territory occupied by that eye's projection to the ipsilateral LGN.

The contralateral projection from the treated eye was modestly increased over normal. The projection from retinas treated with forskolin, CPT-cAMP, or cholera toxin once every 48 hr occupied 93.1% $\pm$ 1.3%, 90.9% $\pm$ 0.9%, or 90.2% $\pm$ 1.7% of the LGN, respectively, compared with 88.9% $\pm$ 1.0% for control retinas from untreated littermates; only forskolin significantly increased the projection over the control value. When CPT-cAMP was injected every 24 hr, the contralateral projection from the treated eye increased occupancy to 93.3% $\pm$ 1.3%, significantly larger than the control projection. Despite these changes, because all of the measurements of contralateral occupancy are confounded by fibers of passage (see Experimental Procedures), we concentrated attention on the ipsilateral projection to the LGN, where consistent and robust increases are observed.

This increase in area occupied by the projection from the more active eye could arise either at the expense of that from the untreated eye, or alternatively because inputs from both eyes fail to segregate completely and instead actually share common territory within the LGN. To distinguish directly between these alternatives, the retinogeniculate projections from both eyes—treated and untreated—were double-labeled at the conclusion of the treatment period, with each eye receiving a different anterograde tracer (see Experimental Procedures and Figure 3). The ipsilateral projection from the CPT-cAMP-treated eye expanded to a similar degree as observed previously, occupying 20.4% $\pm$ 1.3% of the LGN, while the ipsilateral projection from the untreated eye only occupied 11.0% $\pm$ 0.4% of the LGN ($n = 3$; Figure

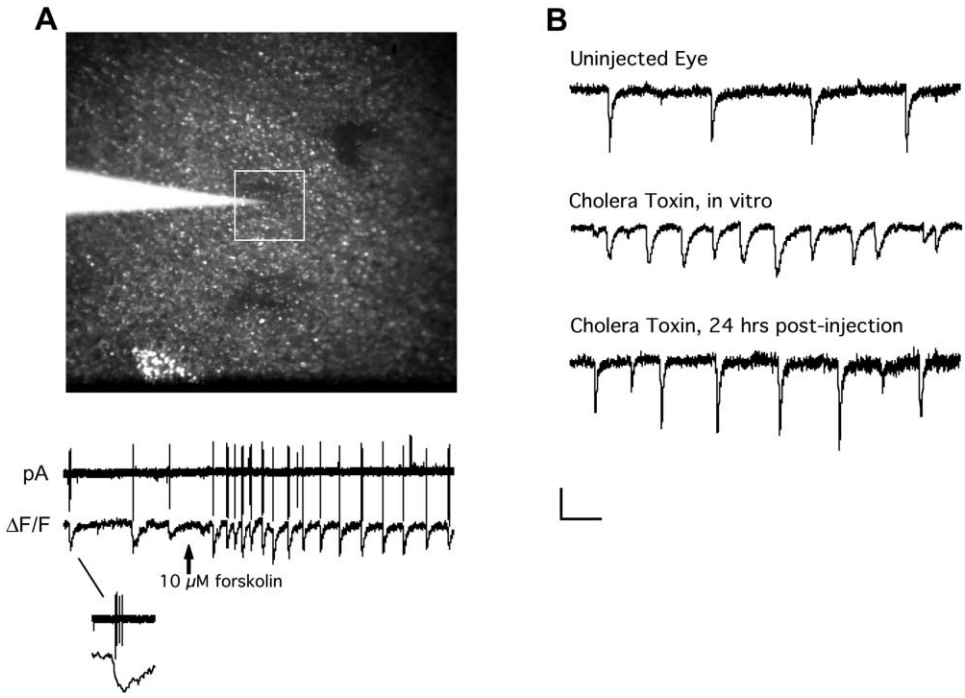

Figure 1. cAMP-Enhancing Agents Increase Levels of Spontaneous Retinal Waves

(A) Ganglion cells generate more frequent bursts of action potential during increases in cAMP. (Top) Fluorescent image of a Fura-2 loaded neonatal ferret retina, where an extracellular recording of a ganglion cell was taken simultaneously with fluorescent monitoring of the surrounding region (white box). (Bottom) Upper trace is an extracellular recording from the cell pictured showing periodic bursts of action potentials. These bursts coincide with decreases in the local fluorescence (lower trace), which signal a propagating retinal wave. Ten micromolar forskolin was bath applied at the arrow. (B) shows fluorescence traces from retinas demonstrating that intraocular injection of cholera toxin in vivo causes a maintained increase in wave frequency. Cholera toxin, if applied acutely in vitro, markedly increases wave frequency (middle trace). When cholera toxin is injected intraocularly in vivo and the retina is removed and imaged 24 hr later, the treated retina (bottom trace) has more frequent waves than occur in the uninjected eye of the same animal (top trace). Scale bar indicates 1 min (expanded to 6 s for the boxed insert) and 5 pA for physiological recordings or 5% $\Delta F/F$ for imaging data.

3D). Thus, the ipsilateral projection from the treated retina significantly expanded in size ($p < 0.001$) while the ipsilateral projection from the untreated retina in fact shrank slightly from saline-injected control values ($p < 0.045$). Animals receiving monocular injections of saline alone had similarly sized ipsilateral projections on both sides: the projection from the saline-treated retina (13.2% $\pm$ 0.1%) was comparable to the projection from the untreated retina (13.9% $\pm$ 0.2%; $n = 3$; $p > 0.35$; Figure 3C). The overall average size of the ipsilateral projections from saline-treated animals was 13.6% $\pm$ 0.2% LGN occupancy, equivalent to the 13.5% observed for untreated control animals.

Figure 3 also shows that inputs from the two eyes are almost completely segregated from each other throughout the LGN. This segregation is particularly evident when layers A and A1 are compared: only red (CtB-TRITC) or green (CtB-FITC) regions are present; no yellow can be observed, which would indicate the presence of overlapping fibers from the two eyes. There is some overlap in the C layers and near the optic tract, but this is difficult to quantitate accurately because of the fibers of passage problem (see Experimental Procedures). We determined the area of overlap between layers A and A1 by measuring, within the same LGN, the area occupied by the labeled projection from the ipsilateral eye to layer A1 and the area left vacant by the labeled projection from the contralateral eye to layers A and C. (If there is no overlap, the difference between these two measurements would be zero; see Experimental Procedures.) In saline-treated animals, there is only a small region of overlap, with just 0.5% $\pm$ 0.4% of the LGN occupied by inputs from both eyes ($n = 6$ LGNs). Similarly, animals treated with monocular CPT-cAMP were not significantly different from saline-treated animals in the extent of overlap: 0.5% $\pm$ 0.4% ($n = 6$ LGNs; $p > 0.85$). Thus, monocular injections of CPT-cAMP do not increase the amount of overlap in the projections from the two eyes to the LGN. Rather, as shown in Figure 3, the area occupied by the CPT-cAMP-treated eye expands at the expense of the projection from the eye with normal activity: for example, compare the small red (untreated) layer A1 in the left LGN with the large green (treated) layer A1 in the right LGN in Figure 3A. Similar results were obtained using other combinations of anterograde tracers. These observations indicate that increasing retinal wave activity in one eye results in segregation of both eye's inputs, but with the more active eye gaining territory at the expense of the eye with normal levels of activity.

Size of Eye-Specific Layers Depends upon Relative Levels of Retinal Activity

The results above are consistent with a model in which an activity dependent competition between inputs from the two eyes drives the process of segregation. If so,

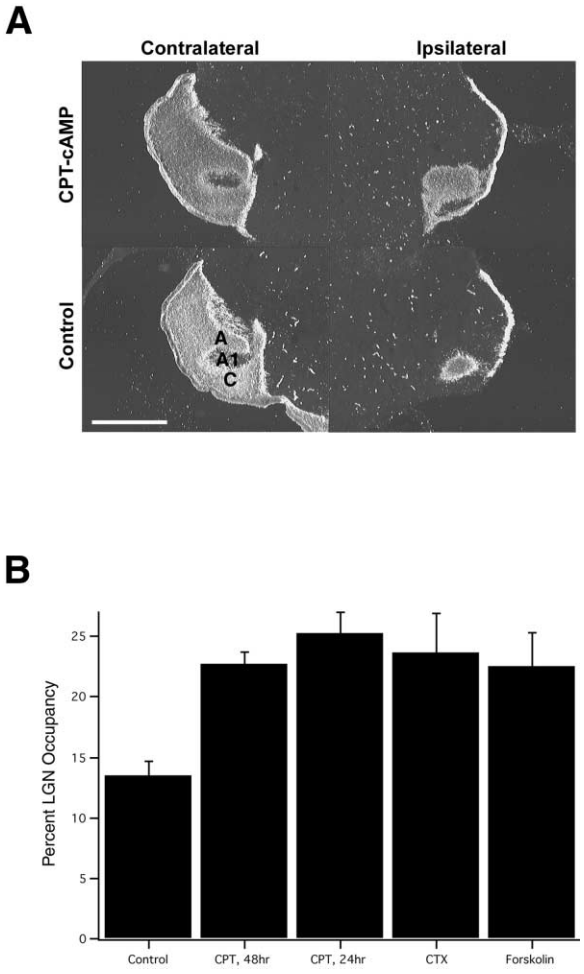

Figure 2. Chronic Treatments that Elevate Retinal cAMP Levels Also Increase Area Occupied by the Ipsilateral Projection within the LGN

(A) Shown are dark-field micrographs of representative horizontal LGN sections from an animal receiving monocular injections of CPT-cAMP every 48 hr from P1–P9 (top row) and from a control littermate that received no treatments (bottom row). The projections to the contralateral and ipsilateral LGN were visualized by TMB reaction of intraocularly injected WGA-HRP. In the CPT-cAMP case, the treated eye was labeled with WGA-HRP. A, A1, and C refer to LGN layers receiving projections from the ipsilateral (A1) or contralateral (A and C) eyes. Scale bar = 500 μm. (B) Histogram of data from all treatment groups, showing the percentage of LGN territory occupied by the ipsilateral projection from the treated eye. The ipsilateral projection following monocular injections of CPT-cAMP every 48 hr (n = 4) or every 24 hr (n = 3), of cholera toxin (CTX; n = 3), or of forskolin (n = 3) all occupied a significantly larger proportion of the LGN than the ipsilateral projection in unmanipulated littermates (control, n = 6).

then binocular, as opposed to monocular, treatments should preserve territorial equality between the two eyes' projections to the LGN. As shown in Figure 4A, the detailed pattern of the retinogeniculate projection following binocular injections of CPT-cAMP once every 48 hr is indistinguishable from that seen in normal animals. Moreover, measurements of the area occupied by the ipsilateral projection following binocular treatments are not significantly different from those of untreated control littermates (Figure 4B; binocular treatment = 14.6% ± 0.9% of the ipsilateral LGN, n = 5 animals, 9 LGNs; control = 13.5% ± 1.2%, n = 6 animals) and both of these results are significantly lower than those following monocular injections of CPT-cAMP (22.7% ± 1.0%, n = 4, $p < 0.005$).

This experiment is crucial for several reasons. First, results shown in Figure 4 rule out the possibility that cAMP has nonspecific effects on retinal neurons unrelated to the increase in wave dynamics. For example, increased cAMP within the retina could dysregulate axon growth, which in turn might prevent the projections from both eyes from segregating into layers. On the contrary, not only do layers of normal size form, but they are located correctly in the binocular zone of the LGN, indicating that despite elevations in retinal cAMP, axons still respond to topographic positional cues within the target. Secondly, Figure 4 indicates that so long as the characteristic spatial and temporal properties of the retinal waves are preserved, eye-specific layers can form normally even if the overall amount of activity in both eyes is elevated. This is a key observation that should be contrasted with all previous experimental attempts to increase intraocular activity by electrical stimulation or strobe rearing (Schmidt and Buzzard, 1993; Weliky and Katz, 1997). Those manipulations, by correlating *all* neuronal activity, caused a degradation in the precision of the retinal projection, which then could not be distinguished from growth dysregulation or other deleterious effects of the stimulation paradigm independent of neural activity patterns.

To verify further that the change in the area of the retinogeniculate projection is due to an imbalance in wave activity between the two eyes, epibatidine was injected with forskolin into the same eye from P1–P9 once every 48 hr. Epibatidine is a potent agonist known to prevent all wave activity by blocking nicotinic cholinergic synaptic transmission (Penn et al., 1998), even in the presence of forskolin (Stellwagen et al., 1999). The consequences of injecting forskolin with epibatidine on the pattern of the retinogeniculate projection from the treated eye are identical to those of injecting epibatidine alone (Penn et al., 1998): the ipsilateral projection is drastically reduced in area (3.1% ± 2.3% occupancy; n = 4), and even the contralateral projection from the treated eye is reduced in favor of the ipsilateral projection from the active eye (Figure 4A). These data argue strongly that the increase in size of the ipsilateral projection seen with forskolin or CPT-cAMP is a direct consequence of changing the balance of wave activity between the two eyes, rather than being due to a nonspecific effect of simply elevating cAMP. Taken together, these observations demonstrate that the increase in the amount of LGN territory occupied by the ipsilateral projection correlates with the difference in activity between the two eyes, not the absolute level of activity within an eye.

Altering cAMP within the Retina Does Not Affect Ganglion Cell Number or Dendritic Morphology

The intraocular injection regimes used here only produce a modest (35%) increase in retinal wave dynamics, which is entirely reversible (and therefore requires that injections be repeated every 48 hr—see Figures 1 and

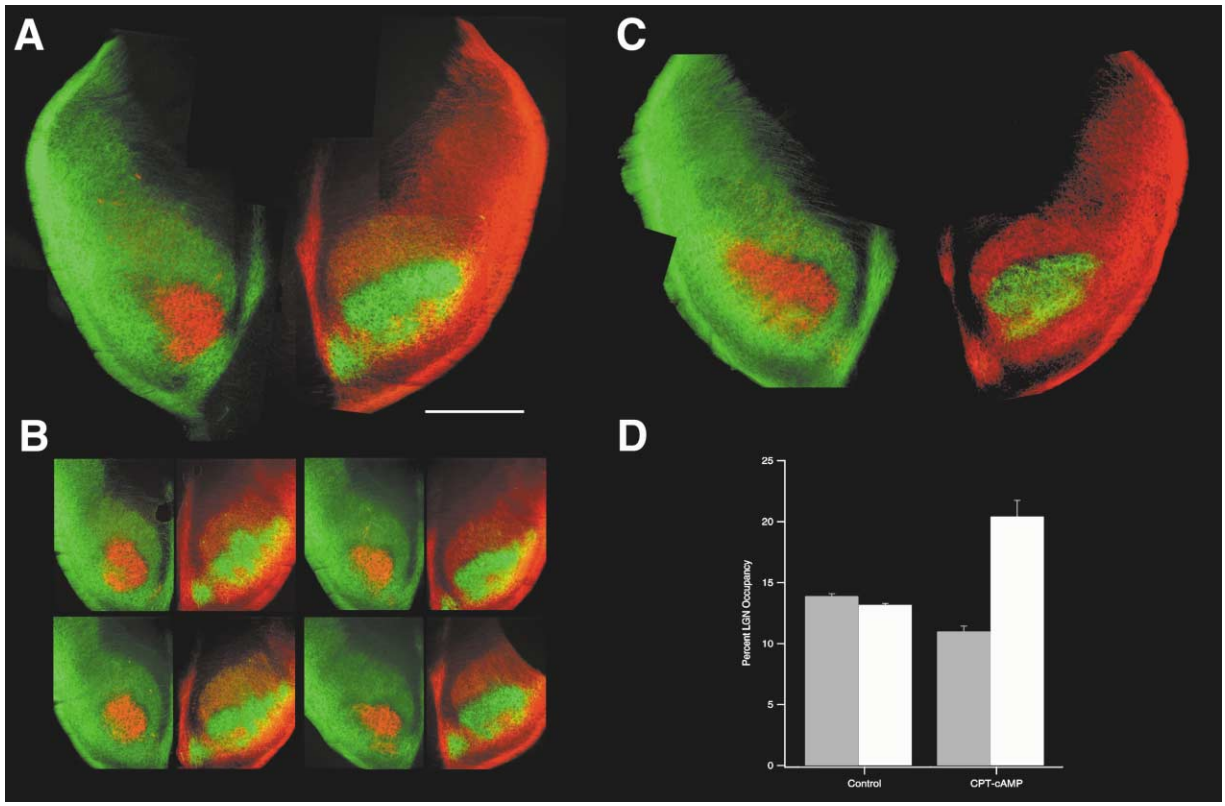

Figure 3. Increase in the Size of the Retinogeniculate Projection from the Treated Eye Occurs at the Expense of that from the Untreated Eye

(A) shows montaged micrographs of a horizontal section taken through P10 ferret LGNs (anterior, top; posterior, bottom). The retina treated from P1–P10 with CPT-cAMP was labeled with FITC conjugated cholera toxin B chain (CtB; green). The untreated retina was labeled with TRITC-conjugated CtB (red). The only areas of overlap (yellow) are near the optic tract and are due to fibers of passage. Projections to layers A and A1 are completely segregated from each other. (B) Shown are four successive sections from the same case as in (A), showing that the expansion of the projection from the treated eye extends over the entire binocular zone of the LGN. Sections are spaced 70 μm apart. (C) shows sections from a P10 ferret that received monocular saline injections from P1–P10, and were then injected with fluorescent CtB. Scale bar = 500 μm. (D) shows group data from all CPT-cAMP and saline-treated (control) retinas labeled with fluorescent CtB (n = 3 CTP-cAMP, three control animals), showing the percent of the LGN innervated by the ipsilateral projection from either the treated (white) and untreated (gray) eye. The projection from the CPT-cAMP-treated retina was significantly greater ($p < 0.001$) and the projection from the untreated retina significantly smaller ($p < 0.045$) than control values.

2). Nevertheless, it is possible that chronically elevating cAMP within the retina could alter retinal ganglion cell development or survival. The segregation of retinal axons into eye-specific layers occurs during the major period of retinal ganglion cell death (Henderson et al., 1988; Lam et al., 1982; Potts et al., 1982), and increasing cAMP can promote ganglion cell survival at least in axotomized retinas (Shen et al., 1999). Thus, a larger retinogeniculate projection could result from having more surviving retinal ganglion cells in the treated eye than the untreated eye, rather than having roughly the same number of ganglion cells projecting to more LGN territory than normal. We consider this possibility quite unlikely because *binocular* injections of CPT-cAMP result in normally sized projections, rather than enlarged projections, as would be expected with enhanced survival. Similarly, monocular injections of epibatidine plus forskolin result in a much smaller than normal projection from the treated eye, whereas if forskolin had been acting only to increase ganglion cell survival, again we would have expected to see an increase in the size of the projection to the LGN.

To assess directly the state of the retina following intraocular treatments with agents that elevate cAMP, we examined cell density in the ganglion cell layer. Following binocular injection of fluorescent CtBs, the number of fluorescently labeled cells in the ganglion cell layer of treated retinas was almost identical to that in the untreated retinas from the same animals (102% $\pm$ 6%; n = 4; $p > 0.77$). To verify that displaced amacrine cells were not obscuring changes in retinal ganglion cell number, we directly assessed the number of ganglion cells by retrogradely labeling them with DiI placed in the optic nerve (Figure 5). Neither the density of retinal ganglion cells nor the overall size of the retinas were significantly different in treated eyes relative to the uninjected eyes from the same animals. Treated retinas had 94% $\pm$ 6% as many RGCs per unit area as the untreated retinas from the same animals (n = 8; $p > 0.27$), and the area of treated retinas was unchanged: 100.2% $\pm$ 0.4% of controls (n = 8; $p > 0.66$). Retinal ganglion cell dendritic morphology also appeared normal, and α- and β-retinal ganglion cells could be clearly identified in treated retinas at P10. Indeed, the dendrites of some β

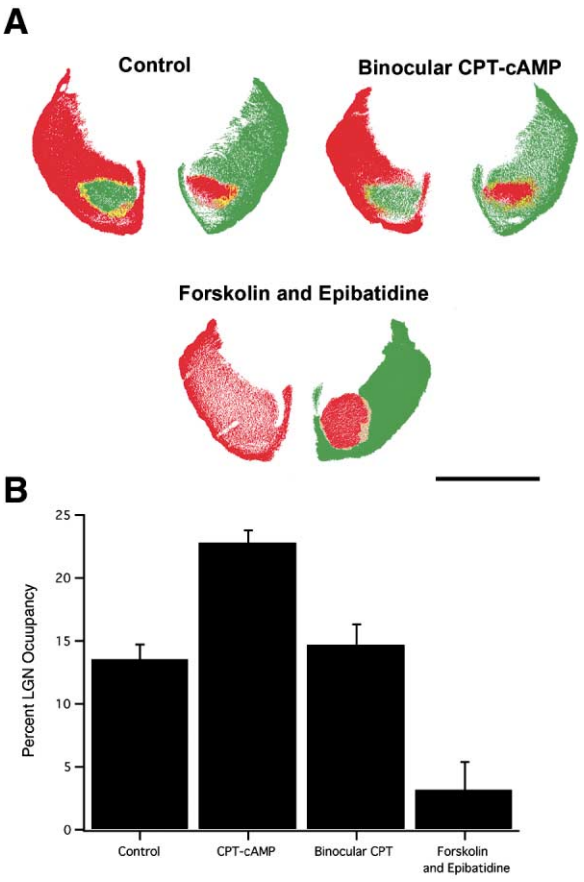

Figure 4. Increase in Size of Ipsilateral Projection Is Caused by Imbalance in Wave Levels between the Two Eyes and Not to Nonspecific Effects of cAMP Increases

(A) shows pattern of retinogeniculate projections in pseudocolored overlays of a pair of adjacent sections from either control, monocular injections of forskolin and epibatidine into the same eye, or binocular injections of CPT-cAMP. In each case, the projection from one eye was labeled with one tracer (green), the other eye (red) with a second tracer (see Experimental Procedures); regions of dual innervation appear yellow. The projection from the eye treated with a combination of epibatidine and forskolin is labeled green; that from the untreated eye in the same animal is labeled red. Scale bar = 500 μm. (B) Histograms showing the percent occupancy of the ipsilateral projection following each treatment. Monocular CPT-cAMP data is taken from Figure 2B (n = 4). When a combination of forskolin and epibatidine is injected into the same eye every 48 hr (n = 4), the ipsilateral projection from that eye is dramatically decreased, as expected from epibatidine injections alone (Penn et al., 1998). Injection of CPT-cAMP into both eyes every 48 hr (n = 5 animals, 9 LGNs) does not alter the size of the ipsilateral projection, indicating that an imbalance of wave activity between the two eyes, rather than an elevation of cAMP, regulates the amount of LGN territory occupied by each eye's ipsilateral projection.

cells were stratified in either the on or off sublayer of the inner plexiform layer (Figures 5C–5E) in treated retinas, as in normals. These observations strongly suggest that under the conditions used here to elevate cAMP levels in the retina, the number of retinal ganglion cells remains unaffected and many aspects of retinal ganglion cell development proceed normally. These data are perhaps not surprising in view of the fact that we have only altered wave dynamics by around 35%, and cAMP has only been observed to enhance cell survival following grave insults, such as axotomy (Shen et al., 1999).

Discussion

Here we have addressed the question of whether the amount and balance of neural activity experienced by retinal ganglion cells influences the pattern of central connections within the LGN. Our results demonstrate that the area occupied by an axonal projection is regulated in accordance with the amount of activity experienced by those inputs, relative to competing inputs. Increasing wave activity of one eye in vivo by intraocular injection of cAMP-enhancing agents permits that eye to acquire more LGN territory than the other eye, which nevertheless has a normal level of wave activity. The gain in territory does not occur if both eyes' activity is similarly augmented. Taken together, these data strongly support the hypothesis that an activity-dependent competition between retinal axons from the two eyes for LGN territory shapes the size of each layer within the binocular zone of the LGN.

An Instructive Role for Retinal Activity in LGN Development

There has been a longstanding discussion, based on previous experiments, about whether neural activity plays a "permissive" or an "instructive" role in patterning axonal connections during development (reviewed in Crair, 1999; Katz and Shatz, 1996). Neural activity could act "permissively," in the sense that a threshold level of activity is required to be able to read or process intrinsic information within LGN neurons, such as a set of molecular cues for each eye-specific layer. Alternatively, activity could act in an "instructive" fashion in the sense that the waves themselves provide the information necessary to determine developmental outcomes. Such information would include both the local correlation in the firing of retinal ganglion cells and the relative levels of activity between the two eyes; each of these parameters would contribute ultimately to the strengthening or weakening of specific synapses with LGN neurons (Crair, 1999; Katz and Shatz, 1996).

Previous evidence, summarized in Table 1, already strongly implicates at minimum a permissive requirement for activity in eye-specific layer formation. Segregation of retinal axons into layers cannot occur without neural activity, in either the retina (Penn et al., 1998) or the LGN (Shatz and Stryker, 1988). When TTX is infused via minipump into the LGN during the normal period of segregation, the eye-specific layers do not form (Shatz and Stryker, 1988) and retinal axons not only remain intermingled, but actually increase their branching (Sretavan and Shatz, 1986). A similar result occurs when retinal waves are blocked in both eyes by interfering with cholinergic synaptic transmission: again, the eye-specific layers fail to form (Penn et al., 1998). In one previous report (Cook et al., 1999), it was noted that binocular TTX treatments did not prevent segregation of retinal axons into eye-specific layers within the LGN. In our experience, it is not possible to obtain a complete blockade of wave activity with retinal injections of TTX without producing lethal systemic effects; this is why

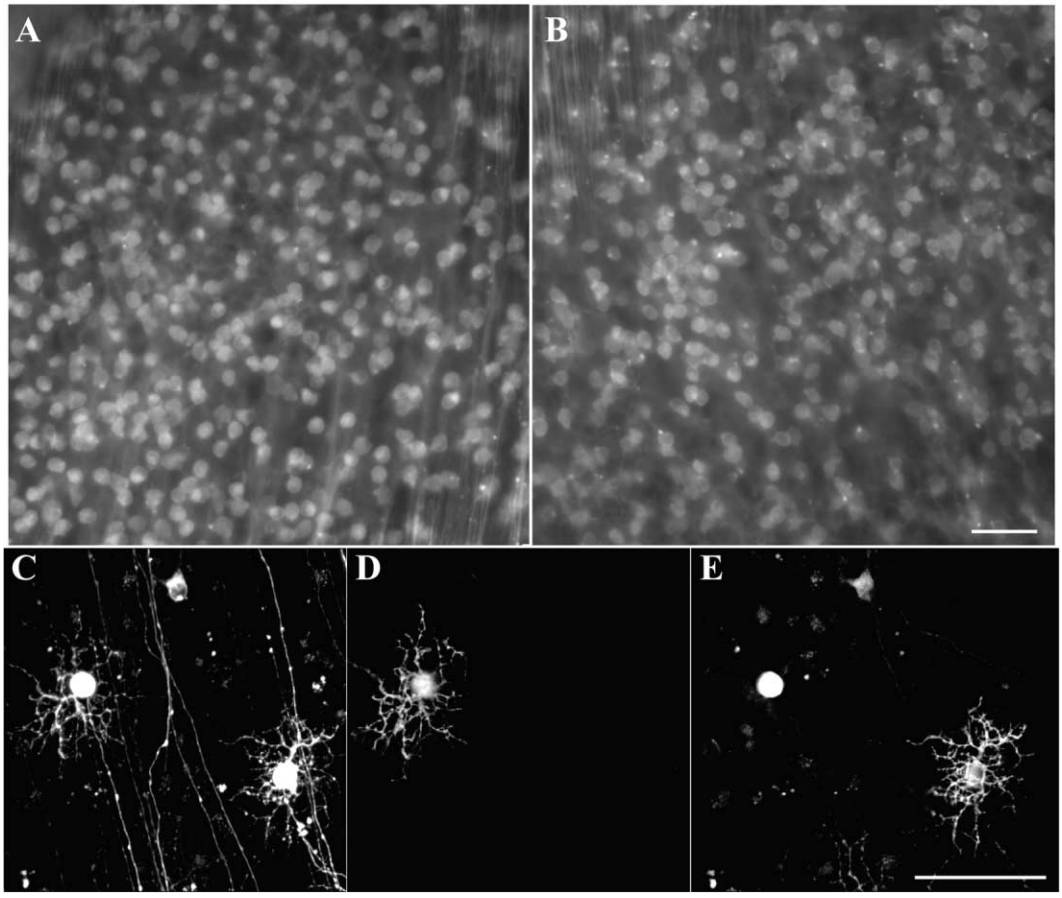

Figure 5. Chronically Elevated cAMP Levels Have No Detectable Effect on Retinal Ganglion Cell Number or Dendritic Morphology

Shown are low power micrographs of retinas treated from P1–P9 with intraocular injections of CPT-cAMP every 48 hr (A) or not receiving treatment (B). Ganglion cells were retrogradely labeled by application of DiI to the optic nerve. There is no significant difference in ganglion cell number between retinas from the two conditions, even though the area occupied by the axonal projection to the LGN has increased. (C) Higher magnification confocal image projection of the dendritic arbors of retinal ganglion cells in a retina treated from P1–P9 with CPT-cAMP every 48 hr. Dendrites appear normal in size and shape (and by inspection, even spine number looks unchanged). Normal dendritic stratification into sublayers within the inner plexiform layer can also be seen: (D) and (E) show single focal planes from (C), demonstrating stratification of dendrites of the cell on the left in the outer half of the IPL (D) while the cell on the right has dendrites stratified in the inner half of the IPL (E). Scale bars = 50 μm.

we used the cholinergic blockers (Penn et al., 1998), which can be confined to the retina and nevertheless completely block retinal waves.

While the observations above argue that neural activity is required for eye-specific segregation, they cannot elucidate how activity works to pattern retinogeniculate

Table 1. Summary of Effects of Monocular or Binocular Manipulations of Retinal Activity during Period of Eye-Specific Layer Formation in the LGN

Treatment	Monocular Injection	Binocular Injection
TTX[1]	ND	No layers; projections from both eyes overlap within the binocular zone of the LGN.
Epibatidine[2]	Projection from activity-blocked eye loses LGN territory; active projection expands.	No layers; projections from both eyes overlap within the binocular zone.
CPT-cAMP[3]	Projection from the more active eye enlarges at the expense of the normally active eye.	Normal refinement and eye-specific layering
Forskolin + Epibatidine[4]	Projection from treated eye loses LGN territory; active projection expands.	ND

[1] Shatz and Stryker, 1988 and Sretavan et al., 1988; TTX infused intracranially to block activity both in LGN neurons and ganglion cell axons; see also Cook et al., 1999.
[2] Penn et al., 1998; intraocular injection of epibatidine into one (monocular) or both (binocular) eyes. Epibatidine blocks all retinal waves.
[3] This study and Stellwagen et al., 1999; CPT-cAMP increases wave dynamics.
[4] This study and Penn et al., 1998, Stellwagen et al., 1999; forskolin increases wave dynamics but not in the presence of epibatidine.

projections. Removing all neural activity could create some kind of pathology that dysregulates axonal growth and promotes sprouting, or could prevent axons from reading and/or responding to eye-specific molecular cues presented by LGN neurons. However, when waves are blocked in one eye only, axons from the active eye actually occupy more LGN territory than normal (Penn et al., 1998). If neural activity were acting permissively to allow retinal axons to read molecular cues on LGN neurons, then the projection from the active eye should have formed a normal sized layer, rather than the expanded layer that was observed; similarly, the projection from the treated, inactive eye should have expanded in a dysregulated growth mode, rather than shrunk as was observed. The observed results are more consistent with an instructive, competitive mechanism in eye-specific layer formation. However, blockades can never fully distinguish between instructive and permissive roles for activity for all the reasons considered above.

The results presented in this present study avoid the caveats raised for experiments involving blockade of activity, and strongly support an instructive role for activity in LGN development. By "instructive," we mean that the patterns of neural activity—both spatial patterns that contain information about firing of nearest neighbors and temporal patterns such as the frequency of wave occurrence—rather than eye-specific molecules in the LGN dictate the formation of the layers within the binocular zone of the LGN. For a simple binary result such as eye-specific layers, even changes in the frequency of spontaneous correlated firing by retinal ganglion cells could be sufficient to alter the relevant informational content of the activity. Here we have shown that when one eye is made *more* active than the other by increasing wave frequency, its projection gains additional territory in the LGN, even though the other eye is also active (rather than silent) and presumably can read available molecular cues if present. If both eyes have increased, but approximately equal, levels of activity, then axonal projections from neither eye gain territory. Instead, each projection occupies the same amount of LGN territory as if both retinas were experiencing normal levels of activity.

This observation provides a crucial missing link to the framework of the historical argument for an instructive role for neural activity. The argument is based on the idea that correlations of activity are required for the strengthening or weakening of synaptic connections, and that retinal activity can provide those correlations in the form of spontaneous waves at early ages or visually driven activity later on (Cline, 1991; Crair, 1999; Katz and Shatz, 1996). As mentioned earlier, experimental support for this idea has come from two sets of experiments in which the activity of *all* retinal ganglion cells is correlated by either electrical stimulation to the optic nerve (Weliky and Katz, 1997) or by strobe rearing (Eisele and Schmidt, 1988; Schmidt and Buzzard, 1993). In each instance, results demonstrate that precise connectivity fails to form and the immature, diffuse pattern is retained. However, these experiments represent the "loss-of-function" case and until our study, the question remained, what is the effect on connectivity if *more* patterned activity is added to the developing system? Our results now demonstrate that if the neural activity is increased equally between the two eyes in a manner that preserves, rather than degrades, spatiotemporal information, then the normal patterns of retinal connectivity result.

A Differential Balance Regulates Activity-Dependent Competition

The increase in projection area resulting from a monocular injection of agents that elevate cAMP is a consequence of activity-dependent competition rather than due to nonspecific effects of cAMP on the growth state of retinal axons for at least three reasons. First, the untreated eye (with normal cAMP levels) yields LGN territory to the treated eye, suggesting that a competitive interaction occurs between the two sets of retinal axons. If growth were simply based on absolute levels of cAMP within the retina, then the untreated eye should have had a normal sized projection to the LGN. Second, combined intraocular injection of forskolin and epibatidine (which blocks retinal waves) decreases the size of the projection from the treated eye to the LGN; an increase would have been expected if elevation of cAMP simply dysregulated growth. Third and perhaps most telling, if cAMP acts to dysregulate growth, then binocular injections of CTP-cAMP should have produced expanded projections to the LGN from both eyes; instead we observed normal sized layers. The only way to reconcile all of these observations is to conclude that it is the *differential* balance between the levels of activity in the two eyes that regulates the amount of LGN territory occupied by the retinal projections from each eye. Similar dependence on relative levels of activity have been observed during the development of ocular dominance columns in the visual cortex (Chapman et al., 1986), although these experiments were conducted after the initial formation of the columns. The overarching principle remains—more activity means more territory; less activity means less territory.

Although each experiment, past or present (Table 1), when taken in isolation may have alternative explanations and drawbacks, it is difficult to reconcile all of the results with any other interpretation. In all cases, the more active eye always gains more territory, whether or not it is treated. The most parsimonious conclusion is that an activity-dependent competition between axons from the two eyes drives the process of binocular segregation and that the layers are not intrinsically defined by unique eye-specific molecules that can only be recognized by active ganglion cell axons. It is also hard to imagine how an activity-independent competition might function, given that the area occupied by retinal projections is always correlated with the differential in levels of retinal activity. We believe that a strong argument can now be made in favor of the hypothesis that neural activity instructs the patterning of connectivity between the retina and LGN during development of the eye-specific layers.

While these experiments argue in favor of an instructive role for neural activity in forming retinal projection patterns within the binocular zone of the LGN, it is worth noting that many aspects of patterning of the retinogeniculate projection can take place not only in the absence of all activity, as when retinal waves are blocked,

but also when one or both eyes have increased levels of activity produced by elevating of cAMP. For example, retinal axons from the temporal retina of the treated eye, while expanding to occupy a larger layer than normal, do not invade the monocular region of the LGN that is normally innervated by axons from the nasal retina of the contralateral eye. Nor do axons from the treated eye ever overgrow the LGN and invade territory belonging to other projection systems. These observations argue that growing axons can still recognize many targeting cues within the LGN even when cAMP is elevated (or activity blocked) and they are entirely consistent with previous suggestions (Flanagan and Vanderhaeghen, 1998; Katz and Shatz, 1996; Tessier-Lavigne, 1995) that both activity-dependent and activity-independent cues cooperate to pattern the retinogenciulate projection.

Instructive Role of Activity beyond Eye-Specific Layer Formation

Retinal waves are thought to function in the development of other features in the visual system. Evidence from turtles suggests that wave activity may affect the size of retinal ganglion cell receptive fields (Sernagor and Grzywacz, 1996). On and off ganglion cells also begin to experience different correlations in firing during retinal wave activity in the third and fourth postnatal weeks in ferret (Wong and Oakley, 1996), during the period of the segregation of on- and off-sublaminae in the LGN (Stryker and Zahs, 1983), a process known to require both neural activity and NMDA receptor activation (Cramer and Sur, 1997; Dubin et al., 1986; Hahm et al., 1991). Whether spontaneous retinal waves, as opposed to activity per se, may also be acting instructively in these situations has not been addressed.

Retinal waves are also well suited to drive the initial refinement of LGN axons into ocular dominance columns in the visual cortex of binocular mammals. In primates, ocular dominance columns are well on their way to forming in utero, when waves rather than visual experience could contribute (Horton and Hocking, 1996; Rakic, 1976). In ferret retina, the waves are present as early as E35 (unpublished observations), and persist until just before eye opening, 5 weeks later (P30) (Wong et al., 1993). Physiological studies in a reduced in vitro preparation in the mouse that contains the entire visual pathway from retinas to LGN indicate that the waves drive LGN neurons to spike at ages even before the eye-specific layers are formed (Mooney et al., 1996). In vivo multielectrode recordings from the ferret LGN at slightly older ages, but still well before eye opening, also demonstrate that retinal waves affect correlations in the spontaneous firing of LGN neurons (Weliky and Katz, 1999). These recordings also showed that even in the absence of the retina, the LGN neurons fire spontaneously. Spontaneous activity from either retinal or geniculate sources would be relayed by LGN axons to the cortex, first to the subplate neurons and at later times to layer 4 neurons, allowing activity to influence the development of cortical columns.

Recent studies using modern physiological and anatomical methods have shown that the system of cortical ocular dominance columns forms much earlier than previously supposed (Crair et al., 2001; Crowley and Katz, 2000), but in all cases, this period is still well within the time frame for retinal wave activity to play a crucial role. Nevertheless, it has been proposed recently that the initial formation of ocular dominance columns does not require neural activity, but rather relies on molecular cues (Crowley and Katz, 2000). This proposal is at odds with the results presented here, in the sense that it seems unlikely that the segregation of retinal axons to form eye-specific layers within the LGN would utilize the information contained in the neural activity patterns, but then the segregation of LGN axons to form ocular dominance columns, which occurs in all mammals *after* the eye-specific layers have formed, would rely solely on molecular cues. Before deciding that the two systems are profoundly different in regard to mechanism, it will be important to manipulate directly neural activity levels and the balance of activity between the two eyes precisely during the times in development that the columns are now known to form. An approach such as the one we have taken here, of increasing activity in one eye by elevating cAMP (rather than by enucleating one eye, as in Crowley and Katz, 2000), would be very informative and might help to reconcile the apparent differences between the initial formation of the eye-specific layers in the LGN and the subsequent development of ocular dominance columns in layer 4 of visual cortex.

Experimental Procedures

All procedures were performed in accordance with approved animal use protocols at UC Berkeley and Harvard Medical School. Day of birth is taken as postnatal day zero (P0).

Retinal Drug Treatments

Following previously published protocols (Penn et al., 1998), 1 μl of solution (or 0.5 μl in the case of cholera toxin) was injected intraocularly with a 30G needle into the posterior chamber at a rate of 0.5 μl/min into P1 ferret kits. Agents injected were: 2 mM CPT-cAMP (Sigma) in 0.9% NaCl saline; 20 mM forskolin (Sigma) in DMSO; 1 μg/ml cholera toxin (List Biological) in 0.9% NaCl saline; 100 μM epibatidine (RBI) in 0.9% NaCl saline; or 0.9% NaCl saline alone. Injections were repeated every 48 hr, increasing the amount by 20% with each injection, with the exception of one experimental group where CPT-cAMP was injected every 24 hr (Figure 2). CPT-cAMP is capable of increasing retinal wave frequency in vitro (to 190% ± 19% of control frequency, n = 3 P1 rat retinas, data not shown).

Anterograde Tracing

To assess the status of the retinogeniculate projection following these treatments, P8 animals received an intraocular injection of anterograde tracer: 2.5 μl tracer, delivered at 1.0 μl/min—5% WGA-HRP (Vector Labs) in 0.9% NaCl saline in one eye, and 1% cholera toxin B chain (CtB; List Biological) in 0.9% NaCl saline in the other. CtB retains no ability to enhance cAMP production. Animals were perfused intracardially 24 hr later with 0.1 M sodium phosphate buffer with 5 U/ml heparin followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer. Brains were removed and post-fixed overnight in fresh fix at 4°C. In some instances, FITC- or TRITC-conjugated CtB's (0.2% CtB in 2% DMSO; List Biological) were injected into each eye and animals perfused after 48 hr (at P10).

Tissue Preparation

Brains were sunk in 25% sucrose in 0.1 M sodium phosphate buffer and sectioned in the horizontal plane at 50 micrometers on the freezing stage of a microtome. Alternate sections were developed according to protocols of Mesulam (1978) for HRP or Angelucci et al. (1996) for CtB. For HRP, sections were rinsed in distilled water (dH_2O; 1 min), reacted in 3,3′,5,5′-Tetramethyl benzidine (TMB; Kirkegaard and Perry) for 1 hr, rinsed in dH_2O (2 times for 1 min) followed

by 30% ethanol (1 time for 2 min), then reacted in 5% sodium nitroprusside in 30% ethanol with 10 mM sodium acetate (pH 3.3) for 1 hr, rinsed in 30% ethanol (2 times for 1 min) and dH_2O (2 times for 2 min). For CtB, sections were quenched of HRP activity for 30 min in 0.5% H_2O_2 in 90% methanol, briefly washed (3 times for 5 min in sodium phosphate buffered saline (PBS)), and placed in blocking solution overnight (2.5% bovine serum albumin (BSA), 0.5% Triton-X, 2% normal donkey serum (NDS) in PBS). Sections were then placed in primary antibody for 2 days at room temperature (goat anti-CtB; (List Biological) at 1:50,000 in 2.5% BSA, 2% Triton, 2% NDS in PBS). Sections were washed (4 times for 15 min in PBS), placed in secondary antibody (biotinylated donkey anti-goat (Jackson) at 1:500 in 2.5% BSA, 1% Triton, 2% NDS in PBS) for 1 hr, washed, treated with avidin-biotin (Vector), washed, and visualized using VIP (Vector). Overlays of the two projections (Figure 4) were created by merging images of adjacent sections in Photoshop (Adobe).

When fluorescently conjugated CtB's were injected intraocularly, brains were embedded in 3% agarose, cut at 70 micrometers on a vibrotome, mounted on slides, and viewed in fluorescence on a Nikon Optiphot microscope. Micrographs were acquired with a color CCD camera (Diagnostic Spot), and images (Figure 3) montaged together in Photoshop (Adobe).

Analysis of Retinal Projection Areas

Slides of anterogradely labeled sections containing superior colliculi and lateral geniculate nuclei were scanned and images acquired into a computer using a CCD camera (Dage) and NIH Image software, using invariant settings. Projection areas of HRP-labeled sections (Figure 2) were determined solely by computer algorithm and thus were not subject to experimenter interpretation. Data were analyzed by setting a threshold of 30% above the background pixel values of unstained tissue, and measuring the percent area of the LGN containing anterogradely transported label. Pixels above threshold were accepted as labeled. Fluorescently labeled LGNs, due to higher background staining, occasionally required some manual resetting of the threshold. However, fluorescent data closely matched HRP data, indicating that no undue experimenter bias was somehow introduced.

Percent LGN occupancy was calculated from four LGN sections containing the largest area of the binocular zone in each animal. Measurement of ipsilateral occupancy included all of the labeled projection to layers A1 and C1. Measurement of contralateral occupancy included Layer A and all of the labeled C layers (C and C2). Note that because the retinal projection from the contralateral eye traverses several layers receiving innervation from the ipsilateral eye, measurements are confounded by a "fiber of passage" contribution. Thus the contralateral occupancy measurements are likely to be overestimates and we consider the ipsilateral occupancy measurements to be more accurate. All data are expressed as average $\pm$ SEM, and the statistical significance of changes was evaluated by use of a one way ANOVA followed by Bonferroni's t test.

To determine the percent overlap between ipsilateral and contralateral projections to the same LGN (e.g., from double-labeling experiments such as that shown in Figure 3), the area occupied by the ipsilateral projection to layer A1 was measured, as was the area left vacant by the surrounding projection from the contralateral eye to layers A and C. The two areas were subtracted and expressed as a percentage of total LGN area—typically about 0.5%. The C layers were not included in these measurements because of the fiber of passage problem mentioned above.

In Vitro Retinal Preparation

Retinas were isolated from newborn ferrets (P0–P10) that had been deeply anesthetized with halothane and then decapitated. Some ferrets received intraocular injections of cholera toxin (1 μl of 1 μg/ml) 24 hr prior to euthanasia. Retinae were rapidly dissected and isolated retinas were incubated with 10 μM Fura2-AM (Molecular Probes) in ACSF containing 1% DMSO and 0.02% pluronic acid for 2–6 hr in an oxygenated chamber at 28°C. All procedures were performed in artificial cerebrospinal fluid (ACSF; 119 mM NaCl, 2.5 mM KCl, 1.3 mM $MgCl_2$, 1.0 mM KH_2PO_4, 2.5 mM $CaCl_2$, 26.2 mM $NaHCO_3$, 11 mM D-glucose). Solutions were buffered with $NaHCO_3$ and oxygenated with a mixture of 95% O2/5% CO_2. For acute in vitro application, cholera toxin (List Biological) was added to the chamber 1–2 hr before recording. Full details of fluorescence imaging, wave data analysis, and electrophysiology are available in Feller et al. (1996, 1997) and Stellwagen et al. (1999).

Optical Recording

Retinas were placed ganglion cell layer up in a temperature-controlled chamber (30°C, Medical Systems) mounted on the stage of either an inverted microscope (Nikon, Diaphot 300) or an upright microscope (Technical Instruments), and were continuously perfused. All experiments were conducted with 380 nm illumination using a 10$\times$ (Nikon) objective. Images were acquired with a SIT camera (Dage, MIT 300). Initially, a background frame was acquired, that was then subtracted on a pixel-by-pixel basis from all subsequent frames to create a difference image. The difference image was averaged over four video frames, giving a time resolution of 120 ms/frame. Movies of fluorescence changes were acquired onto Hi-8 videotape (Sony) with Metamorph Software (Universal Imaging). Three locations, each approximately 1500 μm^2, were monitored on each retina to determine frequency and peak amplitude of fluorescence changes. Fluorescence data are displayed as traces of $\Delta F/F$ versus time, where F is the amount of DC fluorescence corrected for bleaching, and ΔF is the deviation from this baseline.

Physiological Recording

Cell-attached patch clamp recordings were made from ganglion cells using an Axopatch 200A amplifier and pClamp6 software (Axon Instruments). Ganglion cells were identified based upon dendritic morphology, soma size, and presence of an axon. Forskolin (Sigma) was dissolved in DMSO and stored at -80°C until used. Final concentrations of DMSO in ACSF were $<$1%; test solutions of ACSF with 1% DMSO had no effect on the frequency of retinal waves.

Retinal Ganglion Cell Labeling

To determine the number of ganglion cells and to examine their dendritic morphology, DiI crystals were placed on the stump of the optic nerve from fixed eyes, and were allowed to transport for one week at 37°C. In animals double-labeled with fluorescent CtB, the CtB itself was used to determine cell density. Retinas were then removed, flattened, and viewed under a Nikon Optiphot microscope. Cell counts were obtained from identical regions of retina from treated and untreated retinas from the same animals, and expressed as cells per unit area. Images were collected with a color CCD camera (Diagnostic Spot). High magnification pictures were taken on a confocal microscope (Nikon) and projection images created using NIH Image.

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