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Gap junctional regulatory mechanisms in the AII amacrine cell of the rabbit retina

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Abstract

Gap junctions are commonplace in retina, often between cells of the same morphological type, but sometimes linking different cell types. The strength of coupling between cells derives from the properties of the connexins, but also is regulated by the intracellular environment of each cell. We measured the relative coupling of two different gap junctions made by AII amacrine cells of the rabbit retina. Permeability to the tracer Neurobiotin was measured at different concentrations of the neuromodulators dopamine, nitric oxide, or cyclic adenosine monophosphate (cAMP) analogs. Diffusion coefficients were calculated separately for the gap junctions between pairs of AII amacrine cells and for those connecting AII amacrine cells with ON cone bipolar cells. Increased dopamine caused diffusion rates to decline more rapidly across the AII–AII gap junctions than across the AII–bipolar cell gap junctions. The rate of decline at these sites was well fit by a model proposing that dopamine modulates two independent gates in AII–AII channels, but only a single gate on the AII side of the AII–bipolar channel. However, a membrane-permeant cAMP agonist modulated both types of channel equally. Therefore, the major regulator of channel closure in this network is the local cAMP concentration within each cell, as regulated by dopamine, rather than different cAMP sensitivity of their respective gates. In contrast, nitric oxide preferentially reduced AII–bipolar cell permeabilities. Coupling from AII amacrine cells to the different bipolar cell subtypes was differentially affected by dopamine, indicating that light adaptation acting *via* dopamine release alters network coupling properties in multiple ways.

Keywords

Electrical coupling; Gap junction; Retina

Introduction

Connexin expression studies have revealed that gap junctions are found at many sites in the adult nervous system (Condorelli et al., 1998), and recordings from cell pairs indicate that electrical coupling can produce oscillations and synchrony (e.g. Galarreta & Hestrin, 2001; Loewenstein et al., 2001; Landisman et al., 2002; Veruki & Hartveit, 2002a,b). Even different cell types often are connected by gap junctions to form a coupled network. The information processing roles of these networks are determined in part by the conductance of these gap junctions (Chow & Kopell, 2000; Lewis & Rinzel, 2003), which are probably regulated carefully. In this study, we examined the gating of gap junctions in a well-described network made by AII amacrine cells in the rabbit retina. We measured changes in the diffusion coefficients of the tracer Neurobiotin through the two gap junctional pathways made by this neuron in response to stimulation of cyclic nucleotide pathways. The results illustrate how

neuromodulators, intracellular pathways, and connexon properties interact to regulate coupling.

The AII amacrine cell is found in all mammalian retinæ, which have separate pathways for dim signals, collected from rods, and brighter signals, collected from cones. Different receptor subtypes on cone bipolar cells divide the cone pathway into two processing streams that depolarize either at light onset (“ON” types) or at offset (“OFF” types). These cone bipolar cells pass light signals on to ganglion cells of the same polarity. A single type (ON) of rod bipolar cell collects the output from rods, but does not synapse directly onto ganglion cells. Instead, rod bipolar cells pass their signals into ON and OFF channels *via* the AII amacrine cell (see Fig. 1A). The AII cell accomplishes this by making sign-conserving gap junctions with ON cone bipolar cells and sign-inverting inhibitory synapses onto OFF cone bipolar cells. AII amacrine cells also make gap junctions with neighboring AII amacrine cells (Famiglietti & Kolb, 1975; Smith et al. 1986; Sterling et al., 1988; Strettoi et al., 1992).

Because AII amacrine cells make gap junctions both with other AIIs, and also with ON cone bipolar cells, these two pathways differ in the type of cell forming the other hemichannel. The functional differences imparted by the different junctional partners, whether different connexins are contained in the opposing hemichannels, and whether the AII amacrine cell itself makes one or two types of connexin protein are all unknown. Some possible arrangements and their effects on the coupling as determined by the relative permeability of the two gap junctional pathways are shown in Figs. 1B and 1C.

Mills and Massey (1995) estimated the relative efficacy of second messengers on AII–AII coupling by the number of stained AII somas and AII–bipolar cell coupling by the relative brightness of AII and bipolar cells at the same distance from the injected cell. We were concerned that the measurement of relative brightness was dependent on both diffusional paths and could mask some types of changes. Therefore, we have reexamined coupling in this network with a more sensitive method that measures the two diffusion rates independently. These measurements demonstrate that the permeability of both pathways is reduced by dopamine. Further, their relative rates of decline in response to increasing dopamine suggests that dopamine closes only the hemichannel on the AII side. (The term hemichannel is used in this paper only as one-half of a docked pair of connexons, never as a single, undocked connexon.) Both gap junctional pathways are equally closed by application of a membrane-permeant cAMP analog, however. This indicates that (1) the gates on either side of the channels have comparable cAMP sensitivities, (2) the asymmetric effect of dopamine is caused by raising cAMP much more in the AII amacrine cell than in ON cone bipolar cells, and (3) that cAMP does not diffuse across the gap junctions sufficiently to alter the permeability of both hemichannels equally.

Materials and methods

Adult albino rabbits were deeply anesthetized with injections of urethane (1.5 g/kg, i.p.), then humanely killed by intracardial injection of 5 cc urethane following removal of the eyes. All procedures were in accordance with the guidelines of the University of Texas at Houston Animal Welfare Committee. AII amacrine cells were selectively stained (Mills & Massey, 1991) by incubation of the isolated retina in the blue fluorescent dye, DAPI (4,6-diamino-2-phenylindole; Molecular Probes, Eugene, OR). Most of the procedures have been described previously in detail (Mills & Massey, 1998).

Preliminary considerations

The degree to which cells were coupled was determined by measuring diffusion coefficients within a group of coupled AII amacrine and ON cone bipolar cells stained by injecting a single

AII amacrine cell. We have previously established several important points that enabled us to make our diffusion measurements with confidence (Mills & Massey, 1998; 2000). These are (1) passive diffusion is sufficient to account for tracer movement. (2) diffusion across gap junctions is much slower than through the cell and hence rate-limiting. Mills and Massey (1998) found that the diffusion coefficient across the gap junctions of the far more extensively coupled A-type horizontal cell was at most 10% of the diffusion coefficient within the cell. Similarly, tracer diffuses the length of an AII amacrine cell within a few seconds, whereas 15–20 min of diffusion time is required to detect AII amacrine cell–bipolar cell coupling, and 5–10 min required to detect AII–AII coupling. (3) Diffusion coefficients can be accurately measured if the diffusion time is accounted for. For any given cell type and otherwise identical conditions, these diffusion coefficients are stable when measured across a wide range of diffusion times. Additionally, (4) tracer moves in both directions across both sets of gap junctions (Trexler et al., 2001), and (5) tracer continues to diffuse laterally for the entire diffusion time. Therefore, a total equilibrium is never reached, although the relative rate at which AII and ON cone bipolar cells exchange tracer reaches a relative equilibrium that is stable for at least 3 h and facilitates accurate measurement of stable coefficients over time and distance from the injection site (Mills & Massey, 1995). Finally, (6) phosphorylation of channels by cAMP most likely results in a decrease in the open probability of channels. This is discussed in more detail in the Discussion section.

Detailed procedures

Cell injection—Individual AII amacrine cells were filled by iontophoresis (+1 nA, 3 Hz, 10 min) with 4% Neurobiotin (Vaney, 1991; Mills & Massey, 1991). The time elapsed between the start of iontophoresis and fixation was recorded as the total diffusion time, which ranged from 20–180 min. Junctional permeabilities were altered by bath application of dopamine, the D₁ antagonist SCH23390, a nitric oxide donor S-nitrosylpenicillamine (SNAP) (each from Sigma, St. Louis, MO), or Sp-8-CPT-cAMPs (Biolog, La Jolla, CA). Figs. 2A and 2B show examples of tracer coupling following injection of Neurobiotin into a single AII amacrine cell under normal conditions (Fig. 2A) or with 250 μ M dopamine (Fig. 2B). AII amacrine cells and bipolar cells were each imaged with separate stacks of ten 0.5 μ m sections and pseudocolored red or blue for contrast.

Tissue processing—Retinas were fixed in 4% paraformaldehyde (1 h) after several cells were filled. Cells were visualized by overnight incubation in 1:200 streptavidin-Cy3 (Jackson ImmunoResearch, West Grove, PA). Tissue was mounted in 50% 0.2 M phosphate buffer + 50% glycerol + 0.1% para-phenylenediamine (Sigma) to retard fading by fluorescent illumination. Streptavidin-Cy3 gives a linear response range as a function of Neurobiotin concentration, unlike enzyme detection systems such as horseradish peroxidase (Mills & Massey, 1998).

Imaging—Fluorescent images of stained material were acquired using a confocal microscope (Zeiss LSM 410). The optical section through the somas was 1.5 μ m, taken with a 40 $\times$ oil immersion objective. Series of images were acquired with 1-s exposures using laser settings and intensities that excited cells in a range that could be reliably related to previously determined standards, as described below. The image series progressed from low laser intensity through the highest necessary. This minimized fading effects, which were rarely seen.

Measurement of absolute Neurobiotin concentrations within cells—HeLa cells were stained *via* diffusion from patch electrodes that contained specific concentrations of Neurobiotin. Inclusion of the gap junctional blocker carbenoxolone (10 μ M; Sigma) in the medium prevented spread of Neurobiotin to neighboring cells (Fig. 3A, inset). We then processed and imaged these cells identically to the retinal preparations. These measurements

enabled a lookup table to be calculated that converted image pixel intensity to Neurobiotin concentration. Fig. 3A shows the measured pixel intensity of groups of HeLa cells with different Neurobiotin concentrations in the pipette; each point is the mean of 2–5 cells loaded from a particular preparation. Although the 8-bit range of the photodetector should code 2.4 log units, the photodetector was too nonlinear to encode this range. Specifically, the slope of the intensity function in the ranges 0–50 and 200–255 proved too shallow to reliably code intensity. Therefore, the laser brightness was adjusted so that both HeLa and retinal cells were imaged at confocal settings that positioned them in the range 50–200. A single function could be moved along the abscissa to fit HeLa cells with different concentrations of Neurobiotin, and that was predictable from the laser attenuation settings (Fig. 3B). In other words, the same pixel intensities would be produced by a 10-fold decrease in Neurobiotin concentration in the pipette or a 10-fold attenuation of laser intensity.

We used these functions to calculate the Neurobiotin concentrations of all stained retinal cells by using the lookup table in the program SigmaScanPro (SPSS Science, Inc., Chicago, IL), which also converted the distance metric of the image from pixels to microns. Intensity was measured by a 5×5 pixel array placed over the soma. The area occupied by the nucleus was excluded, as it was frequently less intensely stained than the cytoplasm. The output of the SigmaScan program was a table of distances from the injected cell and Neurobiotin concentrations for each cell in a coupled group.

We normalized the actual distance in microns by the spacing density of the group of AII amacrine cells. The diffusion rate then reflects the number of cells traversed rather than actual distance. The unit measure of the diffusion rates is therefore (cell separations)²/s.

Calculation of diffusion coefficients—For each group of stained cells resulting from a single injection, the Neurobiotin concentration for each amacrine and bipolar cell soma was plotted as a function of distance from the injected cell. Two diffusion coefficients were calculated from each such data set. One reflected diffusion between AII amacrine cells (k_{AA}), while the other reflected the diffusion rate from AII amacrine cells to cone bipolar cells (k_{AB}). Assuming that tracer movement occurs by passive diffusion (Zimmerman & Rose, 1985; Mills & Massey, 1998), tracer movement can be calculated by knowing the final concentration gradient across gap junctions (as determined by the intensity measurements), the diffusion time, and a coefficient of diffusion. As before (Mills & Massey, 1998), these coefficients were stable over a wide range of diffusion times, under otherwise comparable conditions (e.g. cell type, pharmacological condition).

These three parameters are sufficient to describe any data set we have obtained. The diffusion coefficient between AII amacrine cells (k_{AA}) and from AII amacrine cells to ON cone bipolar cells (k_{AB}) are determined as described below. However, the greatest variation between data sets was in the total brightness of the coupled group of cells. This is because the amount of tracer directly injected into the target cell differs between injections due to variation of electrode and cell properties following cell penetration. This variation in overall brightness of the coupled group of cells could be accounted for by estimating the total amount of tracer injected into the target cell (see also Mills & Massey, 1998). We did this by adding a term for tracer injection rate into the target cell which was applied for the 10 min of iontophoresis, then set to zero for the remainder of the diffusion time. This third parameter [C_{inj} in eq. (1), which we refer to as iontophoresis rate] was adjusted to fit the measured Neurobiotin concentrations in the coupled group of cells. In Fig. 2C, altering the iontophoresis rate would move the lines fitting the data points up or down on the logarithmic ordinate, without changing their rate of falloff or the distance between the AII amacrine cell data points and those of the bipolar cells.

Separate diffusion coefficients for AII–AII and AII–bipolar cell gap junctions were determined by fitting the slopes of the AII and bipolar cell intensities. Figs. 2C, 2D, and 2F show how the slope of the AII–AII intensity profile changes as the AII–AII diffusion coefficient is changed. The second diffusion coefficient is the average AII–ON cone bipolar cell diffusion rate, which is determined by the distance between the bipolar cell staining intensities and those of the AII amacrine cells (Figs. 2C, 2D, & 2F). In practice, these two coefficients were adjusted iteratively to obtain the best fit.

We calculated diffusion coefficients by choosing parameters that best fit the data curves. Differential equations which related diffusion rates to concentration differences in the various compartments were solved using second- and third-order Runge-Kutta ordinary differential equations in MatLab (The MathWorks, Natick, MA). The equations were of the following form:

$$\frac{dC_{A(0)}}{dt} = C_{inj} + k_{AA} * (C_{A(1)} - C_{A(0)}) + k_{AB} * (C_{B(0)} - C_{A(0)}), \quad (1a)$$

$$\frac{dC_{A(1)}}{dt} = k_{AA} * (C_{A(0)} + C_{A(2)} - 2C_{A(1)}) + k_{AB} * (C_{B(1)} - C_{A(1)}), \quad (2a)$$

$$\frac{dC_{A(i)}}{dt} = k_{AA} * (C_{A(i-1)} + C_{A(i+1)} - 2C_{A(i)}) + k_{AB} * (C_{B(i)} - C_{A(i)}), \quad (3a)$$

$$\frac{dC_{B(i)}}{dt} = \frac{k_{AB}}{V_R} * (C_{A(i)} - C_{B(i)}). \quad (4a)$$

Also tested was a more fully described two-dimensional model wherein the complete geometry of a group of cells was modeled. Although more complex, this model gave qualitatively similar results to the first model.

$$\frac{dC_{A(0)}}{dt} = C'_{inj} + 6 * k_{AA} * (C_{A(1)} - C_{A(0)}) + k_{AB} * (C_{B(0)} - C_{A(0)}), \quad (1b)$$

$$\begin{aligned} \frac{dC_{A(1)}}{dt} = & \frac{1}{2} * k_{AA} * [(2 * (C_{A(0)} - C_{A(1)}) + 2 * (C_{A(1)} - C_{A(1)}) \\ & + 2 * (C_{A(2)} - C_{A(1)}))] \\ & + \frac{1}{2} * k_{AA} * [(1 * (C_{A(0)} - C_{A(1)}) + 2 * (C_{A(1)} - C_{A(1)}) \\ & + 3 * (C_{A(2)} - C_{A(1)}))] \\ & + k_{AB} * (C_{B(1)} - C_{A(1)}), \end{aligned} \quad (2b)$$

which reduces to

$$\begin{aligned} \frac{dC_{A(1)}}{dt} = & k_{AA} * (1.5 * C_{A(0)} + 2.5 * C_{A(2)} - 4 * C_{A(1)}) \\ & + k_{AB} * (C_{B(1)} - C_{A(1)}), \end{aligned} \quad (2c)$$

$$\begin{aligned} \frac{dC_{A(i)}}{dt} = & k_{AA} \times (1.5 \times C_{A(i-1)} + 2.5 \times C_{A(i+1)} - 4 \times C_{A(i)}) \\ & + k_{AB} \times (C_{B(i)} - C_{A(i)}), \end{aligned} \quad (3b)$$

$$\frac{dC_{B(i)}}{dt} = \frac{k_{AB}}{V_R} \times (C_{A(i)} - C_{B(i)}), \quad (4b)$$

where $C_{A(i)}$ represent the tracer concentrations in each (0,1,...,i) AII amacrine cell and $C_{B(i)}$ is the tracer concentration in corresponding bipolar cells. C_{inj} is the rate of tracer injection into the target AII amacrine cell for the 10 min of iontophoresis, and is set to zero thereafter. The diffusion coefficients are k_{AA} for movement between AII amacrine cells and k_{AB} for movement from an AII amacrine cell to ON cone bipolar cells. Eqs. (1)–(2) in each set should contain the volumes of the compartment appearing on the left-hand side of the equations, that is, V_A for eqns. (1)–(3a,b) and V_B for eqn. (4a,b). We have normalized V_A to a value of 1.0 for simplicity and used V_R as the estimated relative volume of the bipolar cell compartments to the AII amacrine cell to which they are coupled. This was because these volumes are difficult to determine accurately. We estimate, from confocal series, that V_B is about 1.1 to 1.4 times V_A , based upon the relative volumes of AII and bipolar cells multiplied by the number of bipolar cell types (4–5). However, the volume of a very poorly coupled cone bipolar cell will be effectively negligible with respect to the distribution of tracer, even if its volume were fairly large. It is likely that the effective volume ratio $VR_{B/A}$ will be dominated by the 2–3 best-coupled cone bipolar cells.

An exact estimate of this value is not critical for the present analysis, however. This is because the analysis and conclusions of this study are based upon relative changes in diffusion coefficients produced by gap junctional modulators. We verified this by refitting data with a range of volume corrections (0.2, 0.5, 1.0, 2.0, & 5.0). Although the coefficients were altered, the changes in response to the experimental variables retained about the same slopes. Nevertheless, the absolute differences mean that the relative magnitudes of the diffusion coefficients, k_{AA} and k_{AB} , are not directly comparable. Further, the diffusion coefficients should be regarded as *effective* diffusion coefficients, as they have been normalized by relative cell volumes.

The fixed numerical coefficients in the second set of equations are determined by the hexagonal array. Although some contact is made with more distant AII amacrine cell, our simulations indicate that the gap junctional area with the nearest neighbors sufficiently dominates tracer movement to allow the simpler analysis. When tracer is injected into the initial AII amacrine cell $A_{(0)}$, it diffuses into six neighbors [eqn. (1b)]. Each cell in the second row of cells is also connected to six neighbors. Half of these neighbors are connected to their six neighboring AII amacrine cells in the following manner [eqn. (2b)]: one cell closer to the injected cell, two cells the same path length from the injected cell, and three cells 1 path length more distant than themselves. The other half were connected to two cells closer to the injected cell, two cells the same path length, and two cells 1 path length more distant than themselves. Path length refers to the number of cells that must be traversed to reach a given cell. Since diffusion within a cell is rapid compared to diffusion across gap junctions, the actual geometric distances within the array were not considered. The numerical coefficients of these two groups were averaged to 1.5, 2.0, and 2.5, as in the eqns. (2c,d). The two cells with the same path length were omitted from the equations since they should have the same concentration of tracer on average. The first three terms should contain the volume of an AII amacrine cell, but this is normalized to 1.0 for our purposes.

This model is based upon well-known anatomical data (Famiglietti & Kolb, 1975; Strettoi et al., 1992). All the data we have acquired can be fit because diffusion through the system is determined by the anatomy, in the following manner. Tracer moves laterally from AII to AII amacrine cell as in other networks comprised of cells of only a single type. However, it enters a bipolar cell only from the AII that contact it directly (Fig. 2E). If there were no gap junction, but cytoplasmic continuity, between the bipolar cell and the nearby AII, then the bipolar cells and AII would have the same decline in intensity as a function of distance from the injected cell and a single diffusion coefficient would describe both types of cell. However, the additional gap junction from AII–bipolar cell produces an additional decline in staining in the bipolar cells.

The ramifications of this are most easily understood graphically (Fig. 2F). Changes in AII–AII permeability produce changes in the slope of AII staining as a function of distance from the site of injection. Changes in AII–ON cone bipolar cell permeability are seen as downward translations of the bipolar cell curves on the logarithmic ordinate, as the permeability of the single AII–bipolar cell gap junction comes into play.

Bipolar cells are coupled to more than one AII amacrine cell and can of course receive or return tracer from many AII amacrine cells. Tracer can also flow in the opposite direction (Trexler et al., 2001), but in general, the concentration gradient will favor flow into the bipolar cell. We have found that including only the closest AII amacrine cell in the analysis produces the best possible fit. This conclusion came from examination of more complicated models with a variety of architectures using scaled contributions of more distant AII amacrine cells to the bipolar cell populations. The simpler model was able to reproduce all the important features of the data with no apparent gain added by the more elaborate models; they were therefore discarded.

Accounting for the heterogeneity of ON cone bipolar cells

Not all bipolar cells are stained across the coupled group. More poorly coupled bipolar cell subtypes are not detectable far from the site of injection. Changes in the diffusion coefficients can change the density of coupled bipolar cells at any given distance. Therefore, the mean brightness of the heterogeneous mix of ON cone bipolar cells cannot be used as a reliable estimate of overall diffusion coefficient, nor are these types completely distinguishable by anatomical means. The following method was therefore used to establish a reliable measure of overall AII–ON cone bipolar cell coupling.

Well-stained groups of coupled AII–ON cone bipolar cells were examined using a large variety of diffusion times and pharmacological manipulations. In regions where ON cone bipolar cells were fully sampled (i.e. even the most poorly coupled bipolar cells were stained), diffusion rates of the brightest bipolar cells was always close to 25 times that of the dimmest bipolar cells. Therefore, a single average ON cone bipolar cell diffusion coefficient (k_{AB}) was chosen for all conditions. A diffusion coefficient that was five times this coefficient fits the most brightly stained bipolar cells. A diffusion coefficient that was 1/5 this coefficient fits the poorly stained bipolar cells in groups where all bipolar cell subtypes were stained. This is illustrated in Fig. 2C (blue lines), where the single fitted coefficient is adjusted to produce a range that fits the entire bipolar cell data set. In conditions where only 1–2 bipolar cell subtypes were stained, all of the stained bipolar cells clustered around the upper line. Usually, all of the bipolar cell subtypes would be stained near the injection site. As distance from the injection site was increased, fewer bipolar cell types could be measured and the lower portion of the range could not be sampled.

Cohen and Sterling (1990) found that one ON cone bipolar cell type in cat retina made gap junctions with another bipolar cell type, but none directly to AII amacrine cells. The range of

bipolar cell staining might be broadened by similar connections in rabbit retina. This type of indirect pathway, as yet undemonstrated, would not affect the analysis significantly.

Immunocytochemistry

Pieces of tissue with injected AII amacrine cells were subsequently processed for calcium binding protein—28 kDa (CaBP; calbindin) immunoreactivity. The monoclonal CaBP antibody (Sigma) was used at 1:500 and visualized with 1:200 donkey anti-mouse secondary, linked to Alexa488 (Molecular Probes). Anti-CaBP selectively labels a single type of ON cone bipolar cell in rabbit retina which is well coupled to AII amacrine cells (Massey & Mills, 1996, 1999).

Results

Neurobiotin injected into a single AII amacrine cell reliably stains coupled cells of two classes: neighboring AII amacrine cells and 4–5 types of ON cone bipolar cells (Vaney, 1991; Hampson et al., 1992; Mills & Massey, 1995; Bloomfield et al., 1997). Figs. 2A and 2B show Neurobiotin coupling between members of the AII amacrine cell mosaic (red somas), and from AII amacrine cells to ON cone bipolar cells (blue) in the presence or absence of exogenous dopamine. As in the prior studies, dopamine (Figs. 2B & 2D) reduced AII–AII coupling compared to the control condition (Figs. 2A & 2C), resulting in a sharply decreased radius of staining.

The intensity of some bipolar cells can exceed the brightness of neighboring AII amacrine cells close to the injection site. The bipolar cell intensities then rapidly decline until they are lower than and parallel to that of the AII population. Detailed figures showing this effect can be seen in Mills and Massey (1995). This temporary imbalance occurs because tracer diffuses more readily between AII amacrine cells than from the AII amacrine cell to bipolar cells, under most conditions. The concentration gradient shortly after iontophoresis fills both AII amacrine cells and ON cone bipolar cells rapidly. As time elapses, the AII amacrine cells continue to rapidly lose tracer laterally across the unbounded AII network. The lower permeability of the AII–ON cone bipolar cell gap junctions and the lower concentration gradient impede the return of tracer from bipolar cells to the AII amacrine cells, which may decline below the concentration of the bipolar cells at this point. This behavior is accurately captured by the model.

Dopamine affects the permeability of both junctional pathways

More detailed examination of the effects of dopamine requires quantitative comparison. We calculated diffusion coefficients separately for the AII–AII and AII–bipolar cell pathways as described in the Materials and methods. We then plotted these constants from experiments where exogenous dopamine concentration ranged from 0 to near saturation (1 μ M; see Hampson et al., 1992). Fig. 4A shows that the diffusion coefficients for both pathways are reduced by increasing concentrations of dopamine.

The diffusion coefficient for AII–AII coupling is clearly reduced more rapidly with increasing dopamine than the diffusion coefficient from AII amacrine cell to ON cone bipolar cells. However, dopamine significantly decreases even AII–ON cone bipolar cell coupling at all volume ratios (for volume ratio $VR_{B/A} = 1$; $t = 2.12$, $df = 47$, $P < 0.05$; other volume ratios were of very similar magnitude and significance). This differs from our prior study using brightness ratios (Mills & Massey, 1995), where no significant effect of dopamine on k_{AB} was found. Similar results were found using the hexagonal model (not shown), although the slope of both functions declined slightly.

Distinguishing changes in permeability of the two different channel types

Fig. 4B more closely examines the relative changes in coupling of the two gap junctional pathways produced by dopamine. Suppose that dopamine affected only AII–AII coupling. Clearly, variation in the diffusion coefficients produced by changes in dopamine concentration would then produce systematic variation along only the AII–AII diffusion coefficient axis (Figs. 1Bb & 1C). No systematic variation would occur parallel to the AII–bipolar cell axis. Similarly, modulation specific to bipolar cell hemichannels would produce a trend parallel to the AII–bipolar cell axis.

An intermediate trend would be predicted by the following simple model, illustrated in Figs. 1Ba & 1C). Suppose that AII amacrine cells produce a single type of connexin protein, which makes homotypic channels with neighboring AII amacrine cells, but heterotypic channels with a different connexin type in ON cone bipolar cells. If only the AII hemichannel were sensitive to cAMP, then the permeability changes produced by cAMP would be very different for the two different channel types. AII–AII channels should have a cAMP-sensitive gate on each of the two hemichannels. Therefore, a cAMP concentration that halved the open probability of a gate would reduce the open probability to $1/2 * 1/2$, that is, $1/4$. The AII/bipolar cell channel would contain only a single cAMP-sensitive gate, on the AII amacrine cell side; its open probability would be $1/2$ of the original value. More generally, the slope of the diffusion coefficients for the two channel types would predict that the AII–AII diffusion coefficient would be reduced as the square of the AII–ON cone bipolar cell diffusion coefficient over the effective range of dopamine concentration. This is exactly what occurs (Fig. 4B, circles/upper line). The slope of the decline in the AII–AII diffusion coefficient relative to the AII–ON cone bipolar cell diffusion coefficient (dark line) was close to 0.5 (mean = 0.37) on the double logarithmic plot, and was significantly different from 1.0 ($t = 9.65$, $df = 24$, $P < 0.001$; volume ratio $VR_{B/A} = 1$). Different volume ratios were parallel and similarly significant. When the hexagonal model was used, the slope of the decline in the AII–AII diffusion coefficient relative to the AII–ON cone bipolar cell diffusion coefficient (dark line) was less than 0.5 (0.25 for volume ratio $VR_{B/A} = 1$; other volume ratios were in the range 0.23–0.36) on the double logarithmic plot. The dotted lines in Fig. 4B show the least-squares fit to the data points fit by the hexagonal model. Again, results were quite similar, with the overall slopes declining slightly (dotted lines) and with changes in the volume ratio producing parallel shifts in the functions, for both linear and hexagonal models (not shown).

The analysis just described assumes that cAMP modulates channels by lowering their probability of being open, rather than changing pore size. There is currently no evidence for change in pore diameter with phosphorylating treatments, although shifts to substates with lower permeabilities can be found (Moreno et al., 1992; Kwak et al., 1995; Mitropoulou & Bruzzone, 2003). However, substate shifts are usually produced by large transjunctional voltages not found *in vivo*. A possible substate of the already low Cx36 conductance (~15 pS) may exist at <10 pS (Srinivas et al., 1999); whether it exists *in vivo* or could support tracer movement is unclear. Mills and Massey (2000) tested retinal neurons for changes in permeability to tracer following application of cAMP or octanol and found that reductions in permeability were consistent with decreases in open probability rather than shifts in permeability of individual channels.

The finding that dopamine altered AII–ON bipolar cell coupling as well as AII–AII coupling requires some modification of our prior model. Mills and Massey (1995) found no significant change in the brightnesses of ON cone bipolar cells relative to their neighboring AII amacrine cells. These results were based on relative brightnesses rather than absolute diffusion coefficients, as in the present study. Therefore, small changes in AII–ON cone bipolar cell coupling would go undetected if AII–AII coupling declined more. The AII–bipolar cell coupling data in Figs. 4A and 4B, by contrast, demonstrate that coupling in both pathways is

reduced by dopamine. The prior model was correct in suggesting that dopamine reduces AII–AII coupling more than AII–bipolar cell coupling, but the more sensitive measurements in this study demonstrate that a significant effect nevertheless also occurs in AII–bipolar cell coupling.

We next tested whether the lower sensitivity of the AII–ON cone bipolar cell gap junction was due to lack of a cAMP-sensitive gate on the ON cone bipolar cell hemichannel, or due to inability of the exogenous dopamine to elevate cAMP at this site. This was done by use of a membrane permeant, hydrolysis-resistant cAMP analog, Sp-8-CPT-cAMPs (4–25 μ M). Addition of this compound to the bath had a much different effect, in that the cAMP analog affected both diffusion coefficients equally. By contrast with the dopamine curve, the slope of the decline elicited in the AII–AII diffusion coefficients relative to the AII–ON cone bipolar cell diffusion coefficients did not differ significantly from 1.0 on the double logarithmic plot (linear model: mean = 0.89; $t = 0.95$, $df = 16$, $P > 0.1$; two-dimensional model: $t = 0.028$, $df = 16$, $P > 0.1$). These values are for a volume ratio $VR_{B/A}$ of 1.0. Other volume ratios (both models) were in the range $t = 0.10$ to 0.41 , all $P > 0.1$. This cAMP slope was, however, significantly different from the dopamine curve [for volume ratio $VR_{B/A} = 1$, $t = 3.55$, $df = 38$, $P < 0.01$ (linear model); $t = 5.50$, $df = 43$, $P < 0.01$ (two-dimensional model); other volume ratios were in the range $t = 3.12$ to 4.94 , all $P < 0.05$]. Therefore, the data support a model whereby dopamine modulates gating on only the AII side of the channel, but cAMP modulates gating in both hemichannels.

A nitric oxide donor has different effects

Mills and Massey (1996) and Xin and Bloomfield (1999) found that AII–ON cone bipolar cell coupling was reduced by a nitric oxide donor, SNAP, or by cyclic guanine monophosphate (cGMP) agonists, while AII–AII coupling was not significantly affected by these drugs. We wanted to confirm these findings with the quantified diffusion coefficient procedure and examine possible interactions between the two systems.

Fig. 4C shows that SNAP produced a much greater reduction in AII–bipolar cell coupling than in AII–AII coupling (linear model: AII–AII: $t = 1.72$, $P > 0.05$, $df = 32$; AII–bipolar: $t = 2.192$, $P < 0.05$, $df = 32$; two-dimensional model: AII–AII: $t = 1.75$, $P > 0.05$, $df = 32$; AII–bipolar: $t = 2.213$, $P < 0.05$, $df = 32$). When applied in conjunction with 500 nM dopamine, the results were similar (linear model: AII–AII: $t = -0.725$, $P > 0.05$, $df = 29$; AII–bipolar: $t = 2.517$, $P < 0.05$, $df = 29$; two-dimensional model: AII–AII: $t = 0.43$, $P > 0.05$, $df = 29$; AII–bipolar: $t = 2.02$, $P < 0.05$, $df = 29$). AII–bipolar cell coupling was significantly reduced, while AII–AII coupling increased (nonsignificantly) on average. The results confirm our prior results (Mills & Massey, 1995), demonstrating that nitric oxide produces changes in permeability of AII–bipolar cell gap junctions, but not the AII–AII gap junctions, and which implicate a site of action located on the bipolar cell side.

Calbindin immunostaining reveals that all bipolar cells are not modulated identically

Multiple types of ON cone bipolar cell are coupled to AII amacrine cells. We used immunostaining for CaBP to look for differential modulation by dopamine of coupling among the bipolar cell pathways. We showed previously that CaBP-positive ON cone bipolar cells were among the best coupled to AII amacrine cells (Massey & Mills, 1996, 1999). We examined the relative intensity of these cells with respect to the brightest group of CaBP-negative ON cone bipolar cells, tested over a variety of concentrations of dopamine. If dopamine-evoked increases in cAMP closed only the AII amacrine cell hemichannel, then there would be no mechanism whereby any single bipolar cell population should be affected more than other bipolar cell subtypes. Instead, the relative sensitivity of all the ON cone bipolar cells should be reduced equally by reduction in the permeability of the AII amacrine cell hemichannel. However, if some or all of the bipolar cells also contained cAMP-sensitive gates,

then they might be modulated more extensively, based upon the dopamine receptors located upon the bipolar cells themselves.

We labeled a subtype of rabbit ON cone bipolar cells with an antibody to CaBP. These cells could then be identified in the Neurobiotin-labeled material (Fig. 5A). It was apparent that dopamine caused CaBP-positive bipolar cells to decline in intensity. They moved from the group of most brightly stained bipolar cells to nearer the middle of bipolar cell brightnesses. We quantified this by measuring the relative brightness of CaBP-positive bipolar cells relative to the upper fitted line of the bipolar cell populations. This line is determined by the brightest of the CaBP-negative bipolar cell subclasses. Under control conditions (Fig. 5B), the CaBP-positive bipolar cells (gray squares) are equal in brightness to the brightest of the CaBP-negative bipolar cells (dark upward triangles). In contrast, high levels of dopamine (Fig. 5D) strongly reduced the staining of CaBP-positive bipolar cells relative to the brightest CaBP-negative bipolar cells.

Fig. 5C plots the log ratio of CaBP-positive bipolar cell staining to that of these other well-coupled bipolar cells. Clearly, the relative staining of CaBP-positive bipolar cells declined relative to the other group at high levels of dopamine/cAMP (250 nM dopamine: $t = 2.2$, $df = 15$, $P < 0.05$; 500–1000 nM dopamine: $t = 5.51$, $df = 16$, $P < 0.001$; cAMP analog: $t = 2.03$, $df = 9$, $P < 0.05$). The D₁ antagonist SCH23390 produced more Neurobiotin staining in CaBP-positive bipolar cells, relative to the next-best stained CaBP-negative bipolar cells, although this effect was not statistically significant.

Nitric oxide, as released by SNAP, had no significant effect on the relative staining intensities of the bipolar cell subtypes examined (Fig. 5C). SNAP (50 μ M) did not alter relative staining of CaBP-positive and CaBP-negative bipolar cells compared to control retina ($t = 0.78$, $P > 0.05$, $df = 21$), nor did addition of SNAP significantly reduce the effect of 500 nM dopamine on relative bipolar cell staining (Fig. 5C; $t = 0.517$, $P > 0.05$, $df = 15$).

Discussion

This paper describes the modulation of coupling in a complex *in vivo* gap junctional network in unprecedented detail. It demonstrates that coupled networks of dissimilar cell types display complexities in regulation of coupling that confer a great deal of plasticity in how the individual cells interact. New details are shown about how a critical and well-characterized retinal circuit alters coupling between its different neural elements to selectively favor different pathways in response to two endogenous neuromodulators, dopamine and nitric oxide. New findings are (1) both AII–AII and AII–ON cone bipolar cell gap junctions are closed by elevations in dopamine. (2) AII–bipolar cell gap junctions are less sensitive to dopamine than AII–AII gap junctions, in a manner consistent with restriction of the dopamine effect to the AII amacrine cell side of the AII–bipolar cell gap junctions (Figs. 1B & 1C). (3) Modulation of the two pathways is equal with a membrane-permeant cAMP analog, suggesting cAMP closes gates equally in both cell types. (4) Nitric oxide modulates only the AII–ON cone bipolar cell hemichannel and acts independently of dopamine.

Possible gating mechanisms

The most direct interpretation of our results is that the connexin in AII amacrine cells produces gap junctional hemichannels whose gates are closed by cAMP. The AII connexin is comprised of connexin36 (Cx36), as found previously by immunocytochemical staining and reporter expression techniques (Feigenspan et al., 2001; Mills et al., 2001; Deans et al., 2002). The gap junctions between pairs of AII amacrine cells are well known to be sensitive *in vivo* to cAMP elevation *via* dopamine receptors (Hampson et al., 1992; Mills & Massey, 1995). Analogs of cAMP have been shown to close hemichannels comprised of Cx35, an ortholog to Cx36

(Mitropoulou & Bruzzone, 2003), and also to reduce the Neurobiotin permeability of Cx35 channels expressed in a HeLa cell line (Winbow et al., 2001).

The close adherence of our data to the line of log slope 0.5 (Fig. 4B, bold line) strongly supports the idea that dopamine regulation occurs on only the AII amacrine cell side. It is therefore likely that dopamine-produced elevations of cAMP occur only within the AII amacrine cell. Cx36 is cation selective. AII amacrine cells do not pass the anionic tracer Lucifer Yellow, although both positive and negative Lucifer Yellow-coupling results have been reported for Cx36 in expression systems (Srinivas et al., 1999; Teubner et al., 2000). The negative charge of cAMP may restrict its movement across these gap junctions such that each cell's hemichannels are significantly affected by only the local concentration in that cell. The equal effectiveness of the membrane-permeant cAMP analog at modulating both diffusion coefficients supports the view that cAMP-sensitive gates are found on both sides of the AII–bipolar cell channel, but cAMP elevations normally occur on only the AII side. Whether this is due to a lack of D₁ receptors on ON cone bipolar cells, excluding possibly the CaBP-positive bipolar cell, or instead due to compartmentalization of signaling in microdomains near the channels is unclear.

How well does tracer coupling reflect functional coupling

The movement of Neurobiotin between cells has served as a good positive indicator for gap junctions, including those in the AII amacrine cell network (Vaney et al., 1998; Massey & Mills, 1999). Recent physiological recordings have confirmed that the gap junctions in the AII/ON cone bipolar cell network are required for normal ganglion cell function (Xin & Bloomfield, 1999; Deans et al., 2002). The link between *changes* in tracer coupling and electrical coupling is more tenuous, as permeability to ions and mid-sized molecules might be affected differently (Kwak et al., 1995; Harris, 2001). While exact correspondence to electrotonic coupling is uncertain, tracer studies are especially useful for modeling the dynamics and connectivity of a complex network, where paired recordings coupled with pharmacology would be difficult.

The advantage of using diffusion coefficients

Mills and Massey (1995) measured coupling from AII amacrine to ON cone bipolar cells by plotting the ratio of their brightnesses as a function of distance from the injected cell. They found that cGMP elevation reduced AII–cone bipolar coupling, but not AII–AII coupling, while cAMP elevation by dopamine closed AII–AII gap junctions, but produced a non-significant elevation of AII–cone bipolar coupling. These effects were robust, repeatable, and consistent with other results (Hampson et al. 1992; Bloomfield et al. 1997). The effect of dopamine on AII–ON cone bipolar cell coupling has not been tested elsewhere, however, and we now provide a clearer picture using independently determined diffusion coefficients.

It is easy to see why the independent measure of two diffusion coefficients used in this study is preferable. Inferences regarding movement of tracer from AII amacrine cells to bipolar cells were dependent on a single measure of relative brightness of the two cell types at a given location. Relative estimates of tracer concentration are not unique for all combinations of flow rate through the two pathways. Absolute tracer flow could decrease in both pathways, but the ratio of tracer concentration in bipolar cells relative to nearby AII amacrine cells could increase if the AII–AII diffusion coefficient decreased more rapidly than the AII–bipolar cell diffusion coefficient. The method used in this paper produces a more constrained solution by substituting measurement of flow rate through each type of channel for relative tracer content.

An additional advantage is gained by using diffusion coefficients. As already noted, the major source of variability between injections is the amount of tracer injected. Because this variation

lies in the iontophoretic rate term and not the diffusion coefficients, it is an efficient measure of tracer diffusion.

Robustness of the findings

Movement of molecules through gap junctions in an array of cells exhibits a stereotypic decline in concentration with distance from the injected cell (Mills & Massey, 1998). The rate of decline from the injected cell is closely related to the permeability of the individual gap junctions. Any model that can closely match this rate of decline will likely be able to compare changes in coupling fairly accurately. It is probable that various nonlinear models such as sums of exponentials would produce the same conclusions as this model. However, we modeled diffusion between interconnected compartments to combine biological plausibility with an ability to easily adapt the equations to compare the many different geometries of coupled networks in the retina.

We implemented our model of diffusion through a linear chain of cells as a simple approximation to the AII amacrine cell array for the following reasons: (1) Its simplicity makes it easily understandable. (2) It can be easily elaborated to include bipolar cells as a biologically reasonable set of independent sinks from the overall AII syncytium. (3) More complicated models can be developed from this skeleton to test the importance of various features, as will be described. We tested the robustness of our results by altering the model in three major ways.

First, because the volumes of the bipolar cell and AII amacrine cell compartments are different, the absolute values of the diffusion coefficients will be directly affected. We fit all of our data with volume ratios $VR_{B/A}$ of AII–bipolar cell compartments ranging from 0.2 to 5.0, a range which must almost certainly include the true value. For simplicity, we normalized the AII volume to 1.0 and expressed the total bipolar cell volume as a ratio relative to that. This relative volume is actually a function of not only the true volume, but also the gap junctional permeability. For example, a bipolar cell with very low gap junctional area with AII amacrine cells would have little effect on overall tracer distribution, even if its volume were large. Therefore, the volume ratio $VR_{B/A}$ is really an *effective* volume ratio, presumably dominated by the bipolar cells with the largest permeabilities.

The effect of changing AII–bipolar cell volume ratios produced changes in the absolute diffusion coefficient from AII amacrine cells to ON cone bipolar cells that were regular and predictable. When the average diffusion coefficients were plotted against the pharmacological treatments, however, the *slopes* of these functions was unchanged, within a small range of variation. In other words, the findings of this study with respect to dopamine and cAMP modulation are unaltered over a wide range of volume ratios.

Other tests of robustness dealt with altering the models of the AII–bipolar cell architecture. The first was to test if different results were obtained with a more elaborate two-dimensional representation of the model. The AII amacrine cell array was modeled as a rigid hexagonal array with independent bipolar cell sinks connecting to each individual AII, as noted in the Materials and methods section.

The quality of the fit for individual data sets differed little between the two types of model. In general, the free parameters could be adjusted to virtually overlay one model's prediction over the other, although the values were of course different. For this reason, the scatter of the data, much of which was produced by natural anisotropy in the cellular mosaics of rabbit retina, was too large to discriminate among the models. On the other hand, the regular decline in staining intensity across the coupled group of cells could be fit well by either model.

The absolute values of the diffusion coefficients were different when using the two-dimensional model, as when using volume ratios. In this case, the slopes of the average diffusion coefficients for each set of drug conditions also declined, although nonsignificantly. More importantly, the slopes of the diffusion coefficients between pairs of AII amacrine cell, k_{AA} , and the slopes of the diffusion coefficients from AII to ON cone bipolar cells, k_{AB} , were changed by the same amount and remained significantly different from one another. This again reinforces the conclusion that it is the differential gating by dopamine and cAMP that produces the differences between the two sets of coefficients, rather than an arbitrary property arising from the choice of models.

A final set of manipulations concerned specific different architectures of the AII-ON cone bipolar cell network. Direct gap junctional contact between bipolar cells can occur (Kolb, 1979) and that can support tracer coupling (Mills, 1999). If a significant direct coupling pathway existed between bipolar cells, the result would be that the bipolar cell populations would pool their tracer in addition to the AII amacrine cell influx. This would result in bipolar cell staining intensities that were shallower than predicted by the simple model. Specifically, the bipolar cell intensity curves would be predicted to have a shallower slope than the AII amacrine cell intensity curves, rather than remaining parallel to it over most of their range. This trend was never observed in the data, and addition of a bipolar cell coupling parameter to the equations reinforced this *a priori* notion. This additional pathway was therefore excluded from the model.

Dopamine differentially affects coupling to bipolar cell subtypes

We also found that dopamine reduced coupling from AII amacrine cells to CaBP-positive ON cone bipolar cells more than to other CaBP-negative ON cone bipolar cells. The CaBP-positive ON cone bipolar cell may be a rabbit homolog of a similarly stratified Cx36-positive bipolar cell found in mouse by Deans et al. (2002). These authors found Cx36 was produced by some bipolar cells, including one ramifying in the ON sublamina and another in the OFF sublamina.

CaBP-positive bipolar cells extensively contact ON α (Y)-type ganglion cells in the rabbit retina (Massey et al., 1996). This ganglion cell initiates the ON magnocellular pathway, which is responsible for the greatest sensitivity to luminance contrast. In cat, the ON α ganglion cell is driven almost exclusively by a single bipolar cell subtype (Freed et al., 1987; Freed, 2000). The CaBP-positive bipolar cell is also one of the best coupled bipolar cells to the AII amacrine cell (Massey & Mills, 1996, 1999). This agrees with the suggestion that the primate homolog of the rabbit ON α ganglion cell is a primary carrier of the rod signal in scotopic vision (Lee et al., 1997). The preferential decrease in coupling of the CaBP-positive ON cone bipolar cell to AII amacrine cells in response to dopamine increases suggests that it is maladaptive for this particular type of bipolar cell to be coupled to the AII amacrine cell at high levels of background. In daylight, it would be deleterious for the detection of fine spatial contrast to have spatial acuity degraded and have a bipolar cell's signal strength dissipated through lateral gap junctions. There are other bipolar cells which are extensively coupled to one another (Umino et al., 1994; Mills, 1999; Jacoby & Marshak, 2000). These presumably have functions requiring less spatial acuity.

If the CaBP-positive bipolar cell were to contain Cx36, and other ON cone bipolar cells contained another connexin, then some additional questions are raised. Coupling to all bipolar cell types would be reduced by dopamine due to increased cAMP on the AII amacrine cell side of the gap junction. However, since cGMP elevation does not modulate Cx36 in AII amacrine cells, then either (1) Cx36 hemichannels do not contain a cGMP-sensitive gate, in which case the CaBP-positive bipolar cell should be insensitive to cGMP, or (2) nitric oxide is ineffective in elevating cGMP in AII amacrine cells. Fig. 5C indicates that the CaBP-positive bipolar cell is affected about equally by the SNAP treatment. This would favor the possibility that Cx36

channels might also be sensitive to both second messengers, but regulation is dependent on local intracellular regulation.

Our findings indicate that cAMP can modulate cAMP-sensitive gates on both AII and bipolar cell hemichannels. Although dopamine diminishes coupling from AII amacrine cells to the CaBP-positive bipolar cell more than to other bipolar cells, a similar effect was also found with the cAMP agonist. There would be no reason to find a differential effect if all bipolar cells were equally sensitive to cAMP. We have no clear explanation of this result, and can only speculate that differences in cAMP sensitivity may be found across the bipolar cell population.

Are AII/ON cone bipolar cell gap junctions heterotypic?

This paper suggests that differential effects of cAMP and cGMP are likely due to different receptors or internal regulation, rather than structural differences. However, electron microscopy reveals an asymmetrical structure described as dense or fluffy appearing on the AII cell side of the bipolar cell–AII amacrine cell gap junction, but not in AII–AII gap junctions (Kolb, 1979; Strettoi et al., 1992). Cx36 immunoreactivity is found at AII–bipolar cell contacts (Feigenspan et al., 2001; Mills et al., 2001), but confocal resolution is insufficient to distinguish if puncta are symmetrically placed. At present, the most compelling evidence for heterotypic AII–cone bipolar cell connexins is the distinctly lower permeability to large tracers of AII–cone bipolar cell channels compared to AII–AII channels. These measurements were made under conditions where open probability and plaque size should not differ (Mills & Massey, 2000).

While the findings of Deans et al. (2002) strongly suggest that at least one of the bipolar cell subtypes in each sublamina contains Cx36, the data for the other bipolar cell subtypes remains equivocal. Our data (Mills, 1999; Mills et al., 2001; this study) agree that at least one ON and one OFF cone bipolar cell subtype could use Cx36. Recently, Cx45 has been suggested as an ON bipolar cell connexin in mouse retina (Kirsch et al., 2003). However, no connexin type has yet been identified that will form heterotypic channels with Cx36 *in vitro* and, notably, the conductance of Cx45 channels in transfected HeLa cells were not modulated by a cGMP analog (van Veen et al., 2000).

Functional consequences

Dopamine is released by light stimulation and produces many different effects in the retina (reviewed by Witkovsky & Deary, 1991). These effects, in general, promote the transition from rod to cone vision. If dopamine did not reduce signal transmission from AII amacrine cells to ON cone bipolar cells (Mills & Massey, 1995), then AII–ON cone bipolar cell coupling would reduce acuity and dilute the photopic signal. This paper, by measuring the AII–AII and AII–bipolar cell diffusion coefficients independently, establishes that dopamine does in fact reduce photopic flow from ON cone bipolar cells into AII amacrine cells when dopamine levels rise, as well as reduce the impact of the scotopic AII pathway as light level (and dopamine) increases.

Further reduction might occur with independent rises in cGMP in ON cone bipolar cells (Mills & Massey, 1995; Xin & Bloomfield, 1999), possibly arising from nearby nitric oxide generators. Using the new method of independently determining diffusion coefficients for AII–AII and AII–bipolar cell pathways, we verified our prior result that putative cGMP stimulation *via* incubation in the nitric oxide donor SNAP reduced AII–ON cone bipolar cell coupling without significant effect on AII–AII coupling. There is precedent in the retina for modulation of gap junctional channels and glutamate receptor sensitivity by both cAMP and cGMP (Lu & McMahon, 1997; Lu et al., 1998; McMahon & Schmidt, 1999), and we presume the bipolar cell hemichannel is sensitive to both neuromodulators. We cannot determine whether these

modulate the same or different gates or channels. Nevertheless, the complex response of AII amacrine cells to increasing levels of light and neuromodulators suggest that the retina has a great deal of plasticity in adapting its circuitry to match the signal-to-noise ratio of the environment, even at the level of gap junctional connectivity in subcircuits.

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References

- Bloomfield SA, Xin D, Osborne T. Light-induced modulation of coupling between AII amacrine cells in the rabbit retina. *Visual Neuroscience* 1997;14:565–576. [PubMed: 9194323]
- Chow CC, Kopell N. Dynamics of spiking neurons with electrical coupling. *Neural Computation* 2000;12:1643–1678. [PubMed: 10935921]
- Cohen E, Sterling P. Demonstration of cell types among cone bipolar neurons of cat retina. *Philosophical Transactions of the Royal Society B (London)* 1990;330:305–321.
- Condorelli DF, Parenti R, Spinella F, Trovato Salinaro A, Belluardo N, Cardile V, Cicirata F. Cloning of a new gap junction gene (Cx36) highly expressed in mammalian brain neurons. *European Journal of Neuroscience* 1998;10:1202–1208. [PubMed: 9753189]
- Deans MR, Volgyi B, Goodenough DA, Bloomfield SA, Paul DL. Connexin36 is essential for transmission of rod-mediated visual signals in the mammalian retina. *Neuron* 2002;36:703–712. [PubMed: 12441058]
- Famiglietti EV, Kolb H. A bistratified amacrine cell and synaptic circuitry in the inner plexiform layer of the retina. *Brain Research* 1975;84:293–300. [PubMed: 1111833]
- Feigenspan A, Teubner B, Willecke K, Weiler R. Expression of neuronal connexin36 in AII amacrine cells of the mammalian retina. *Journal of Neuroscience* 2001;21:230–239. [PubMed: 11150340]
- Freed MA. Rate of quantal excitation to a retinal ganglion cell evoked by sensory input. *Journal of Neurophysiology* 2000;83:2956–2966. [PubMed: 10805691]
- Freed MA, Smith RG, Sterling P. Rod bipolar array in the cat retina: Pattern of input from rods and GABA-accumulating amacrine cells. *Journal of Comparative Neurology* 1987;266:445–455. [PubMed: 3693619]
- Galarreta M, Hestrin S. Electrical synapses between GABA-releasing interneurons. *Nature Reviews: Neuroscience* 2001;2:425–433.
- Hampson ECGM, Vaney DI, Weiler R. Dopaminergic modulation of gap junction permeability between amacrine cells in mammalian retina. *Journal of Neuroscience* 1992;12:4911–4922. [PubMed: 1281499]
- Harris AL. Emerging issues of connexin channels: Biophysics fills the gap. *Quarterly Review of Biophysics* 2001;34:325–472.
- Jacoby RA, Marshak DW. Synaptic connections of DB3 diffuse bipolar cell axons in macaque retina. *Journal of Comparative Neurology* 2000;416:19–29. [PubMed: 10578100]
- Kirsch T, Schubert T, Janssen-Bienhold U, Soehl G, Maxeiner S, Willecke K, Weiler R. Cellular expression of Cx45 in the mouse retina. *Investigative Ophthalmology and Visual Science* 2003;44E-Abstract 4133
- Kolb H. The inner plexiform layer in the retina of the cat: Electron microscopic observations. *Journal of Neurocytology* 1979;8:295–329. [PubMed: 490185]
- Kwak BR, van Veen TAB, Analber LJS, Jongsma HJ. TPA increases conductance but decreases permeability in neonatal rat cardiomyocyte gap junction channels. *Experimental Cell Research* 1995;200:456–463. [PubMed: 7556455]
- Landisman CE, Long MA, Beierlein M, Deans MR, Paul DL, Connors BW. Electrical synapses in the thalamic reticular nucleus. *Journal of Neuroscience* 2002;22:1002–1009. [PubMed: 11826128]

- Lee BB, Smith VC, Pokorny J, Kremers J. Rod inputs to macaque ganglion cells. *Vision Research* 1997;37:2813–2828. [PubMed: 9415362]
- Lewis TJ, Rinzel J. Dynamics of spiking neurons connected by both inhibitory and electrical coupling. *Journal of Computational Neuroscience* 2003;14:283–309. [PubMed: 12766429]
- Loewenstein Y, Yarom Y, Sompolinsky H. The generation of oscillations in networks of electrically coupled cells. *Proceedings of the National Academy of Sciences of the USA* 2001;98:8095–8100. [PubMed: 11427705]
- Lu C, McMahon DG. Modulation of hybrid bass retinal gap junctional channel gating by nitric oxide. *Journal of Physiology (London)* 1997;499:689–699. [PubMed: 9130165]
- Lu C, Zhang DQ, McMahon DG. Differential modulation by SNP and dopamine of Vj-dependent and Vj-independent electrical synapses in retinal horizontal cells. *Society for Neuroscience Abstracts* 1998;24:135.
- Massey SC, Mills SL. A calbindin-immunoreactive bipolar cell type in the rabbit retina. *Journal of Comparative Neurology* 1996;366:15–33. [PubMed: 8866843]
- Massey SC, Mills SL. Gap junctions between AII amacrine cells and calbindin-positive bipolar cells in the rabbit retina. *Visual Neuroscience* 1999;16:181–189.
- Massey SC, Kittila CA, See TP. ON α ganglion cells receive potential input from calbindin cone bipolar cells in the rabbit retina. *Investigative Ophthalmology and Visual Science (Suppl)* 1996;37:S950.
- McMahon DG, Schmidt KF. Horizontal cell glutamate receptor modulation by NO: Mechanisms and functional implications for the first visual synapse. *Visual Neuroscience* 1999;16:425–433. [PubMed: 10349964]
- Mills SL. Unusual coupling properties of a cone bipolar cell in mammalian retina. *Visual Neuroscience* 1999;16:1029–1035. [PubMed: 10614585]
- Mills SL, Massey SC. Labeling and distribution of AII amacrine cells in the rabbit retina. *Journal of Comparative Neurology* 1991;304:491–501. [PubMed: 1850763]
- Mills SL, Massey SC. Differential properties of two gap junctional pathways made by AII amacrine cells. *Nature* 1995;377:734–737. [PubMed: 7477263]
- Mills SL, Massey SC. The kinetics of tracer movement through homologous retinal gap junctions. *Visual Neuroscience* 1998;15:765–777. [PubMed: 9682877]
- Mills SL, Massey SC. A series of biotinylated tracers distinguishes three types of gap junction in retina. *Journal of Neuroscience* 2000;20:8629–8636. [PubMed: 11069972]
- Mills SL, O'Brien JJ, Li W, O'Brien J, Massey SC. Rod pathways in the mammalian retina use connexin36. *Journal of Comparative Neurology* 2001;436:336–350. [PubMed: 11438934]
- Mitropoulou G, Bruzzone R. Modulation of perch connexin35 hemichannels by cyclic AMP requires a protein kinase A phosphorylation site. *Journal of Neuroscience Research* 2003;72:147–157. [PubMed: 12671989]
- Moreno AP, Fishman GI, Spray DC. Phosphorylation shifts unitary conductance and modifies voltage dependent kinetics of human connexin43 gap junction channels. *Biophysical Journal* 1992;62:51–53. [PubMed: 1376174]
- Smith RG, Freed MA, Sterling P. Microcircuitry of the dark-adapted cat retina: Functional architecture of the rod–cone network. *Journal of Neuroscience* 1986;6:3605–3617.
- Srinivas M, Rozental R, Kojima T, Dermietzel R, Mehler M, Condorelli DF, Kessler JA, Spray DC. Functional properties of channels formed by the neuronal gap junction protein connexin36. *Journal of Neuroscience* 1999;19:9848–9855. [PubMed: 10559394]
- Sterling P, Freed MA, Smith RG. Architecture of rod and cone circuits to the on-beta ganglion cell. *Journal of Neuroscience* 1988;8:623–642. [PubMed: 2828567]
- Strettoi E, Dacheux RF, Raviola E. Synaptic connections of the narrow-field, bistratified rod amacrine cell (AII) in the rabbit retina. *Journal of Comparative Neurology* 1992;325:152–168. [PubMed: 1460111]
- Teubner B, Degen J, Söhl G, Güldenagel M, Bukauskas FF, Trexler EB, Verselis VK, DE Zeeux CI, Lee CG, Kozak CA, Petrasch-Parwez E, Dermietzel R, Willecke K. Functional expression of the murine connexin36 gene coding for a neuron-specific gap junctional protein. *Journal of Membrane Biology* 2000;176:249–262. [PubMed: 10931976]

- Trexler EB, Li W, Mills SL, Massey SC. Coupling from AII amacrine cells to ON cone bipolar cells is bi-directional. *Journal of Comparative Neurology* 2001;437:408–422. [PubMed: 11503143]
- Umino O, Maehara M, Hidaka S, Kita S, Hashimoto Y. The network properties of bipolar-bipolar cell coupling in the retina of teleost fishes. *Visual Neuroscience* 1994;11:533–548. [PubMed: 8038127]
- Vaney DI. Many diverse types of retinal neurons show tracer coupling when injected with biocytin or Neurobiotin. *Journal of Neuroscience Letters* 1991;125:187–190.
- Vaney DI, Nelson JC, Pow DV. Neurotransmitter coupling through gap junctions in the retina. *Journal of Neuroscience* 1998;18:10594–10602. [PubMed: 9852595]
- van Veen TA, van Rijen HV, Jongsma HJ. Electrical conductance of mouse connexin45 gap junction channels is modulated by phosphorylation. *Cardiovascular Research* 2000;46:496–510. [PubMed: 10912460]
- Veruki ML, Hartveit E. AII (Rod) amacrine cells form a network of electrically coupled interneurons in the mammalian retina. *Neuron* 2002a;33:935–946. [PubMed: 11906699]
- Veruki ML, Hartveit E. Electrical synapses mediate signal transmission in the rod pathway of the Mammalian retina. *Journal of Neuroscience* 2002b;22:10558–10566. [PubMed: 12486148]
- Winbow V, O'Brien J, Mills SL. Mutations in retinal connexin35 in the intracellular loop or carboxyl terminus block or decrease direct phosphorylation by protein kinase A. *Investigative Ophthalmology and Visual Science (Suppl)* 2001;42:S193.
- Witkovsky P, Dearry A. Functional roles of dopamine in the vertebrate retina. *Progress in Retinal Research* 1991;11:247–292.
- Xin D, Bloomfield SA. Comparison of the responses of AII amacrine cells in the dark- and light-adapted rabbit retina. *Visual Neuroscience* 1999;16:653–665. [PubMed: 10431914]
- Zimmerman AL, Rose B. Permeability properties of cell-to-cell channels: Kinetics of fluorescent tracer diffusion through a cell junction. *Journal of Membrane Biology* 1985;84:269–283. [PubMed: 4032457]

Fig. 1.

(A) AII amacrine cells (AII) relay input from rod bipolar cells (RB) to cone bipolar cells, which then contact ganglion cells (GC). Each AII amacrine cell makes gap junctions both with neighboring AII amacrine cells and with a variety of ON cone bipolar cells (ON CB). (B) Three models of gap junctional gating in the AII–ON cone bipolar cell circuit. Gap junctions between neighboring AII amacrine cells are assumed to use a single connexin type and are closed by elevations in cAMP in each model. The models differ in their view of the AII–bipolar cell channels. In (Ba), the AII–bipolar cell channel consists of an AII hemichannel with a cAMP-sensitive gate identical to that between pairs of AIIs, while the bipolar cell hemichannel is insensitive to cAMP. In (Bb), both the AII and bipolar cell hemichannels are insensitive to

cAMP. In (Bc), both AII and bipolar cell hemichannels are sensitive to cAMP. Therefore, the models predict two cAMP-sensitive gates between pairs of AIIIs, and 0, 1, or 2 cAMP-sensitive gates between the AII and ON cone bipolar cells. For convenience, gates are depicted as directly modulated by local cAMP binding, but modulation is almost certainly indirect *via* protein kinases. Each hemichannel is drawn as if comprised of a single connexin type, although this relationship is neither proven nor obligatory for the models. (C) These models predict that the permeability changes in response to elevations in cAMP must be a function of the number of cAMP-sensitive gates in the AII–ON cone bipolar cell channels. The k_s are diffusion coefficients (cell separations²/s), plotted arbitrarily on these axes.

Fig. 2.

(A & B) Injection of Neurobiotin into an AII amacrine cell stains many neighboring AII amacrine cells (red), and also stains many ON cone bipolar cells (blue). Dopamine reduces AII–AII coupling, thereby producing smaller stained groups (B) than in retina not exposed to dopamine (A). (C & D) The fluorescent intensity of the somas stained following injection of Neurobiotin into an AII amacrine cell declines as a function of distance from the injected cell, but more rapidly for dopamine-treated retinas (D) than in control (C) retinas. The lines are the best fit of a model which uniquely determines diffusion coefficients between the AII–AII pairs (red points and line) and between AII's and the population of bipolar cells (blue triangles; blue lines predict the range). For visual clarity, only one-third of the data points, randomly selected,

are shown. (E) Tracer injected into an AII amacrine cell (left, with pipette) diffuses into distant AII cells through a series of AII–AII gap junctions. By contrast, tracer in a bipolar cell must have diffused through a series of AII–AII gap junctions, and also through a single AII–ON cone bipolar cell gap junction. (F) The effect of reducing gap junctional permeabilities in the two different pathways reflects the model in (E). Tracer concentration in AII amacrine cells (red line) and bipolar cells (whose range is shown by blue lines) is plotted as a function of distance from the injected cell. Reducing AII–AII permeability only (middle panel) increases the rate of decline for both AII and bipolar cells, as tracer must traverse the same number of AII–AII gap junctions for either cell type at any given distance. Bipolar cell staining is further reduced by the constraint of crossing the AII–bipolar cell gap junction, but remains the same fixed decrement as in the left panel. Reducing AII–bipolar cell permeability only (right panel) leaves the decline in AII amacrine cell brightness almost unchanged, but bipolar cell intensities decrease proportionately to the reduction in AII–bipolar cell coupling.

Fig. 3.

HeLa cells filled by diffusion of known concentrations of Neurobiotin from a patch pipette were imaged on a confocal microscope. (A) The photodetector intensity increased with increasing concentration of Neurobiotin in the pipette. The inset shows an example of a HeLa cell filled and imaged in this manner. (B) The brightness of HeLa cells at each Neurobiotin concentration was measured at different laser attenuation settings of the confocal microscope. The resulting function could be shifted to also fit the brightness values of HeLa cells with a different Neurobiotin concentration.

Fig. 4.

Exogenous dopamine lowered the diffusion coefficient (cell separations²/s) for both gap junctional pathways. (A) As dopamine concentration was increased, the diffusion coefficient from AII amacrine cells to bipolar cells (triangles) was reduced less than that between pairs of AII (circles). Values shown are mean + 1 S.E.M. To test the effect of volume differences between the compartments, diffusion coefficients were calculated with volume ratio $VR_{B/A}$ that ranged from 0.2 to 5.0. These regression lines are shown for k_{AB} for volume ratios of 0.2, 1.0, and 5.0, and the data for 1.0. Differing volumes do not significantly change the slope of the functions. The AII–AII diffusion coefficients changed only marginally because their relative volume was set to 1.0, and are therefore not shown. (B) The model of Fig. 1Ba predicts that

the diffusion coefficient from AII to ON cone bipolar cell (ordinate) will decline at half the diffusion coefficient found between pairs of AII (abscissa), consistent with cAMP action at two gates/channel for AII–AII coupling, as opposed to only one at channels from AII to bipolar cells. The dark solid line is the prediction of this model. The dark dashed line is the least-squares regression to the data and lies close to the model prediction. However, the membrane-permeant cAMP analog, Sp-8-CPT-cAMPS produced data (triangles) that is fit by the dashed gray regression line and the model prediction of slope 1 (solid gray line), as would be produced by an equal decline in both diffusion coefficients. This indicates equal cAMP sensitivity at each site and suggests that differential modulation is not based upon differences in the types of gates on the two types of channel. The dotted lines are the predictions of the hexagonal model. Although the slopes were less, the overall findings were quite similar. (C) The nitric oxide donor SNAP produced significant changes in AII–bipolar coupling, but not in AII–AII coupling, either when administered alone, or in combination with 500 nM dopamine.

Fig. 5.

(A) Some bipolar cells stained from injection of an AII amacrine cell are immunopositive for CaBP (light and dark asterisks). Other bipolar cells of similar intensity but CaBP-negative can be found throughout the field. This image was inverted in intensity. (B & D) In control experiments (B), CaBP-positive bipolar cells (light squares) are among the best-stained bipolar cells. There is no difference (solid line) between the upper envelope of CaBP-negative bipolar cells (dark upward triangles) and the CaBP-positive bipolar cells. (D) High levels of dopamine (250 nM—1 μM) reduce the brightness of CaBP-positive bipolar cells (squares) relative to other well-coupled ON cone bipolar cells (upward triangles). AII amacrine cells (circles) and other CaBP-negative bipolar cells (downward triangles) are shown for comparison. (C) The fluorescent intensity of CaBP-positive bipolar cells relative to the brightest CaBP-negative bipolar cells decreases in the presence of high dopamine or cAMP. In contrast, the D_1 antagonist SCH23390 slightly elevates CaBP-positive bipolar cell staining relative to other bipolar cells. The nitric oxide donor SNAP had no significant effect on the relative intensities of the bipolar cell subtypes, with or without dopamine. Data is the $\log_{10}$ of the ratio of the mean intensity of the CaBP-positive bipolar cells to the intensity of the brightest CaBP-negative bipolar cells.