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The kinetics of tracer movement through homologous gap junctions in the rabbit retina

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Abstract

Observation of the spread of biotinylated or fluorescent tracers following injection into a single cell has become one of the most common methods of demonstrating the presence of gap junctions. Nevertheless, many of the fundamental features of tracer movement through gap junctions are still poorly understood. These include the relative roles of diffusion and iontophoretic current, and under what conditions the size of the stained mosaic will increase, asymptote, or decline. Additionally, the effect of variations in amount of tracer introduced, as produced by variation in electrode resistance following cell penetration, is not obvious. To examine these questions, Neurobiotin was microinjected into the two types of horizontal cell of the rabbit retina and visualized with streptavidin-Cy3. Images were digitally captured using a confocal microscope. The spatial distribution of Neurobiotin across the patches of coupled cells was measured. Adequate fits to the data were obtained by fitting to a model with terms for diffusion and amount of tracer injected. Results indicated that passive diffusion is the major source of tracer movement through gap junctions, whereas iontophoretic current played no role over the range tested. Fluorescent visualization, although slightly less sensitive than peroxidase reactions, produced staining intensities with a more useful dynamic range. The rate constants for movement of Neurobiotin between A-type horizontal cells was about ten times greater than that for B-type horizontal cells. Although direct extrapolation to ion conductances cannot be assumed, tracer movement can be used to give an estimate of relative coupling rates across cell types, retinal location, or modulation conditions in intact tissue.

Keywords

Neurobiotin; Lucifer Yellow; Gap junction; Connexin; Retina; Tracer coupling

Introduction

Since its introduction less than a decade ago, Neurobiotin has rapidly become one of the favored tools for inferring the presence of gap junctions. The reasons are easily seen. Electron microscopy, the only certain method, is laborious and time-consuming. If junctional coupling is not the primary focus of the reconstruction, minute gap junctions can easily go undetected.

Other methodologies using patch-clamp recording in isolated cells or expression systems have led to an enormous growth in understanding of fundamental properties of gap-junctional connexins (Bennett et al., 1991). Nevertheless, many cell types cannot be isolated or coupled *in vitro*, and network properties cannot be investigated without use of intact tissue. These constraints often dictate that tracer coupling is the only practical method of assaying junctional connectivity.

The biotinylated tracers Neurobiotin and biocytin, on the other hand, are easily introduced into cells in intact tissue. Spread of tracer to neighboring cells of the same or different types suggests a patent pathway, usually believed to be through gap junctions. Following its introduction into the retina, Neurobiotin coupling was found where none had been seen previously with fluorescent tracers such as Lucifer Yellow (Vaney, 1991) and led to confirmation at the ultrastructural level of gap junctions where none had been reported previously. For this reason, Neurobiotin is the tracer of choice for delimiting gap junctions. The superior passage of Neurobiotin relative to Lucifer Yellow has often been attributed to its smaller size (286 Da), but is more likely to be due to cationic selectivity of the channels (Veenstra et al., 1995), as biotin-X cadaverine (442 Da; +1) will easily pass gap junctions impermeable to Lucifer Yellow (443 Da; -2) (Mills & Massey, 1995a).

Neurobiotin coupling is not without its limitations, however. Tracer coupling is often taken as evidence of electrical coupling, but evidence that many of these tracer-coupled cells are also functionally electrically coupled is lacking. Although a variety of ganglion cells show tracer coupling (Vaney, 1991; Dacey & Brace, 1992; Xin & Bloomfield, 1997), ganglion cell receptive fields closely match the dendritic field of the injected cell (Yang & Masland, 1994; Xin & Bloomfield, 1997). This is despite the fact that Neurobiotin labeling can sometimes be seen in neighboring ganglion cells (Vaney, 1991; Xin & Bloomfield, 1997). Questions have persisted about whether many of the Neurobiotin-demonstrated gap junctions in the retina are functional *in situ*, or whether they are some artifact of iontophoresis.

The extent of spread of Neurobiotin throughout a coupled network of cells should in theory tell something about the degree to which those cells are coupled. Networks containing large and numerous open gap junctions should clearly show more spread than networks with more limited junctions. Quantification has been difficult, however. The size of the stained patches often asymptotes some time after introduction of Neurobiotin (Hampson et al., 1992; Bloomfield et al., 1995). The relative roles of diffusion and iontophoretic current have not been thoroughly tested nor is the effect of amount of injected tracer on patch size understood. Although the literature is rich in examples of estimating coupling parameters from spread of fluorescent dye in various vertebrate and invertebrate tissues (Weidman, 1966; Safranyos & Caveney, 1985; Zimmerman & Rose, 1985; Brink & Dewey, 1978, 1980; Imanaga, 1989; Jaslove & Brink, 1989; Rae et al., 1996), quantification of Neurobiotin coupling has thus far relied solely on counting the number of stained somas (e.g. Hampson et al., 1992, 1994; Bloomfield et al., 1995). Beyond this, there have thus far been no quantitative dye studies in neural tissue, nor quantitative studies of permeability to cationic tracers. Since most connexons have a selectivity for cations over anions (Veenstra et al., 1995), and the positively charged Neurobiotin is the most popular tracer today, a study is needed that examines the roles of diffusion, iontophoretic current and the amount of tracer injected on Neurobiotin spread through neural gap junctions.

To examine the manner in which Neurobiotin moves through coupled networks of neurons of the same type ("homologous" coupling), Neurobiotin coupling was studied in the two horizontal cell networks of the rabbit retina. While both A-type and B-type horizontal cells show tracer coupling with biotinylated tracers (Vaney, 1991; Mills & Massey, 1994), only the A-type shows coupling when injected with Lucifer Yellow (Dacheux & Raviola, 1982; Mills & Massey, 1994). The goals of the experiments described were to establish the relative roles of diffusion and iontophoretic current in movement of Neurobiotin and to establish a basis for quantification of relative coupling rates for nonfluorescent tracers such as Neurobiotin. Establishing a suitable quantitative measure would enable comparison of relative coupling rates across gap junctional networks and across conditions that alter the permeabilities in a single network. Comparison of the rate constants among the two types of horizontal cell is a logical place to begin, as their receptive-field sizes and space constants have already been

measured in the rabbit retina (Dacheux & Raviola, 1982; Bloomfield & Miller, 1982; Bloomfield et al., 1995).

Methods

The primary methods used have been described previously (Mills & Massey, 1992), but will be recapitulated briefly.

Retinal preparation

Eyes were removed from New Zealand White rabbits deeply anesthetized with urethane. After removal of the vitreous and pigment epithelium, the isolated retina was placed on Whatman 40 filter paper with the photoreceptor side exposed. To label horizontal cells, the retina was incubated for 12–15 min at 37°C in oxygenated Ames medium (Ames & Nesbett, 1981) containing 15 μ M DAPI. After rinsing in Ames medium, the retina was placed vitreal-side up on filter paper. It was subsequently placed in a chamber on the microscope stage and continuously perfused with oxygenated Ames medium.

Visual identification of somas

A Zeiss microscope modified for fixed stage and fitted with epi-fluorescence was used to examine pieces of tissue for DAPI labeling. A filter set with a bandpass of 400–440 nm for excitation and which passed wavelengths above 470 nm for viewing was used for viewing both DAPI and Lucifer Yellow fluorescence.

Dye injection

Cells were impaled under visual control using a long working distance objective (Nikon, SLWD, 40 $\times$). Micropipettes were pulled on a Brown-Flaming horizontal micropipette puller (Sutter P-80), tip-filled with 4% NeurobiotinTM (Vector Labs, Burlingame, CA) and 1% Lucifer Yellow-CH (Molecular Probes, Eugene, OR) in 50 mM Tris and backfilled with 3 M lithium chloride. When the cell of interest was contacted by the electrode tip, penetration was forced and Lucifer Yellow ejected long enough to determine if a quality penetration was made. Neurobiotin was then injected intracellularly with 3 Hz positive current (1 nA; 4 min except as noted). Tissue with filled cells was removed from the chamber and fixed in 4% paraformaldehyde for 1 h.

Visualization

The biotinylated tracer Neurobiotin (Kita & Armstrong, 1991) was visualized by reacting it overnight with streptavidin-indocarbocyanine (Cy3) (Jackson ImmunoResearch, West Grove, PA; dilution 1:200) in 0.1 M phosphate buffer containing 0.5% Triton X-100. Some tissue was incubated overnight in 1:100 streptavidin-HRP in the same buffer. HRP was visualized with 0.003% hydrogen peroxide in the presence of 0.05% DAB.

Quantification

Coupling rate was determined by comparing the rate of spread of dye from the injected cell to its coupled neighbors. Fluorescent images were acquired using a Sony CCD camera (model AVC-D7) or a confocal microscope (Zeiss LSM 410). For the CCD camera, the autogain capability was switched off and images obtained with different neutral density filters placed in the light path. A lookup function of pixel intensity *versus* relative brightness was obtained from these images and applied to data images. Images of horizontal cells were acquired from the confocal microscope (20 $\times$ objective; 8- μ m optical section). Because the range of intensities routinely exceeded the 8-bit coding capacity of the acquisition system, images were captured using two or more intensity ranges. The intensity of each cell could then be matched with a

standard measured under identical conditions. These standards were nitrocellulose blots or gelatin slices containing known concentrations of Neurobiotin and visualized with streptavidin-Cy3. In practice, intensities were measured by the program SigmaScan using a lookup table of intensities, along with distance from the site of injection calibrated against a standard image.

The primary basis of comparison was obtained by plotting staining intensity of somas as a function of distance from the injected somas, which we call the “spatial profile.” Random factors such as quality of penetration and electrode tip characteristics can produce large variations in relative brightness. Data obtained from similar experimental conditions described similar curves if they were normalized to the total amount of injected dye. This was estimated by cumulating the amount of staining intensity in all cells in the patch and hereafter referred to as bolus size. To average curves from different injections, cell distances were placed into bins of 50 μm width. The mean and standard error of intensities within these distance bins were then computed.

Modeling

Movement of tracer by diffusion through gap junctions generates time-intensity profiles in connected cells that are related to the factors governing tracer movement, namely, the junctional resistance, cytoplasmic diffusion rates, and internal binding and sequestration. Fig. 1A shows a series of theoretical curves modeled after Zimmerman and Rose (1985). The uppermost curve represents the fluorescent intensity of the injected cell as a function of elapsed time. Each succeeding curve represents the intensities of coupled cells progressively more distant from the injected cell. A brief injection of a bolus of fluorescent tracer into the first cell will produce a single intensely fluorescent cell, with no fluorescence in the neighboring cells. As time elapses, dye moves into adjoining cells at a rate proportional to the coupling rate constant and distance from the injected cell. The injected cell and its near neighbors peak rapidly, then decline in intensity due to loss of tracer to more distant cells, which accumulate tracer slowly. Cells beyond some distance from the site of injection will never become detectable, due to limited amount of dye and a threshold for detection. For the purposes of this figure, junctional flux is assumed to be much smaller than cytoplasmic diffusion rates and is strongly rate-limiting. Loss due to membrane permeability and compartmental sequestration (Zimmerman & Rose, 1985) did not improve fits and were therefore not further considered.

With a nonfluorescent biotinylated tracer such as Neurobiotin, changes in staining intensity of individual cells cannot be sampled across multiple time intervals. Only the relative intensities of the cells at a single time interval, that elapsed between injection and fixation, is measurable following visualization of the tracer. A sample case is illustrated by the vertical lines in Fig. 1A, where the relative amounts of tracer in adjoining cells are indicated at 10 and 30 min following injection. Fig. 1B reproduces these two intensity profiles along the spatial axis by plotting the intensity of each curve in Fig. 1A as it intersects the two vertical lines. The 10- and 30-min time samples produce quite different profiles of intensity of staining *versus* distance from the site of injection. In this simple diffusion model, increasing the amount of time for tracer to diffusion clearly broadens the spatial profile, so that less tracer is evident in the region nearest the injection site and more has moved to the distant cells. Given the diffusion time, a unique combination of injected amount of tracer and coupling rate will fit each profile. Changes in the amount of tracer injected produce multiplicative changes in the intensity profiles, resulting in parallel translations along a log-intensity axis. Changes in coupling rate k_j produce changes in the rate of decline of the function.

Results

Two estimates of coupling

The most common method of modeling tracer flux is to estimate the effective diffusion coefficient D_e (Weidman, 1966; Safranyos & Caveney, 1985; Ramanan & Brink, 1990; Rae et al., 1996). In complex networks, estimation is dependent on adaptation of the complex equations to the specific geometry of the system to be studied. This is sometimes unknown. For this reason, we have used the method of Zimmerman and Rose (1985) to estimate coupling rate k_j between cell pairs. In horizontal cell networks, where coupling is between cells of a single type, both methods were used for the sake of comparison.

Estimating coupling rate k_j

In cases where a bolus of dye was injected, usually over a 4-min interval, coupling rate was estimated by fitting the decline in fluorescent intensity in somas as distance from the injected cell increased. Following Zimmerman and Rose (1985), a series of differential equations relating tracer flux to a coupling rate constant k_j and the difference in concentration C between coupled cells were fitted to the data [eqn. (1)]. The fitting procedure was performed in MatLab (The MathWorks, Inc., Natick, MA) using second- and third-order Runge-Kutta ordinary differential equations to iteratively solve for the coupling rate constant, k_j . In practice, this proved to be a two-parameter system, with the amount of dye injected and k_j determining the height of the initial curve and the rate of dropoff, respectively. Empirical data consisted of series of points declining in intensity with distance from the injected cell. The best-fitting curve to each set of points was produced by adding tracer to the initial cell for a duration corresponding to the length of iontophoresis and no further tracer thereafter. The diffusion equations were applied over the entire duration. The amount of tracer that fit the curve when added over the iontophoretic interval was called the delivery rate, in moles/s. This rate times the duration of iontophoresis was the modeled total amount of tracer, termed the bolus size.

$$\begin{aligned}\frac{dC_1}{dt} &= k_j(C_2 - C_1) \\ \frac{dC_2}{dt} &= k_j(C_1 + C_3 - 2C_2), \text{ etc.}\end{aligned}\tag{1}$$

One of the primary goals was to compare coupling rates in different types of homologous networks. It was also desirable to determine how coupling rate changes across the retina for a given cell type. Because most retinal cells show location-dependent changes in size, dendritic-field area, and sometimes amount of overlap, a coupling rate based on a pure distance measurement would impede meaningful comparison. If junctional diffusion is strongly rate-limiting compared to cytoplasmic diffusion, then tracer movement is more directly proportional to the number of gap junctions that must be crossed, rather than the absolute distance. For this reason, horizontal cells were injected at only a few representative eccentricities and the density of the injected patch was recorded. This number was used to correct for changes in radial diffusion that reflected changes in size and density of cells rather than changes in junctional diffusion rate. In the modeling procedure, the empirically determined displacements were divided by the average between-cell spacing to produce a rate constant k_j that reflected cell²/s rather than a more traditional cm²/s.

Estimating the effective diffusion coefficient D_e

Estimates of D_e were obtained as the second method, so as to establish a basis for comparison between Neurobiotin data, obtained most easily *via* the Zimmerman and Rose (1985) method, and more traditional D_e measurements, obtained by sampling over both space and time. To facilitate the comparison, both D_e and k_j were estimated in Lucifer Yellow-coupled cells. A

direct method of estimating coupling was used following Safranyos and Caveney (1985). These researchers filled cells continuously, rather than injecting a single bolus. When the intensity of cells is plotted against log time, t , elapsed since initiation of injection, a family of parallel sigmoidal curves is produced, displaced according to distance from site of injection, r . Fig. 1C illustrates this for a sample injection of Lucifer Yellow (4 nA, 15 min) into an A-type horizontal cell. Iontophoresis was terminated after 15 min. To show the effect of termination of iontophoresis, dye spread was measured for 10 additional minutes. The fluorescent intensities rapidly began to decline below the fitted lines. There is evidence from these curves that the full iontophoretic intensity began to decline after about 13 min, as the curves began to fall below the fitted slopes at this time. This suggests at least partial blockage of the electrode has occurred, a phenomenon commonly observed in these and other physiological experiments when monitoring the bridge balance. In all the quantitative experiments reported here, care was taken to exclude observations where electrode resistance increased more than marginally.

Note also that the most distant cells only began to measurably increase in fluorescent intensity after iontophoresis ceased, as there is a time lag between activity at the electrode and coupled cells. Nevertheless, after the initial time lag, the rate of increase in fluorescent intensity matches that of the more directly coupled cells.

When data from continuous injections are instead plotted against $\log(4t/r^2)$, the linear portion of the curves (filled symbols) all lie along a single template, as seen in Fig. 1D (see also Fig. 4C from Safranyos & Caveney, 1985). The effective diffusion coefficient, D_e , can be calculated from the slope m and y-intercept b of the linear portion of this curve [eqns. (2–5)].

$$m = \frac{S}{4\pi D_e h} \quad (2)$$

$$b = m[\ln(D_e) - \gamma] \quad (3)$$

$$D_e = e^{[(b/m) - \gamma]} \quad (4)$$

Because m is estimated graphically, no estimate is needed of the tracer delivery rate S or h , the thickness of the two-dimensional sheet of processes through which the tracer is diffusing. γ is Euler's constant (0.5772). The advantage of this method, besides yielding direct estimates rather than iteratively determined free parameters, is that the superposition of the family of curves onto a single template overcomes the primary disadvantage of the biotinylated tracer approach. This is that visualization of biotinylated tracers can only occur following fixation and tissue processing, so that only a single time interval of diffusion is available. Most dye kinetic studies have used fluorescent tracers, whereby intensity values are recorded over a complete set of both time and displacement. The graphical method allows data points from different time-displacement curves to still determine the same basic template that provides the estimate of D_e . The effectiveness of this method is shown by the gray circles in Fig. 1D, which resulted from a 15-min continuous injection (4 nA) of Neurobiotin. Despite the fact that the points represent cells from a range of over 800 μm and only 1 time interval, the method again yielded a straight line, except near the injection site, where some saturation of the function occurs. Comparison of the D_e of Lucifer Yellow and Neurobiotin from these normalized curves suggests that Neurobiotin diffuses through the A-type horizontal cell network at 5.7 times the rate of Lucifer Yellow. The Lucifer Yellow value ($4.5 \times 10^{-7} \text{ cm}^2/\text{s}$) is quite similar to that found by Safranyos and Caveney (1985) in the epidermal junction of the larval beetle. However, this value is not likely to be generally applicable across all gap junctions, as binding and sequestration of Lucifer Yellow will play greater roles in circuits where movement across the gap junctions does not occur so quickly.

The effective diffusion coefficient, D_e , is not the same as the coupling rate constant, k_j , however. D_e is the apparent diffusion of a molecule from its site of injection and is determined not only by its permeability through gap junctions, but also its mobility through the cytoplasm. This relationship is given by $1/D_e = 1/D_c + 1/D_j$, where D_c is the cytoplasmic mobility and D_j is the rate of diffusion through the gap junctions, determined by junctional permeability to that tracer and the distance between successive gap junctions (Weidmann, 1966, with appendix by A.L. Hodgkin; Imanaga, 1989; Ramanan & Brink, 1990). D_e is influenced by a number of factors, including loss, binding, and sequestration of tracer, as well as the tortuosity and viscosity factors arising from the composition of the cytoplasm and organelles. If tracer mobility through the gap junctions is strongly rate-limiting compared to cytoplasmic diffusion, i.e. $D_j \ll D_c$, then D_e will be directly proportional to the junctional permeability to a good approximation. D_j , like D_e , is measured in cm^2/s , and is equal to the cell permeability P_j (cm/s) multiplied by constants for the average distance between gap junctions (cm) and the dimensionless ratio of their area to that of the cell cross-sectional area. Despite the fact that the methods of estimating k_j and D_e are not formally equivalent, the correlation coefficient for D_e with k_j in continuous dye injection experiments was 0.96, calculated over a large number of different types of cell, biotinylated tracers, and pharmacological conditions. This lends credence to the idea that D_j is the strongly rate-limiting step to the flow of Neurobiotin, as measured by D_e and that k_j is an effective measurement of coupling.

Horizontal cell staining patterns

For each horizontal cell type, the spread of dye was qualitatively similar to that previously reported in rabbit retina (Vaney, 1991, 1993, 1994; Hampson et al., 1994; Mills & Massey, 1994; Bloomfield et al., 1995). Fig. 2 shows examples of Neurobiotin coupling for A-type horizontal cells (A) and B-type horizontal cells (B). Note that the gradient of intensity is qualitatively steeper for B-type horizontal cells (B) than for A-type horizontal cells (A) (cf. Figs. 4A and 4B), implying a lower coupling rate for Neurobiotin between B-type horizontal cells.

Comparison of fluorescent and peroxidase staining

To compare relative staining profiles of the fluorescent and peroxidase detection systems, A-type horizontal cells were injected along the midline of the inferior retina. Following fixation, the retina was cut with a razor along this axis. One piece was visualized with Cy3, the other with HRP/DAB, following the procedures outlined in the Methods section. Four coupled patches bisected approximately equally were analyzed further. Relative intensity of HRP staining was determined by calibrating intensity values against known changes in intensity, using neutral density filters. An edge artifact existed whereby tissue within $40\ \mu\text{m}$ of an edge was stained above background. Consequently, coupled somas in this region were discarded. Cy3 calibrations were as previously described. There was no edge artifact for Cy3 material beyond the very border itself. Fig. 3 shows a comparison of Cy3 and HRP average staining intensities for the four coupled patches. Following averaging, each of the mean curves was normalized to 1.0.

Several important points are suggested by Fig. 3. The amount of Neurobiotin should be nearly equal in all cells the same distance from the site of injection, regardless of the method of visualization. Fig. 3 clearly shows that Cy3 has a much larger dynamic range than the HRP/DAB visualization technique, in the sense that small increments in Neurobiotin concentration can be accurately related to measurable changes in fluorescent intensity. Cells stained with HRP/DAB tend to be better stained with respect to background, but saturate quickly to a very small dynamic range over most of the coupled patch. Relative detection efficiency was in general somewhat superior for the HRP/DAB method, resulting in overall larger patches. However, some of the Cy3 curves also had somas detectable out to $1500\ \mu\text{m}$, so that both

methods seem equally sensitive in this average curve. The important point, however, is that the extended and linear dynamic range of Cy3 enables effective coding of intensity and Neurobiotin concentration. Due to the enzyme-based saturation of the peroxidase reaction, these studies could not have been done with HRP.

The role of diffusion in spread of Neurobiotin

The first hypothesis to be tested was whether diffusion plays a major role in spread of tracer, or whether tracer ceases movement after termination of iontophoresis. Fig. 4A shows the spatial profiles for three A-type horizontal cells injected for 4 min, then allowed to perfuse for various amounts of time following tracer injection. The progressive broadening of the spatial profile with increased diffusion time clearly indicates that spread of dye continues for durations at least as long as those investigated. The fitted lines are calculated from the differential equations [eqn. (1)] relating concentration differences to diffusion time and coupling rate. A similar family of curves is shown for three B-type horizontal cells in Fig. 4B. The data points for 60-min diffusion time lie mostly below those for the 45-min diffusion time. This is because relatively less dye was injected into the initial cell, as often happens within the variability of iontophoresis. Nevertheless, this curve is relatively broader than the 45-min curve, in that proportionately more dye is in the distant cells than in the 45-min curve.

Fig. 4C shows average coupling rate constants, k_j , determined for each average diffusion time and horizontal cell type. The k_j s do not change with changes in diffusion time, indicating that relative flux across the gap junctions is unchanged over the whole of the duration of diffusion time. As seen in this figure, A-type horizontal cells typically are coupled at least 10 times as well as B-type horizontal cells. The 5-min time interval in A-type horizontal cells was nonsignificantly higher than the other points. This elevation could possibly be due to increased coupling rate during the period of iontophoresis. It could also be due to continued movement of Neurobiotin for some brief time after transfer to fixative. Any such added diffusion time could be a significant increment for the 5-min time interval, but negligible for longer intervals. We favor the latter hypothesis, as increasing the iontophoretic intensity did not increase the coupling rate.

The gap junctions of A-type horizontal cells are unique in the mammalian retina in passing detectable amounts of Lucifer Yellow, save for a recent brief report of an amacrine cell in cat retina (Vaney, 1996). The two types of horizontal cell probably have different connexin types, since A-, but not B-type horizontal cells will pass Lucifer Yellow (Dacheux & Raviola, 1982; Mills & Massey, 1994), and they also display different relative permeabilities to larger cationic tracers (Mills & Massey, 1995b). It might therefore be argued that diffusion dominates tracer movement only where there are large, charge-insensitive gap junctions, but perhaps not in other cell types that utilize other gap-junctional proteins with different properties. However, entirely analogous results were obtained for the less well-coupled and Lucifer Yellow-impermeant gap junctions of B-type horizontal cells, suggesting diffusion governs Neurobiotin movement in each case.

The role of iontophoretic current in spread of Neurobiotin

To test whether the iontophoretic current also plays a role in spread of dye, A-type horizontal cells were filled with Neurobiotin with nominal iontophoretic current values of 1 or 10 nA for 15 min total injection time. Following iontophoresis, each piece of tissue was immediately transferred to fixative. After streptavidin-Cy3 visualization, the intensity profiles were compared (Fig. 5A). Although the higher level of current naturally led to more intense staining, the profiles were essentially identical when normalized by the intensity of staining. This indicates no greater relative spread of Neurobiotin with the higher iontophoretic value. The

same value for k_j (solid line) fit both conditions equally well, although the modeled delivery rates were naturally higher for the 10-nA group (cf. Fig. 5D).

Figs. 5B–5D further compares the effects of diffusion and iontophoretic intensity on tracer movement. A-type horizontal cells were filled with Neurobiotin with nominal iontophoretic current values of +0.25, 1, 4, and 16 nA. To approximately equalize the amount of dye injected, injection times were 16, 4, 1, or 0.25 min, respectively, for a constant product of 4 nA-min. Additional diffusion time beyond the length of iontophoresis was 0, 12, 15, and 15.75 min, for a total diffusion time of 16 min from the beginning of iontophoresis. Fig. 5B shows that the spatial intensity profiles for these conditions are nonetheless similar. This indicates the 16-min diffusion time rather than the magnitude of iontophoretic current is the main determinant of patch size and staining profile. Despite the identical diffusion times and roughly equal amount of dye injected, the diffusion model still predicts less staining at the periphery for the longest iontophoretic durations, as the latter part of these injections do not have time to diffuse over the full area. The two data sets using long iontophoretic durations (circles and diamonds) do in fact have less staining in the most distant cells. To further contrast iontophoresis and diffusion as mechanisms of movement, some cells injected for 15 s at 16 nA were transferred to fixative immediately after iontophoresis, rather than diffusing for the full 16 min. These are shown as filled inverted triangles, together with the predicted curve from the model. The rapid decline strongly contrasts with the other broader curves and reinforces the notion that more time for diffusion is needed to reach the patch size seen with 16 min total diffusion. Fig. 5C shows the coupling rate constant k_j , averaged by separately fitting k_j for each cell. The average k_j did not increase significantly over the 64× factor of nominal current investigated. Next, a delivery rate parameter was estimated as the amount of tracer injected over the duration of iontophoresis that, together with k_j , fit the curve. By contrast with k_j , the delivery rate of dye rose consistently with increased iontophoretic current (Fig. 5D). Hence, the magnitude of the iontophoretic current influences the delivery rate, as it should, but has no significant effect on transjunctional rate constants.

Relative coupling rates in horizontal cell networks

Fig. 6 compares the Neurobiotin-coupling rates between the two horizontal cell types at different retinal eccentricities. Each cell was filled with Neurobiotin for 4 min; diffusion time was variable. Rates were determined by fitting the experimental data, varying the free parameters k_j and delivery rate, and entering the total diffusion time as a fixed parameter. Coupling is strongest between A-type horizontal cells, with $k_j = 0.0026$ and varies little across the retina. As is well known from receptive-field measurements (Dacheux & Raviola, 1982; Bloomfield & Miller, 1982; Bloomfield et al., 1995), rabbit B-type horizontal cells are coupled about a log unit less well than their A-type counterparts. There is about a twofold variation in the amount of coupling between pairs of B-type horizontal cells across the retina, with the most coupling in the superior retina, but the least in the inferior retina. This is perhaps a bit surprising, as those in the inferior retina have the highest coverage factor (Vaney, 1993; Mills & Massey, 1994), hence the most opportunity for overlap.

Counting stained somas to estimate relative coupling

While increasing the amount of tracer introduced into a cell will increase the overall intensity of the stained patch of cells, its effect on the size of the patch, as determined by number of stained somas, is less consistent. The reason for this is that the major determinant of patch size is the time allowed for the dye to spread. Were there no threshold for detection, all patches allowed to diffuse for the same amount of time would be of equal size, though different intensities. As dye diffuses, however, the cells at the periphery of the field will take some time to accumulate sufficient dye to be detectable. Cells beyond the perimeter of the patch will accumulate and lose dye without ever crossing the threshold of detection. Because of this, patch

size will eventually stabilize at some asymptote and even eventually decrease in size. Examination of the relative intensities of the visible somas indicates that diffusion is continuing, but with a steady decrement of intensity in the central region compared to more distant cells. This relationship is made more explicit by applying the differential equations used to predict k_j to model systems. Fig. 7 shows how varying coupling rate and threshold of detection can dramatically alter the form of the functions relating patch size to diffusion time.

These simulations indicate that many different functions relating number of coupled cells to diffusion time can be found with variations in k_j and detection efficiency. This may partially explain why the data of Bloomfield et al. (1995) (their Fig. 2B) asymptote after only 15 min of iontophoresis, with no more coupling observable with added diffusion time. These investigators not only used HRP with DAB visualization, which had a greater detection efficiency than Cy3 in our hands, but also intensified staining with cobalt, which further increases detection efficiency (Adams, 1981). The function relating number of stained somas to diffusion time is therefore dependent on factors that influence k_j , including choice of tracer, cell type, and adaptive state, and on method of visualization. Functions ranging from linear to asymptotic and declining can be expected, based on these parameters. These factors cannot explain the findings in Fig. 4A of Bloomfield et al. (1995) that continual iontophoresis up until time of fixation is ineffective after about 8 min. In our model, continual addition of tracer must label progressively further cells equally effectively. It is only after tracer ceases to be added by termination of iontophoresis or electrode blockage that an asymptote or decline can be seen. For example, in Fig. 1D, where Lucifer Yellow is continuously added to an A-type horizontal cell, a departure from the steady increase in fluorescence occurs after 13 min, due to an apparent increase in electrode resistance.

Discussion

This study had three major results. The first was to establish a framework for measuring coupling rates with nonfluorescent tracers. The second was to determine the mechanism of tracer movement in homologous neural networks connected by gap junctions. The last purpose was to establish a basis for comparison of relative coupling across cell types and pharmacological conditions. Movement of tracer in the absence of iontophoretic current was conclusively demonstrated, and the contribution of current to the movement of tracer was proven marginal over the parametric range employed. Finally, relative coupling rates for Neurobiotin for the two rabbit horizontal cell networks were measured. It is less clear to what degree relative tracer coupling reflects relative electrical coupling (Kwak et al., 1995; Veenstra et al., 1995) and to what degree different tracer coupling rates reflect differences in number of gap junctions *versus* type of connexin. Additionally, the major role played by some junctions may be metabolic, rather than electrical, so that movement of mid-sized tracers may be most meaningful. The cyclic nucleotide second messengers bear a net negative charge in solution, and most gap junctions are selective for cations (Veenstra et al., 1995). Nevertheless, Brink (1996) calculated that typical second messengers should have considerable flux across gap junctions consisting of known connexins.

Estimation of the coupling rate parameter k_j has several advantages. First, the relationship between number of stained somas and injection parameters such as iontophoretic current level and diffusion time is controversial and largely untested (but cf. Bloomfield et al., 1995). At best, comparison of relative coupling rates between cell types is qualitative. Two parameters, the relative coupling rate k_j , and delivery rate were adequate to fit all of the spatial intensity curves produced by our injections. The delivery rate parameter had the virtue of compensating for the large variation in amount of injected tracer that occurs even in ostensibly identical protocols, due to variations in electrode characteristics and quality of penetration. The coupling parameter k_j can be used as a pure measure of rate of tracer movement, subject to the constraints

of unequal cell volume. A further advantage of quantifying tracer spread by this means is that the rate constant k_j is implicit in all pairs of neighboring cells. This means that the same coupling information can be gained from examining any pair of cells across the entire mosaic of coupled cells, so that examining the spatial profile as a whole provides a robust and highly redundant estimate of k_j .

Possible mechanisms of tracer movement

Diffusion—Passive diffusion adequately accounts for all movement of tracer across gap junctions in our study. Tracer continues to move long after iontophoresis is stopped. There is no evidence that diffusion ceases until the time of fixation. Other experimenters have found that the size of cell patches often reaches a plateau after some fixed period of diffusion (Hampson et al., 1992) or iontophoretic time (Bloomfield et al., 1995). Patch size cannot increase without bounds due to the finite supply of dye and the existence of a threshold for detection. Nevertheless, the spatial profile of any patch continuously broadens with time elapsed since injection, so that the relative intensity of somas in the center of a patch declines relative to more peripheral somas. Further support for this interpretation comes from observations we have made that an A-type horizontal cell perfused for 4 h postinjection produced a patch in which only the injected soma was visible. After photochromic intensification (Vaney, 1992), however, the threshold of detection was dramatically lowered and coupled somas could be seen to extend for a few millimeters.

Iontophoresis—The ability of tracer to move *via* passive diffusion does not by itself preclude the possibility that iontophoresis also aids in dye spread. It is of more than theoretical interest whether or not iontophoretic current aids in spread of tracer. Because Neurobiotin and Lucifer Yellow are membrane-impermeant, it is generally assumed that tracer spread reflects only changes in junctional resistance, not membrane resistance. As pointed out by Kamermans (1989) and Vaney (personal communication), if tracer movement is significantly affected by applied current, the value of membrane resistance will affect not only spread of electrical current, but less directly, movement of tracer also.

Bloomfield et al. (1995) speculated that spread of Neurobiotin through gap junctions might be driven by the iontophoretic current, rather than by passive diffusion. They found an enlarged radius of coupling with injection time up to 8 min, but no increase with further iontophoresis or diffusion time. In many other tissues, by contrast, movement of negatively charged tracers appears to occur *via* passive diffusion through the gap junctions (Brink & Dewey, 1978; Brink & Ramanan, 1985; Zimmerman & Rose, 1985; Imanaga, 1989). This approach is limited in neural systems because their smaller unitary conductances seldom pass fluorescent tracers. Safranyos and Caveney (1985) tested the hypothesis that dye movement through gap junctions is driven by the iontophoretic current, but found that carboxyfluorescein coupling rates were not significantly affected by intensity of iontophoresis, from 0.5 nA to 10 nA. Further, Saltarelli and Ball (1995) found that use of pressure injection rather than current was sufficient to move Neurobiotin through gap junctions in fish retina.

Similarly to Safranyos and Caveney (1985), we found no clear influence of current over the range tested in this study. This applies only to the *rate* of movement, which is proportional to concentration gradient. Staining intensity is the product of current and injection time, and increases with increased iontophoretic current as a straightforward result of a higher rate of delivery (Fig. 5D).

Nevertheless, it is not entirely clear in what form possible effects of current magnitude would take. The current, which clearly drives tracer out of the electrode, might aid in driving tracer some distance from the electrode. This distance could theoretically span several gap junctions. Higher current would presumably drive tracer further than a lower current, but since a 10-nA

current theoretically injects 10 times the amount of tracer into the cell than 1 nA, 10 times the charge would be required to move the total amount of tracer over the same distance. This could then still result in qualitatively similar spatial profiles.

Alternatively, the applied current could open relatively more gap junctions, although steady-state conductance is typically at a minimum at a transjunctional potential of 0. This would require that k_j increase with increased iontophoretic intensity. We have obtained no evidence for this, as the same k_j fits both 1-nA and 10-nA curves in Fig. 5A. These two curves did show an increase in k_j (to 0.0035) over those in Figs. 4C and 5C. Since the 1-nA and 10-nA curves were identical in shape, the increase in k_j could not have been due to the increase in current magnitude *per se*. It could presumably be due to the added length of iontophoresis (15 min) compared to the more usual period of 4 min, followed by added diffusion time. We regard this unlikely. In all other experiments, coupling rates were found to be about 0.0025 over a variety of conditions varying magnitude and duration of iontophoresis and length of diffusion time. In Fig. 5C, for example, where iontophoretic duration and intensity was co-varied, there was no effect on k_j . The 1-nA and 10-nA, 15 min iontophoresis data of Fig. 5A were taken from only a few retinas at a later time, in an experiment specifically designed to compare spatial profiles under these two limited conditions with a minimum of variation due to other sources. It is possible that these few retinas were relatively better coupled than the average over the course of the entire study. Fig. 5B emphasizes that a large current (16 nA) applied for a brief interval (15 s) has dramatically different profile than other combinations of current magnitude and diffusion time.

It is difficult to design an experiment to directly study the effect of iontophoretic intensity while holding all other potentially influential parameters constant. If one ejects two levels of current for the same duration (Fig. 5A), the total amount of tracer will vary. If one varies the duration of iontophoresis to hold total tracer amount constant (Figs. 5B–5D), the total amount of charge delivered will be a constant, so that perhaps the most important parameter for the current-driven hypothesis is not varied. Nevertheless, we have shown that under a variety of conditions, the magnitude of iontophoretic current does not influence the relative distribution of dye (Fig. 5A) or the overall coupling rate (Figs. 5A and 5C), but only the delivery rate (Fig. 5D). The relative distribution of tracer across the coupled patch is accounted for by the total diffusion time, with no obvious contribution from magnitude of iontophoresis.

The relationship between tracer coupling and electrical coupling

It is tempting to try to estimate electrical coupling from tracer coupling data, but the task is formidable. If gap junctions are of a single connexin type and of unitary conductance, a linear relationship between electrical coupling and tracer coupling (Verselis et al., 1986; Bloomfield et al., 1995) may exist. Even with these constraints, electrical coupling is also a function of membrane resistance, which may be practically infinite for tracers. It is doubtful that a given linear relationship would be unchanged by parameters that affect both coupling and membrane resistance, such as neuromodulators. On the other hand, given the constraints noted, a decline in coupling rate as estimated by tracer coupling could predict corresponding decreases in electrical coupling without concerns about changes in membrane resistance. In this case, tracer coupling could give a clearer view of junctional changes than electrical coupling.

Unfortunately, the burden of proof for this scenario is steep. Gap-junctional plaques may contain more than a single connexin type (Sosinsky, 1995). Examination of connexons in expression systems usually shows the presence of more than a single conductance state, which may be differentially favored by intracellular messengers (Moreno et al., 1994; Kwak et al., 1995). Finally, the relationship between conductance and permeability to tracers is not straightforward across connexin types (Veenstra et al., 1995).

Comparing permeabilities across different cell types

We have shown tracer coupling patterns resulting from injection into individual somas of two homologous neural networks. Since physiological characterization of coupling is forbidding, it is useful to obtain some estimate of relative coupling rates in an effort to understand the functional considerations. The results here establish a framework for comparing degrees of coupling across different cell networks and give an initial estimate of relative coupling rates.

With only a single tracer, differences in connexin type cannot be distinguished. Total permeability rates (G_j) represent the product of mean open time, total number of connexons, and individual connexon permeability (g_j). When two cells contain the same connexin type, a lower k_j implies fewer connexons, as g_j is unchanged, and the ratio of G_j s directly reflects the relative decline in number of open connexons. Without information about connexin type, individual connexon permeabilities and average open time of the connexons are confounded across cell types and cannot be separated.

To what extent do relative Neurobiotin-coupling rates in different cell types mirror relative permeabilities and conductances to other molecules? Extrapolation from Neurobiotin coupling is particularly difficult for ions and other small molecules that have significant membrane leakage or if the channels display multiple conductance states, some of which are impermeant to Neurobiotin. Bloomfield et al. (1995) found the space constants of rabbit A- and B-type horizontal cells to be 642 and 159 μm (ratio = 4.1). The relative coupling rates of A- to B-type horizontal cells to Neurobiotin across the retina ranged from 13 in superior retina to 25 in inferior retina. Near the visual streak, the ratio was about 19. The space constant λ is given by

$$\lambda = \sqrt{\frac{R_m}{R_s}}$$

so

$$\frac{\lambda_A}{\lambda_B} = \sqrt{\frac{R_{mA}/R_{sA}}{R_{mB}/R_{sB}}}$$

where R_m is the membrane sheet resistance and R_s the junctional resistance. If $R_{mA} \cong R_{mB}$ and junctional permeabilities are $G = 1/R_s$, then

$$\frac{\lambda_A}{\lambda_B} \cong \sqrt{\frac{G_A}{G_B}}$$

A space constant ratio of 4 (Bloomfield et al., 1995) would thereby predict a total conductance ratio of about 16 near the visual streak, as compared with our value of 19.

Cell geometries and tracer movement

For A-type horizontal cells, tracer moves exclusively between cells of the same type. B-type horizontal cells are more complicated, with homologous coupling between somas, a long axon serving as a high-impedance (but probably transport-facilitated) sink for tracer, and finally homologous coupling between the extensive arbors of the axon terminals. Despite these differences in cell geometry and coupling rate, we have found that a simple diffusion model adequately accounts for movement of positively charged tracer in both systems. This model can be extended to the homologous coupling of AII amacrine cells, with adequate provision for the loss of tracer to heterologously coupled ON cone bipolar cells. A subsequent paper will extend the analysis to coupled systems consisting of these and other unlike cell types. We

predict that other homologous systems of coupled cells, such as cone pedicles and some other amacrine cells can also be described by this approach. The exact function of coupling in these different homologous coupled networks may differ, however, based on the magnitude of the coupling and other parameters that affect current flow in the different types of cell.

Coupling between homologous cell types and its effect on receptive fields has served as the model for understanding the function of gap junctions. Examination of other roles will be aided by understanding the relative magnitude of coupling efficiencies and the permeability to mid-sized molecules. For the present, we conjecture that movement of tracer and similarly-sized bioactive molecules in homologically coupled neural systems should occur similarly for similar combinations of time and distance. Prediction of movement of small ions must wait for determination of the exact connexin characteristics.

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Fig. 1.

Quantification of tracer spread across coupled cell networks. (A) Following brief injection of a bolus of tracer into a cell, fluorescence of the initial cell declines (upper curve), while its successively more distant neighbors (succeeding curves) slowly gain in fluorescence as dye moves through gap junctions. The rate of transfer between cells is proportional to the coupling rate constant k_j . The dashed and dotted lines mark the relative fluorescence of groups of cells at 10- and 30-min postinjection. (B) The relative staining intensity of the group of cells marked by the dashed and dotted lines in (A). Increased diffusion time leads to broadening of the curve. The rate of decline is defined by reciprocity between diffusion time and coupling rate. (C) Continuous injection of Lucifer Yellow (4 nA; 15 min) produces a family of parallel curves when plotted against logarithmic time. The rate of growth is similar over different periods of time, as the straight lines of fixed slope demonstrate. See (D) for symbol key. (D) Replotting each curve against $4 * \text{time} / r^2$, where r is distance from site of injection, leads to superposition of the linear portion of these curves. Estimation of coupling rate from superposition of such curves. Lucifer Yellow was injected for 15 min into an A-type horizontal cell. The fluorescent intensity of all visible horizontal cells was plotted at 1-min intervals. For each time interval,

cells a given distance from the site of injection were averaged into 50- μm bins and this mean intensity plotted *versus* $4 * \text{time}/\text{distance}^2$. After some initial latency, during which cells only slowly accumulate fluorescence (unfilled symbols), the fluorescent intensity of each cell (filled symbols) falls on a line whose slope and intercept determine the apparent diffusion coefficient, D_e . Points after cessation of iontophoresis are omitted from the plot. Data from a 15-min Neurobiotin injection (gray circles) demonstrate that cells with different displacements, but the same diffusion time, nevertheless plot linearly.

Fig. 2. Neurobiotin coupling in the two horizontal cell mosaics. (A) A-type horizontal cells. (B) B-type horizontal cells. Each micrograph is a stack of 1.0- μ m optical sections from a confocal microscope.

Fig. 3. Relative staining intensity across patches of A-type horizontal cells stained with Neurobiotin. Each patch was hemisected, with the halves visualized with HRP/DAB or Cy3. The HRP/DAB technique produced curves with strong saturation and small dynamic range, compared to Cy3.

Fig. 4.

Neurobiotin diffuses at a constant rate in horizontal cells. The spatial staining profiles broaden with increased diffusion time, both in A-type horizontal cell (A) and B-type horizontal cell (B). The 45-min condition for the A-type horizontal cell combines two data sets. All other sets are from a single injection whose coupling rate was near the mean for that condition. (C) The estimated coupling rate constant k_j is constant across diffusion time, for both horizontal cell types. Error bars are ± 1 S.E.M.

Fig. 5.

Effects of changes in the magnitude of the iontophoretic current. (A) The spatial intensity profile for injections of 4% Neurobiotin for 15 min at 1 ($n = 7$) or 10 nA ($n = 11$). No differential effect of the iontophoretic current on relative distribution of tracer can be seen. The solid line is the best fitting prediction of the diffusion model. (B–D) A-type horizontal cells were filled with Neurobiotin at nominal currents of 0.25 nA for 16 min, 1 nA for 4 min, 4 nA for 1 min, or 16 nA for 15 s. Total perfusion time was always 16 min, except for 16 nA FI, which was fixed immediately (FI) following the 15-s iontophoresis. (B) All data except FI follow approximately the same spatial profile. The solid curve is the prediction of the diffusion model for the FI data. (C) The coupling rate constant k_j was not increased by magnitude of iontophoretic current. (D) The rate of delivery of tracer was increased by increasing current.

Fig. 6.

Relative rate constants of the two horizontal cell types at different retinal eccentricities. The rate constant, k_j , represents the total coupling between neighboring cells and reflects individual channel permeabilities, number, size, and density of gap-junctional plaques, and average open time of the individual channels. There is little difference of retinal location on the average coupling of A-type horizontal cells. B-type horizontal cells are significantly better coupled in the superior periphery. Error bars are ± 1 S.E.M. Number of observations, from left to right, are 6, 18, 5, 15, 5, and 8.

Fig. 7.

Changes in number of stained somas as a function of diffusion time, coupling rate, and detection threshold. (A) Varying coupling rate over a factor of only 10 changed the function relating number of cells stained above detection threshold to diffusion time from rapidly asymptotic, then declining to nearly linear functions of widely differing slope. (B) Varying threshold of detection over a factor of 100 changes the number of stained somas from nearly linear (best detection) to quickly declining (poorest detection). A moving average was applied to all functions to remove a stairstep appearance resulting from adoption of a rigid hexagonal mosaic in the cell geometry.