Adhesive Recognition and Retinotectal Specificity  
(chick/selective adhesion/neuronal specificity)

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ABSTRACT An assay has been developed to test the hypothesis that neurons from a limited area of the retina will adhere preferentially to that part of the optic tectum near their normal synaptic termini. The method measures the adherence of 32P-labeled cell bodies from either the dorsal or ventral half of the neural retina of chick embryos to dorsal and ventral tectal halves. When a labeled, single-cell suspension is prepared from dorsal half-retina, more cells bind to the ventral half of the tectum. When the labeled cells are from ventral half-retina, more bind to dorsal half-tecta. This preferential adhesion mimics the retinotectal projection found in vivo. Dorsal retinal cells show this preference shortly after dissociation with crude trypsin, and maintain it for at least 9 hr. Ventral retinal cells, however, require incubation in nutrient medium after trypsinization in order to display this selectivity. Comparable results are obtained when the cell suspension is prepared from pigmented retina. The data support an interpretation of neuronal specificity dependent on cell-surface interactions and demonstrate a clear correlation between selective adhesion and biological function.

Development of the nervous system is characterized by ordered patterns of specific interconnections between classes of neurons. An elegant example of this specificity is the formation of the topographically ordered projection of the neural retina onto the optic tectum. This projection occurs by way of the optic axons which extend from their cell bodies in the retina to the tectum. Once on the tectum, they migrate to their appropriate positions and form synapses with the superficial layers of tectal cells.

In pigeons, the connections between neurons of the retina and those of the optic tectum have been studied using electrophysiological techniques by Hamdi and Whitteridge (1). They observed a continuous, spatial representation of the retina on the surface of the tectum. Axons from the nasal region of the retina innervated the posterior region of the tectum, while axons from the temporal region of the retina innervated the anterior region of the tectum. In a similar fashion, the dorsal retina innervated the ventral tectum and the ventral retina innervated the dorsal tectum. This retinotectal projection was verified histologically in pigeons (2), and in chicks (3), and is consistent with the projection observed in other vertebrates (4).

The way in which these connections are made is not known, although several models have been suggested (5-7). One widely accepted hypothesis, due primarily to Sperry (5), is that neuron surfaces possess qualitatively distinct chemical groups. These specific surface components would allow the optic axons to recognize and adhere to their proper termination points in the tectum. If specific adhesion is involved, then increased adhesion might be detectable between retinal and tectal cells from physiologically connected areas compared to unconnected areas. Although adhesive differences at this level have not previously been reported, Garber and Moscona (8) demonstrated that cells dissociated from various parts of chick brain formed aggregates with differing characteristics. They also showed (9) that a cell-free "factor" from mouse cerebrum cultures stimulated the aggregation of both mouse and chick cerebral cells, but did not stimulate aggregation of other brain cells.

Our approach to retinotectal specificity is an outgrowth of the studies of Roth and Weston (10). They developed an assay that could demonstrate differences in adhesions between cells from different tissue types. They circulated aggregates of different types in suspensions of labeled cells. The numbers of labeled cells that adhered to the various aggregate types were determined radioautographically and compared. In further work (11), embryonic cells were labeled with [32P]-phosphate, and the collecting aggregates were counted in a scintillation counter. This assay was modified further for the present study. Here a 32P-labeled cell suspension made from either the dorsal or the ventral region of the retina was placed in petri dishes containing both dorsal and ventral tectal halves. After incubation for a short time, the tectal halves were washed and individually counted in a scintillation counter.

The results show a preferential adhesiveness of cells from the dorsal region of the retina to the ventral half of the tectum and of cells from the ventral region of the retina to the dorsal half of the tectum.

MATERIALS AND METHODS

Media. Nutrient medium was prepared aseptically and contained the following components (v/v): Dulbecco's Modified Eagle's Medium (GIBCO), 90%; calf serum (GIBCO), 10%; penicillin G (Parke-Davis), 100 units/ml. The medium was equilibrated with 5% CO2 in air to a pH of 7.2-7.4. Labeling medium consisted of 3 ml of nutrient medium containing 0.5 mCi of carrier-free 32P (International Chemical & Nuclear Corp.). Dissociating medium contained Difco 1:250 trypsin, 0.1% (w/v) in Ca++, Mg++, glucose-free Hanks' Balanced Salt Solution.

Preparation of Labeled Neural Retina Suspension. Dorsal or ventral halves of neural retina were dissected aseptically from both eyes of 7- to 12-day [stage 31-38 (12)] White Leghorn chick embryos. Four to eight intact, dorsal or ventral neural retina halves were placed in 25-ml Delong flasks containing 3 ml of labeling medium for 2-4 hr at 37°C. After they were labeled, the half-retinas were rinsed in Hanks' Balanced...
Salt Solution. Dissociation was begun by incubation of the tissue in 3 ml of dissociating medium. After 25 min, this was decanted and 4 ml of nutrient medium was added. Gentle pipetting through a small-bore Pasteur pipette resulted in a suspension of single cells. The suspension was centrifuged at 170 × g for 8 min, and the pellet was suspended in nutrient medium.

Preincubation. An important parameter in this study is the time between trypsinization and the exposure of the tecta to the retinal suspension. A series of 25-ml Delong flasks, each containing 3 ml of the cell suspension, was kept at 37°. At given intervals, cells were removed from one flask, centrifuged at 170 × g for 8 min, and suspended in fresh nutrient medium.

Preparation of Tectal Halves. Intact optic tecta were dissected aseptically from 12- to 14-day (stage 38-40) chick embryos immediately before use. The outer membranes including the pia mater were removed and the tecta were split into dorsal and ventral halves, as defined by the electrophysiological mapping of Hamdi and Whitteridge (1). Dorsal tectal halves can be definitively distinguished from ventral tectal halves by the geometries of their internal surfaces. Therefore, it was possible in every experiment to treat all halves identically and in the same containers until they were ready for scintillation counting.

Incubation. An equal number of dorsal and ventral tectal halves were placed in a 35-mm plastic Falcon tissue-culture dish with their exterior surfaces facing up. Incubation was begun by addition of 4 to 6 × 10⁶ labeled cells from either dorsal or ventral half-retinas suspended in 4 ml of nutrient medium to the culture dish. The dish was incubated at 37° for 30 min unless specified otherwise.

At the end of the incubation, the tectal halves were washed, identified as either dorsal or ventral, and counted individually in a Nuclear Chicago Liquid Scintillation System.

Experiments with Tecta Uninnervated by Retinal Axons. Control experiments were performed with tecta that had never been innervated by optic nerve fibers. Windows were prepared in eggs at day 3½-4 (stage 21-23). Both eyes of each embryo were destroyed with a pair of fine needles. The eggs were then sealed with cellophane tape and replaced in the incubator. At stage 38-39, these tecta were used in the usual experimental protocol.

Preparation of Pigmented Retina Suspension. Other experiments used labeled cell suspensions prepared from pigmented retina. Dorsal or ventral halves of pigmented retina along with the adhering choroid coat were dissected from stage-34 embryos and labeled. A 30-min exposure to dissociating medium separated the choroid coat from the pigmented cells. A further 20 min in dissociating medium, followed by gentle pipetting, resulted in a cell suspension of small clumps of labeled pigmented cells. The experiments were then continued in the usual manner.

Preparation of Other Nonretinal Suspensions. The appropriate tissue was dissected from stage-35 animals and labeled and dissociated as described for neural retina.

Treatment of Data. Three to six dorsal tectal halves and an equal number of ventral tectal halves were used in each experiment. Data will be reported as the mean number of cells adhering per tectal half ± the standard error of the mean. Number of tecta will be given in parentheses. Some of the data will be further reduced and presented as a ratio of: [cells adherent to the physiologically “matching” tectal half] /[cells adherent to the “nonmatching” tectal half]. For instance, when the cell suspension is made from ventral retina, the ratio will be: (cells adherent to dorsal half-tecta)/(cells adherent to ventral half-tecta). Therefore, ratios significantly greater than 1.0 indicate specific adhesion that correlates with retinotectal mapping. Various parameters affect the absolute number of cells that adhere to the tectal halves, but seem not to affect the ratios. These parameters include cell concentration, time of incubation, and rigor of the washing procedure, and are constant only within each experiment. Therefore, although ratios from different experiments are directly comparable, the absolute number of cells may not be.
Student's $t$-test was used to calculate the significance levels between the numbers of cells collected by dorsal and ventral tectal halves.

**RESULTS**

**Retinal Cell Adhesion to Tectal Halves.** Fig. 1 shows the ratio of cells adhering to ventral and dorsal tectal halves as a function of preincubation time of the retinal cells after trypsinization. Each curve represents a dorsal half-retina suspension that was divided into three or four parts and preincubated for the designated time. For each time point, a portion of the preincubated cells was centrifuged, the supernatant was discarded, and fresh nutrient medium was added to the cells. This suspension was incubated with the tectal halves for 30 min. The results show a consistent preferential adhesion of dorsal retinal cells to ventral half-tecta regardless of the length of preincubation.

In Fig. 2, the cell suspensions were made from the ventral region of the retina. All other conditions were the same as those in Fig. 1. A dependence on preincubation time after trypsinization was observed. The ratio of the numbers of cells adhering to dorsal and ventral half-tecta starts at a value smaller than one. As the preincubation time is lengthened, the ratio continually increases to values near two. Qualitatively similar results have been obtained in over 100 additional experiments involving dorsal and ventral retinal suspensions.

**Retinal Cell Adhesion to Noninnervated Tecta.** As a control for the possibility that the retinal cells were adhering to the optic axons on the surface of the tecta, tectal halves devoid of optic axons were used in several experiments. Table 1 presents the results of four experiments with dorsal and ventral retina suspensions with noninnervated tectal halves. Again, a 30-min incubation is used with various preincubation times. Preferential adhesion of dorsal retina cells to ventral tectum is demonstrated. Also ventral retina specificity for dorsal tectum increases with increasing preincubation time. These results with noninnervated tecta give ratios similar to those obtained with innervated tecta. Because of variations in the parameters of the assay, the absolute number of cells that adhered is difficult to compare, but the data show that the preferential adhesion is not greatly affected by the presence of the optic axons.

**Nonretinal Cell Adhesion to Tectal Halves.** Cells from either retinal half adhere preferentially to ventral half-tecta shortly after trypsinization. To ascertain if ventral half-tecta are generally more adhesive to freshly trypsinized neural tissue, brain structures other than retina were trypsinized and used as cell suspensions. Preincubation time was 20 min, followed by a 30-min incubation with tectal halves. Cell suspensions were prepared from cerebrum, cerebellum, brain stem, and optic tectum. None of these showed preferential adhesion to either tectal half.

**Pigmented Epithelium Adhesion to Tectal Halves.** Cell suspensions were made from pigmented epithelium from either the dorsal or ventral region of the eye. When these cells were radioactively labeled, they showed the same preferential adhesiveness seen with neural retina cell suspensions. In addition, these cells are sufficiently pigmented to be observable on the surface of the tectum. Fig. 3 is a micrograph of a dorsal and a ventral half-tectum that have been incubated with a pigmented cell suspension from the ventral half of the eye. After washing, many more cells can be seen adhering to the dorsal half than to the ventral half of the tecta.

Fig. 4 shows two micrographs, one of a dorsal and the other of a ventral half tectum that have been incubated with a pigmented cell suspension from the dorsal half of the eye. The magnification is greater than in Fig. 3, showing single cells and small clusters of pigmented cells adhering in a uniform fashion over the surface of the tectum. There is a significantly higher density of pigmented cells on the ventral than on the dorsal half tectum after washing.

**Effects of Washing on Adhesions.** The amount of washing of the tectal halves after incubation affects not only the number of retinal cells adhering to them but also the ratio of retinal cells between tectal halves. After incubation, there are about the same number of cells on both tectal halves regardless

<table>
<thead>
<tr>
<th>Labeled cell suspension</th>
<th>Retinal age, cell concentration, specific activity</th>
<th>Preincubation (min)</th>
<th>No. of retinal cells adhering to dorsal half-tectum</th>
<th>No. of retinal cells adhering to ventral half-tectum</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dorsal retina</td>
<td>7 days, $8 \times 10^6$ cells/ml, 2.0 cpm/cell</td>
<td>20</td>
<td>$1750 \pm 320$ (5)</td>
<td>$3710 \pm 430$ (4)</td>
<td>$&lt;0.05$</td>
</tr>
<tr>
<td>Dorsal retina</td>
<td>11 days, $8 \times 10^6$ cells/ml, 0.3 cpm/cell</td>
<td>300</td>
<td>$4720 \pm 60$ (3)</td>
<td>$7640 \pm 310$ (3)</td>
<td>$&lt;0.05$</td>
</tr>
<tr>
<td>Ventral retina</td>
<td>7 days, $1.9 \times 10^6$ cells/ml, 0.5 cpm/cell</td>
<td>20</td>
<td>$3350 \pm 90$ (4)</td>
<td>$3720 \pm 450$ (4)</td>
<td>$&gt;0.05$</td>
</tr>
<tr>
<td>Ventral retina</td>
<td>8 days, $2.5 \times 10^6$ cells/ml, 0.2 cpm/cell</td>
<td>240</td>
<td>$16000 \pm 600$ (4)</td>
<td>$8400 \pm 1700$ (4)</td>
<td>$&lt;0.05$</td>
</tr>
</tbody>
</table>
of which suspension was used. A large number of these cells are removed with light washing. The remaining cells display the preferential adhesiveness mentioned above. Through subsequent washings this specificity remains until virtually all the cells are removed by the washings. These results are independent of cell suspension density in the range tested (5 x 10⁶ to 6 x 10⁶ cells per ml).

**DISCUSSION**

These results demonstrate an adhesive selectivity between neural retina and optic tectum which mimics the innervation of the tectum by the retina. Isotopically labeled cells prepared from dorsal half-retinas adhere preferentially to ventral half-tecta. Cells from ventral half-retinas adhere preferentially to dorsal half-tecta. This adhesion correlates with the final position of the axonal tips of retinal ganglion cells as they synapse with their target cells in the tectum. A simple explanation of these data is that moieties on the cell surface participate in a process of recognition and specific adhesion, and that these molecules play a major role in an early selectivity that ultimately determines the retinotectal projection.

Cells from dorsal and ventral half-retinas display different behavior as a function of the time between trypsinization and the beginning of incubation with the tectal halves. Soon after trypsinization, cells from both half-retinas show an adhesive preference for ventral half-tecta. The cells from dorsal half-retinas maintain this specificity for up to 9 hr when preincubated in nutrient medium. However, the cells from ventral half-retinas lose their preference for ventral half-tecta and develop selectivity that favors adherence to dorsal half-tecta, in accordance with the retinotectal map. This finding may suggest that the molecules that identify dorsal half-retinas are only slightly affected by the trypsin solution or are replaced at a relatively rapid rate. The surfaces of the cells from ventral half-retinas may be extensively altered by the crude trypsin and require more modification before they exhibit selectivity paralleling neuronal specificity in vivo.

Experiments with optic tecta that had never been in-

**Fig. 3.** Adhesion of cells from ventral pigmented retina to tectal halves in 40 min. (A) A dorsal half-tectum; (B) a ventral half. The cells were not labeled isotopically and are visible because of their intense pigmentation. The pigmented retinas were from 10-day embryos; the tecta from a 12-day embryo. The cells were preincubated for 6 hr. Bar = 1 mm.

vated by the optic nerve show selectivity similar to those with innervated tecta. These experiments serve two purposes: First, they act as a control against the possibility that the preferential adherence is actually due to the labeled retinal cell bodies adhering to the retinal ganglion axons on the surface of the tectum. The specificity is still exhibited even when these fibers are absent. Second, these experiments favor an interpretation of neuronal specificity in which both the retina and the tectum are independently specified. That is, the tectum seems not to be dependent on retinal innervation to show differential adhesive properties.

This assay detects preferential adherence by cell bodies taken from the retina, yet the topographic map is actually created by the tips of the retinal ganglion axons. If this observed specificity plays a role in vivo, at least some of the identity-conferring molecules present on axonal tips are also present on cell bodies in the retina. Furthermore, the adhesion we observe involves the tectal surface and not the less superficial cells with which the retinal axons ultimately form synapses. This may imply a distinction between positional specificity and synaptogenesis.

Experiments were performed with pigmented retina in place of neural retina, and demonstrated visually the selectivity shown by 125I-labeled neural retina. This result suggests that many cell types in a region of retina bear the same specificity even though only the ganglion cells send axons to the optic tectum. Although the pigmented retina is not a neural tissue, it has the potency, in Triturus, to regenerate a viable neural retina that sends a topographically correct projection to the optic tectum (13). In chicks before stage 9, the pigmented retina will also regenerate a neural retina, but the resulting retina is radially inside-out and will not regenerate an optic nerve that reaches the tectum (14).

Although this assay only divides the retina and the tectum into dorsal and ventral halves, the in vivo specificity is much more precise. The locus for termination of a given retinal cell's axon is small compared to the area of the tectal surface (1). Therefore, the probability in our assay that a retinal cell will have the opportunity to adhere to its exact locus of termination is correspondingly small. Yet a relatively large fraction of the retinal cells do adhere. One can conclude that most

**FIG. 4.** Adhesion of cells from dorsal pigmented retina, shown at a higher magnification. (A) A dorsal half-tectum; (B) a ventral half. Again, the retinas were from 10-day embryos and the tecta from a 12-day embryo. Preincubation was for 20 min followed by a 30-min collection period. Bars = 0.5 mm.
of these cells are adhering to the tecta at positions other than the normal locus for termination of their axons. The data show that the retinal cells are more likely to adhere to the half of the tecta into which they map. They show more affinity for tectal sites near their normal terminus than to sites farther away. This result suggests gradual changes in the entities responsible for this selectivity as one moves across the tectum.

Results of experiments in which retinotectal maps have been determined after surgical manipulations of the retina or the tecta have been interpreted as evidence that retinotectal specificity is not absolute but capable of regulation (for review, see ref. 7). Other likely alternatives, however, have not been ruled out (15). The data presented here are consistent with either absolute or context-dependent specificities. A combination of surgical manipulation and an adhesive assay might aid in the analysis of this problem.

We have no information as to which elements on the tectal surface the retinal cells are adhering, nor do we know whether all of the retinal cells or only a subpopulation show this specificity. It is possible, also, that the crude trypsin solution used to dissociate the retinal cells may be unmasking adhesive selectivity that, in vivo, does not exist. Nonetheless, the data show that cells from the neural retina will preferentially adhere to the surface of the optic tectum in a manner that mimics the retinotectal mapping. This finding supports an interpretation of neuronal specificity dependent on cell-surface interactions and demonstrates a clear correlation between selective adhesion and biological function.

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