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In vitro experiments on axon guidance demonstrating an anterior-posterior gradient on the tectum

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Axonal growth cones originating from explants of embryonic chick retina were simultaneously exposed to two different cell monolayers and their preference for particular monolayers as a substrate for growth was determined. These experiments show that: (1) nasal retinal axons can distinguish between retinal and tectal cells; (2) temporal retinal axons can distinguish between tectal cells that originated from different positions within the tectum along the antero-posterior axis; (3) axons originating from nasal parts of the retina have different recognizing capabilities from temporal axons; (4) the property of the tectal cells, which is attractive for temporal axons, has a graded distribution along the antero-posterior axis of the tectum; and (5) this gradient also exists in non-innervated tecta.

Key words: axon/tectal gradients/growth cones/cell recognition

Introduction

During the development of the nervous system, growing axons find their ways over long distances to their specific targets. How these axons are guided along specific paths and how the axons come to terminate in specific areas within the target organ are central questions in neuroembryology. A variety of possible mechanisms has been discussed. These include mechanical guidance along pre-existing channels, electrical guidance, chemotaxis due to substances with graded distribution, and specific recognition of the membranes of cells or neurites by the growth cone. If cell recognition by the growth cone is essential in axon guidance, axonal growth cones may also be capable of distinguishing *in vitro* different cell types or cells of a given type derived from different spatial positions of the target tissue.

Previous investigations have shown that *in vitro* growth cones have a preference for certain cell types as substrates supporting axonal growth demonstrating that cell type differences can be recognized *in vitro* by growth cones (Letourneau, 1975; Bonhoeffer and Huf, 1980).

In this paper evidence will be presented that axonal growth cones of temporal retinal ganglion cells have recognizing properties different from those of growth cones from nasal retinal axons. Axons from temporal retina, which *in vivo* project to anterior tectum, show *in vitro* a marked preference for anterior tectal cells. Further, it will be shown that tectal cells carry position-dependent markers which have a graded distribution along the antero-posterior axis and which are recognized *in vitro* by growth cones originating from temporal retina.

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Results

The assay

The design of the experimental assay is depicted in Figure 1. A retinal explant – normally a 0.3-mm strip extending from the nasal to the temporal part of the retina of a 6-day-old chick embryo – is placed on a cell monolayer (A) which is attached to a coverslip. Since the retinal tissue from which the fibers originate has been stained with rhodamine isothiocyanate (RITC) before explantation, the outgrowing fibers can be detected by their fluorescence (Bonhoeffer and Huf, 1980). Axons grow along monolayer (A) until they meet a second monolayer (B) attached to the edge of another coverslip which has been bevelled at an angle of 20°. At this point each axon must decide along which of the two monolayers it prefers to grow. The number of fibers which remain on monolayer (A) and the number of fibers which grow along monolayer (B) are determined by observation with an inverted fluorescence microscope.

Description of axonal growth on monolayers

About 6–8 h after the explant has been placed on the monolayer, axons start to invade the monolayer and after 25–30 h the density of fibers leaving the explant ranges up to 150 per mm of the explanted strip of retina. The maximum velocity of axonal growth is 0.1 mm/h as measured in time-lapse recording. The direction of growth corresponds in general to the original *in vivo* direction (Bonhoeffer and Huf, 1980). The axons leave the temporal retinal explant in a highly bundled form in contrast to axons from the nasal part (Halfter *et al.*, 1981). All axons tend to maintain a constant direction of growth for reasons which are not understood. It has not yet been clearly demonstrated whether the observed axonal growth is due to regeneration or to *de novo* outgrowth. No branching of axons has been observed. After ~36 h axons slowly begin to deteriorate and decay. While the maximum length of the axons observed is ~3 mm, most of the axons are <1 mm in length. The number of outgrowing axons varies with the age of the retina used for the explant. In particular, there are striking age-dependent differences in the number of outgrowing axons between the nasal and temporal part of the retinal explant, which has also been observed by Halfter *et al.* (1981).

Control experiment on the equivalence of the two monolayers

The result of a control experiment in which growing axons are offered the choice between two identical monolayers is presented in Figure 2a. Monolayer (A) and monolayer (B) were prepared from retinal cells. The fibers show no strong preference for the upper or lower monolayer. The average of five experiments shows that ~53% of the fibers stay on monolayer (A), while 47% grow along monolayer (B) (Table I, line a). The reason for this slight asymmetry could possibly be the tendency of axons to grow straight ahead or to stay on the continuous layer with which they are in contact. Aside from these minor effects, the decision between upper and lower layer at the choice point is spatially neutral. Major differences detected in systems with different cells in the two

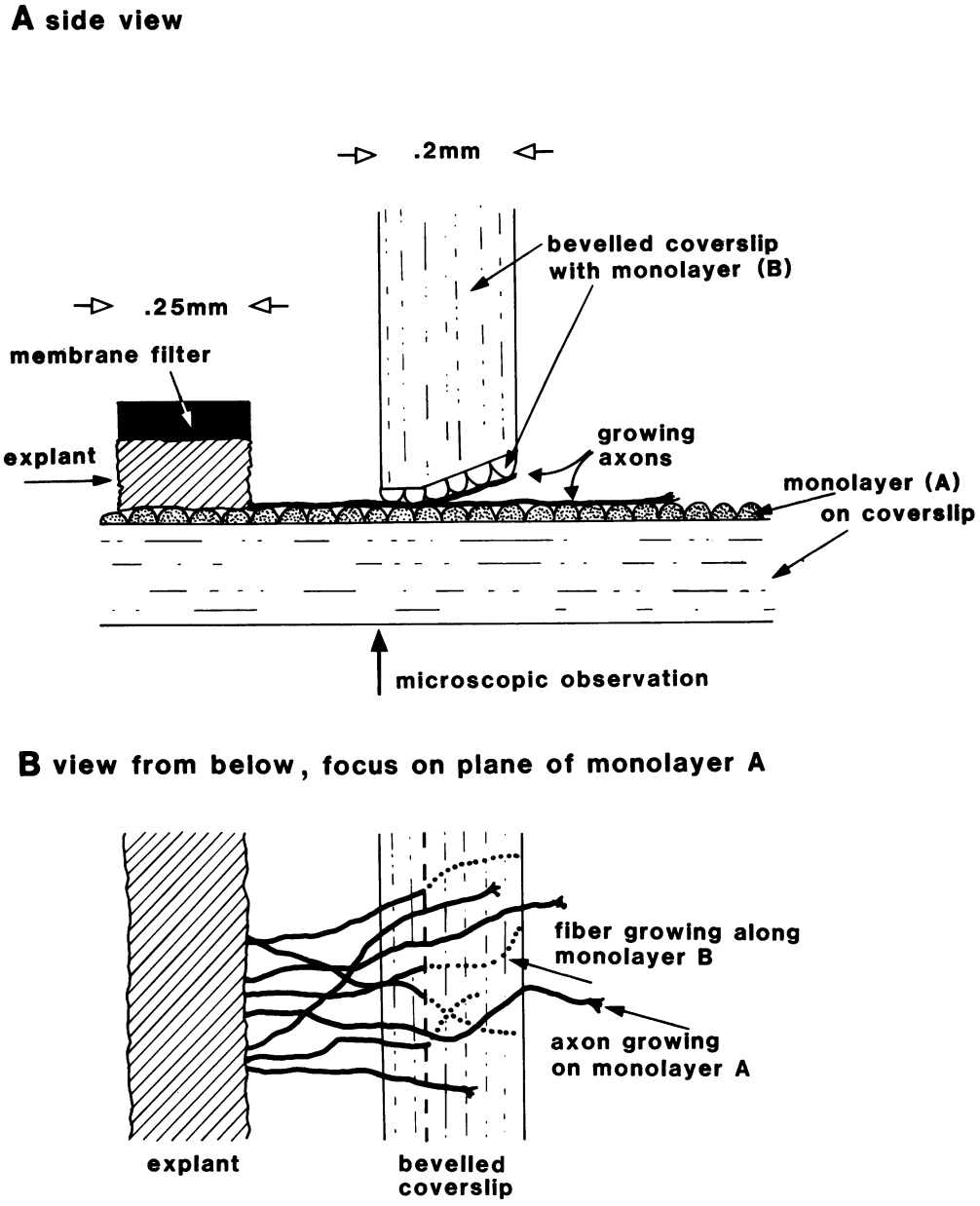

Fig. 1. Design of the experiment.

layers must therefore be due to biochemical differences between the layers.

Choice between retinal and tectal cell monolayers for axonal growth cones originating from nasal or temporal retina

If one of the cell layers is composed of retinal, the other of tectal cells, the axons originating from the nasal part of the retina clearly prefer the tectal monolayer, whereas temporal axons do not show such a clear preference. An example is presented in Figure 2b and numbers averaged over several experiments are listed in Table I (lines b and c). About 90% of the nasal retinal axons have preferred the tectal monolayer; only 56% of the temporal fibers showed preference for the upper tectal monolayer. This shows that axons of nasal retina have different properties from those of temporal retina.

Choice between cell monolayers prepared from anterior and posterior tectum

To determine whether retinal axons prefer cells from one part of the tectum to cells from another part, monolayers derived from anterior and posterior parts of the tectum were prepared. Tecta of 8-day chick embryos were divided into three approximately equal parts along the antero-posterior axis. The middle part was discarded and the anterior and posterior parts were used to prepare the monolayers. As seen in Figures 2c and 2d and from the numbers averaged over several experiments and presented in Table I (lines d and e), temporal axons clearly prefer an anterior tectal monolayer over the corresponding posterior monolayer, independent of whether anterior tectal cells were used for the upper or the lower monolayer. Nasal axons do not show this strong preference.

Attractivity of tectal cells as substrate for temporal retinal axons decreases gradually from the anterior to the posterior pole of the tectum

Tecta of 8-day embryos were isolated and divided into five

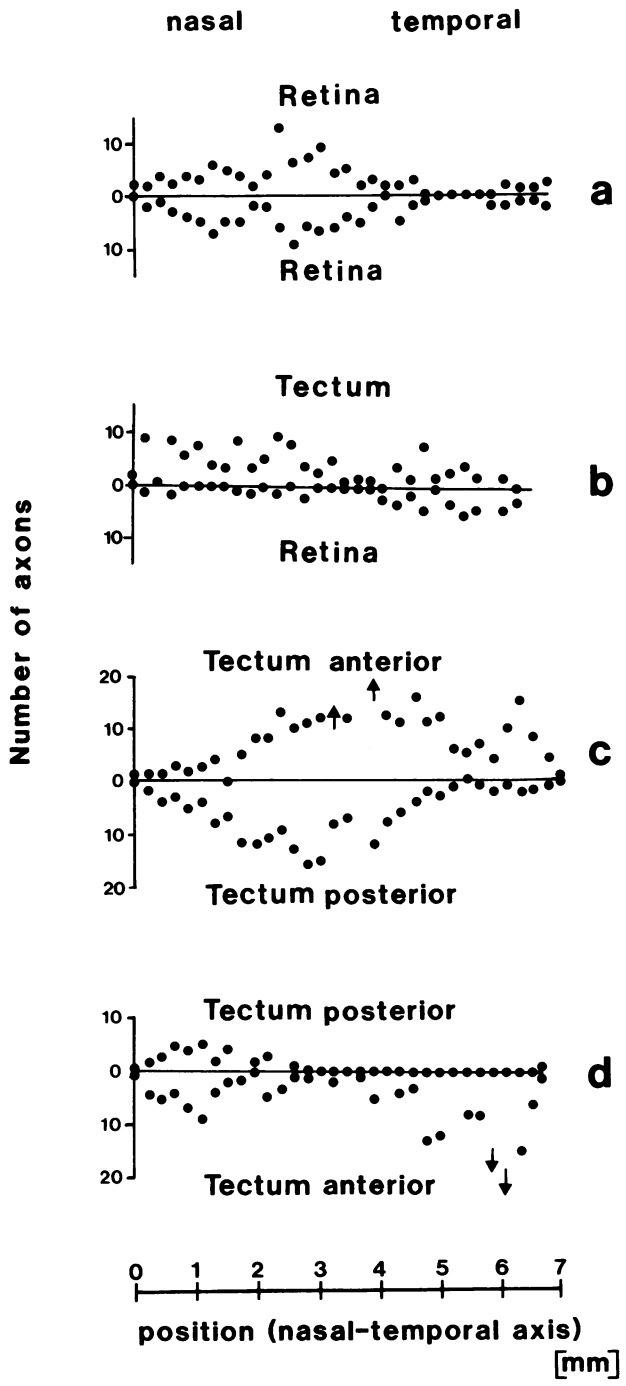

Fig. 2. Choice by retinal axons between cell monolayers of various origins. In each of the four parts of the figure the name of the tissue above and below the line indicates the origin of the cell used for the upper and lower monolayer of cells. The abscissa indicates the position along the naso-temporal axis of the explant. The numbers of axons growing along the upper and lower monolayers within adjacent fields of microscopic observation are determined and plotted above and below the abscissa, respectively. When the field of observation contained too many fibers only minimal numbers (arrows) could be determined.

approximately equal pieces along the antero-posterior axis. They are sequentially designated with the numbers 1–5, number 1 being the most anterior part. Choice experiments were performed with different combinations of monolayers (Table II). In all experiments all temporal axons clearly prefer the more anterior cell type, i.e., monolayer 1 is preferred to 2,

Table I. Retinal axons observed on upper and lower monolayer in various choice experiments

Line	Cells on upper monolayer / Cells on lower monolayer	Retinal axons on upper monolayer / Retinal axons on lower monolayer: Nasal explant	Temporal explant	No. of experiments
a	retina / retina	$\frac{475}{525}$ (48 ± 3)		5
b	tectum / retina	$\frac{640}{78}$ (89 ± 6)	$\frac{724}{562}$ (56 ± 9)	13
c	retina / tectum	$\frac{91}{935}$ (8.8 ± 1.1)	$\frac{105}{234}$ (31 ± 8)	5
d	tectum anterior / tectum posterior	$\frac{477}{699}$ (41 ± 7)	$\frac{332}{96}$ (78 ± 8)	6
e	tectum posterior / tectum anterior	$\frac{161}{236}$ (40 ± 4)	$\frac{14}{360}$ (3.7 ± 3)	3

The percentage of fibers growing along upper monolayer is calculated as an average over several experiments. It is given in brackets together with the standard deviation.

Table II. Number of axons growing along cell monolayers derived from cells from various positions

Cells on upper monolayer / Cells on lower monolayer	Retinal axons on upper monolayer / Retinal axons on lower monolayer: Nasal explant	Temporal explant	No. of experiments
$\frac{T1}{T2}$	$\frac{216}{249}$ (46 ± 4)	$\frac{293}{89}$ (77 ± 4)	2
$\frac{T2}{T1}$	$\frac{106}{221}$ (32 ± 7)	$\frac{21}{329}$ (6 ± 1.5)	3
$\frac{T2}{T4}$	$\frac{542}{638}$ (46 ± 1.5)	$\frac{706}{301}$ (70 ± 12)	3
$\frac{T4}{T2}$	$\frac{259}{324}$ (44 ± 9)	$\frac{52}{373}$ (12 ± 9)	2
$\frac{T3}{T4}$	$\frac{83}{126}$ (40 ± 7)	$\frac{122}{33}$ (79 ± 13)	2
$\frac{T4}{T3}$	$\frac{198}{478}$ (29 ± 7)	$\frac{54}{549}$ (9 ± 5)	2
$\frac{T4}{T5}$	$\frac{738}{702}$ (51 ± 3)	$\frac{790}{344}$ (70 ± 3)	2
$\frac{T5}{T4}$	$\frac{310}{539}$ (37 ± 10)	$\frac{152}{530}$ (22 ± 3)	2

Number of retinal axons observed on upper and lower monolayers in experiments in which tectal cells of various positions along the antero-posterior axis have been offered for choice (T1 most anterior, T5 most posterior tectal cells). The percentage of fibers growing along upper monolayer is calculated as an average over several experiments. It is given in brackets together with the standard deviation.

3 is preferred to 4, etc. The nasal fibers do not exhibit a clear preference. The data given in Table II show that, for temporal axons, the attractivity of tectal cells decreases gradually from the anterior to the posterior pole of the tectum.

Cells from non-innervated tecta reveal the same position-dependent differences as those from innervated tecta

The higher attractivity of the monolayer originating from the more anterior cells could be due to the fact that the anterior tectum is more strongly innervated than the posterior part in an 8-day embryo (Rager and von Oeynhausen, 1979). To exclude this possibility, tecta were prepared from embryos whose eyes had been removed at day 3. These tecta were divided into three parts. Choice experiments with monolayers

Table III. Number of retinal axons observed on upper and lower monolayer

Cells on upper monolayer / Cells on lower monolayer	Retinal axons on upper monolayer / Retinal axons on lower monolayer		No. of experiments
	Nasal explant	Temporal explant	
T1 / T2	645 / 680 (49)	560 / 299 (65)	3
T2 / T3	392 / 414 (49)	285 / 94 (75)	2
T1 / T3	220 / 327 (40)	237 / 71 (77)	1

The observations were made in experiments in which the tectal cells on the monolayers stem from different positions of the tecta and in which the tecta have not been innervated (T1 most anterior, T3 most posterior tectal cells). The percentage of fibers growing along upper monolayer is calculated as an average over several experiments and is given in brackets.

derived from such tectal cells also show that temporal axonal growth cones prefer to grow along a more anterior monolayer, whereas nasal fibers do not exhibit this preference (Table III).

Experiments on age dependence of the expressed positional differences of tectal cells

The development of the tectum proceeds from the anterior to posterior pole (LaVail and Cowan, 1971a, 1971b; Cowan *et al.*, 1968). It would be desirable to know whether the observed positional differences described above are due to the difference in developmental age of the various parts of the tectum or whether they are true functions of position in the tectum which are not reducible to the age of nerve cells since the last mitosis. If the latter is correct, one would like to know at which developmental stage the positional effects become observable and how long they persist. We have performed choice experiments with tectal cells of embryos between 6 and 9 days old. In these experiments, temporal retinal axons prefer anterior tectal cells from even the youngest embryos (6 days) to posterior tectal cells of 9-day embryos. When the upper monolayer contains the 6-day anterior and the lower the 9-day posterior cells, 76% of the temporal fibers grow along the anterior monolayer; in the reversed monolayer arrangement of the monolayers as many as 94% grow along the 6-day anterior cell monolayer. Unfortunately, so far we have been unable to prepare cell monolayers from tecta younger than 6 days or older than 9 days, since young cells showed a strong tendency to form clusters and tecta older than 9 days could not easily be dissociated into viable cells. Although age dependence has to be investigated more extensively, we take the results as an indication that the observed differences between anterior and posterior tectal cells are real positional effects rather than secondary effects caused by differences between developmental age of the anterior and posterior parts of the tectum.

Discussion

Recently it has been demonstrated that *in vitro* growing axons are able to distinguish between different cell types (Bonhoeffer and Huf, 1980). Encouraged by this finding, we attempted to use the same experimental design to show that growing axons not only distinguish between different cell types but that they are also capable of discriminating between cells of the same type but originating from different positions.

We used the visual system of the chick and hoped to find *in vitro* an analogy to the situation *in vivo* where the axons of retinal ganglion cells produce a highly ordered topographic projection to the tectum opticum: the more nasal (temporal) the retinal origin of the axons, the more posterior (anterior) is their terminal field in the tectum, the more dorsal (ventral) their origin, the more ventral (dorsal) their projection on the tectum.

If the *in vitro* system reflects the *in vivo* situation, retinal axonal growth cones should distinguish between tectal cells from different positions within the tectum and the preference of a growth cone for a particular tectal cell should depend on the retinal position of the cell body belonging to that growth cone. To some extent this situation exists: (1) Retinal axons from the temporal retina can distinguish between tectal cells from different tectal positions. In the *in vitro* assay, temporal fibers preferentially grow over monolayers of the most anterior tectal cells as demonstrated in Figures 2c and 2d and Table I, lines d and e. This corresponds to the *in vivo* situation where temporal fibers project to the anterior tectum. (2) Nasal fibers show, in contrast to temporal fibers, a clear preference for tectal cells when they are offered the choice between retinal and tectal monolayers. This is demonstrated in Figure 2b and Table I (lines b and c). Nasal and temporal fibers also behave very differently with respect to their preference for anterior tectal cells over posterior tectal cells and further with respect to their tendency to form fascicles and to bind tectal membrane vesicles (Halfter *et al.*, 1981).

These results encourage us to believe that the *in vitro* system and the *in vitro* guidance of the axons reflect, to some degree, the *in vivo* situation. Thus, an *in vitro* system of the type described may be useful for studying the cues which play a role in the formation of specific neuronal connections.

Current theories for the formation of the topographic retino-tectal projection involve the interaction of retinal and tectal components which are graded with respect to the position of the cells within the tissue. One of these models (Fraser, 1980) explains axonal guidance by differential adhesion, another (Gierer, 1981) by more general mechanisms of directional activation of growth cones. According to such models the projection of retinal ganglion cells along the naso-temporal axis onto the postero-anterior axis of the tectum could, for example, be achieved by graded distributions of positional markers within the retina along the temporo-nasal axis and two counteracting gradients of positional markers within the tectum along the antero-posterior axis. One of the two tectal gradients involved for projection would tend to direct an incoming fiber to the anterior pole of the tectum, whereas the other one would tend to direct this fiber to the opposite, posterior pole. The directing strength of both gradients would depend on the positional marker which the incoming fiber carries and thus lead to a balanced final position of the axon on the target.

The experiments described have clearly demonstrated the existence of a tectal gradient. Some cell property attractive for temporal retinal axons decreases gradually along the tectum in the antero-posterior direction. This gradient is also found on non-innervated tecta and might well represent one of the two gradients postulated in the theories above, namely the one which shifts the fibers towards the anterior pole of the tectum. We do not yet know when in development this gradient first appears or for how long it persists. It is, however,

clearly present in tecta at between 6 and 9 days of embryonic development. We also do not know whether this gradient is expressed by all tectal cells or only by certain cell types.

We have not observed any preference of nasal axons for posterior tectal cells. Furthermore, as far as we can tell, axons from the middle part of the retina do not show any preference for central tectal cells. A possible explanation of these findings is that the second postulated gradient on the tectum is not expressed in the *in vitro* system because its components are destroyed during the dissociation of the tissue by trypsin when cells are prepared for the monolayers. In a similar *in vitro* assay system (Kern-Veits and Bonhoeffer, in preparation) in which this dissociation step is eliminated and a layer of cell membranes is used instead of the upper cell monolayer, nasal axons grow preferentially over membranes of posterior tectal cells.

A dorso-ventral gradient expressed in cell-cell adhesion of perikarya has been found previously in retina (Gottlieb *et al.*, 1976) and Trisler *et al.* (1981) found a dorso-ventral gradient within the retina by immunological methods. It is, however, an open question whether these dorso-ventral gradients are involved in axonal guidance during the formation of the retino-tectal projection. Experiments with our assay system on dorso-ventral gradients did not reveal a clear preference of any retinal axons for dorsal or ventral monolayers. This is, perhaps, not surprising as retinal fibers seem to sort out according to their dorso-ventral position before they reach the tectum and not after arrival on the tectum (Rager and von Oeynhausen, 1979). Thus, dorso-ventral gradients on the tectum may be weak and difficult to detect.

It seems to us likely that the observed specific preferences of axons for particular cell types or cells from particular areas reflect properties of the guiding mechanisms *in vivo*.

Materials and methods

The culture medium is a modification of Dulbecco's modified Eagle's medium as described by Claviez (1980) which contains 10% fetal calf serum (FCS), 2% chicken serum, 10 units/ml streptomycin, and 10 units/ml penicillin. The major modification concerns the buffer, which has been changed to 28 mM $NaHCO_3$ and 5 mM N-2-hydroxy-piperazine-N'-2-ethane sulfonic acid (HEPES).

The wash medium is Hank's balanced salt solution without Ca^{2+} and Mg^{2+}. 3-(Triethoxysilyl)propylamine and dimethylsulfoxide (DMSO) were purchased from Merck. Concanavalin A (ConA) was bought from Serva; streptomycin, penicillin, HEPES, FCS, and chicken serum from GIBCO. RITC was obtained from Sigma. Trypsin (Cat. No. 37 07 TRL 3) and DNase I (Cat. No. 2007) were supplied by Worthington. Coverslips were bevelled with Ceri 600 (Pieplow & Brandt, Henstett) or obtained from Hellma (Müllheim/Baden) in bevelled form.

Cell preparation for monolayers

Tecta and retinae were surgically isolated from chick embryos, washed in Hank's solution, and incubated in Hank's solution (~50 mg tissue/ml) containing trypsin (1 mg/ml for retina, 2 mg/ml for tectum). Incubation was carried out for 10 min at room temperature and subsequently for 10 min at 37°C. The tissue was then thoroughly washed, taken up in culture medium containing 25 μg/ml of DNase I at a concentration between 2×10^7 and 5×10^7 cells/ml, incubated for 5 min at 37°C, and carefully dispersed by slowly forcing (1 ml/3 s) the tissue twice through a capillary (10 cm long, 0.4 mm in diameter). This cell suspension was diluted with an equal volume of calcium- and magnesium-free Hank's solution and centrifuged for 10 min at 600 r.p.m. and 4°C. The sediment was taken up in Hank's solution at a concentration of 2.2×10^7 retinal cells/ml or 1.3×10^7 tectal cells/ml. About 0.25 ml of the cell suspension was placed on top of a pretreated coverslip (20 mm in diameter) and the cells were allowed to settle at room temperature. After 15 min the coverslip was carefully rinsed with prewarmed culture medium and kept in the culture medium until use. The pretreatment of the coverslips was a modification of a method described by Gottlieb and Glaser (1975). The coverslips were cleaned in concentrated nitric acid, washed in water, dried, put into a solution of 10% triethoxysilylpropylamine in DMSO and kept there for 60 min at 115°C. Then they were rinsed first with DMSO, then with water and dried. Before being used for the production of monolayers the derivatized coverslips were incubated in a ConA solution (1 mg/ml ConA in Hank's solution) at room temperature for 1 h and finally rinsed in Hank's solution.

Preparation of stained retinal explants

The retina of a 6-day chick embryo was surgically isolated. A black membrane filter was soaked in Ca-Mg-free Hank's solution containing ConA (0.1 mg/ml) for 1 h and subsequently washed with the same buffer. The retina was spread on the membrane filter in a Ca-Mg-free Hank's solution. The membrane filter containing the retina preparation was placed briefly on a dry filter paper. Thus, the retina becomes firmly attached to the membrane filter. This was then placed in the staining solution prepared as follows: 1 mg of RITC is dissolved in 20 μl DMSO. This solution is diluted 1000–5000-fold with vigorous shaking into Ca-Mg-free Hank's solution. After 15 min of incubation in staining solution at room temperature the retina and its supporting membrane filter were washed three times with wash medium and then incubated in culture medium for 1 h at 37°C. The supporting filter with the retina was again briefly placed on a filter paper to remove the liquid and chopped into strips 0.2–0.3 mm wide with a McIlwains Tissue Chopper. For an alternative staining procedure, 20 μl of the RITC solution in DMSO was diluted in 1 ml warm (~60°C) agar solution (1% agar in Ca-Mg-free Hank's solution). The agar was cooled between two microscope slides kept 0.3 mm apart; the agar sheet was washed in wash medium for 50 min, freed from wash medium and kept in humid atmosphere for ~2–5 h after which it was put on top of the retinal explant for 5 min. Washing and slicing of the explant followed as above. The strips of stained retinal explants were placed on the monolayer as indicated in the figure. The layer of ganglion cells touches the monolayer. The strips were held in position by small stainless steel holders at the ends of the strips where they were not covered by the retina. The distance between explant and monolayer (B) (Figure 1) varied between 0.05 and 0.25 mm which did not affect the experimental results. Monolayers and explants were incubated at 37°C in 4% CO_2 until microscopic observation or fixation in 4% paraformaldehyde. Care was taken that surgery and handling of the retinal explants, including the tissue chopper procedure, was carried out under sterile conditions in a sterile hood.

Microscopic observation

Microscopy was carried out with epifluorescence (wavelength of incident light 545 nm, observation at >590 nm) in an inverted microscope (Axiomat, Zeiss) equipped with an image-intensifying television camera (Siemens).

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