Avoidance of posterior tectal membranes by temporal retinal axons

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Summary
Membrane carpets consisting of alternating membrane stripes were prepared from plasma membranes of anterior and posterior chick optic tectum. Axons from retinal explants extend neurites on these carpets. Axons of the nasal retina do not distinguish between the stripes. Axons of the temporal retina prefer to extend neurites on anterior tectal membranes.

Treatment of the membrane fragments with high temperature interferes with the pattern of neurite outgrowth from temporal axons. When growing on carpets consisting of treated anterior and posterior tectal membranes, temporal retinal axons no longer distinguish between the stripes. Treatment of posterior membranes alone is sufficient to abolish the preference of temporal axons to extend neurites on anterior tectal membranes. Treatment of the anterior membranes alone has no effect.

This result is best explained by a repulsive component in the posterior tectal membranes. Temporal, but not nasal, axons specifically recognize and avoid that component, with the result that they do not extend neurites on posterior tectal membrane stripes. Once the repulsive component is destroyed, temporal axons are able to extend neurites on posterior tectal membranes.

Key words: retinotectal, chick, cell membranes, in vitro, recognition.

Introduction
Axons originating from the temporal retina which project in vivo to the anterior part of the optic tectum grow also in vitro preferentially on cell membranes derived from anterior tectum when they are offered the choice of anterior and posterior membranes simultaneously (Walter et al. 1987). It is conceivable, but not yet proven, that this correspondence is not accidental and that the in vitro preference reflects the axon behaviour in vivo. It, therefore, seemed to us of interest to understand the basis of this preference in more detail.

An assay system for substrate preferences of growing axons has been presented in the accompanying paper (Walter et al. 1987). In this paper, we investigate whether the preference of temporal retinal axons for growth on anterior membranes is due to a special attractivity of these membranes or to a repellent property of posterior membranes. The results indicate that the choice reflects not so much a preference for anterior as an avoidance of posterior membranes.

Materials and methods
The preparation of plasma membranes, striped carpets and the preparation of retinal explants are described in the accompanying paper (Walter et al. 1987). Optic tecta were dissected from 9-day-old chick embryos.

Chemical and physical inactivation of membrane fragments
All treatments were performed in a suspension of membrane fragments before the formation of the carpets on the filter.

Heat inactivation
0.5 ml of membrane suspension in PBS+ was adjusted to 100–200 μg protein ml⁻¹ as measured by the extinction of the suspension at 220 nm after diluting 1:15 in 2% SDS. In order to find conditions optimal for inactivation, this suspension was heated in a water bath for various lengths of time and to various temperatures. Inactivation was stopped by cooling the suspension to 0°C. The optimum was found to be heating at 63°C for 8 min. These conditions were used for all experiments.
Inactivation by aldehyde fixation

40 % paraformaldehyde (PFA) and 0-01 % glutaraldehyde (GA) were used as fixatives. Paraformaldehyde was dissolved in PBS+ by boiling. The pH was adjusted to 7-0 with KOH. Glutaraldehyde (Merck) was purchased as a 25 % solution which was diluted 2500-fold in PBS+. Membranes were suspended in the fixative either for 90 min (PFA) or 30 min (GA) at 37°C. After treatment, membranes were pelleted by centrifugation (Eppendorf centrifuge), washed twice in PBS+ and suspended in PBS+ for the preparation of membrane carpets.

Inactivation by pronase treatment

Pronase C (Serva) was diluted in buffer (0.1 M-Tris-HCl, 1 mM-CaCl₂, pH 8.0) and preincubated at 60°C for 30 min. Membranes at a concentration of 100 μg protein ml⁻¹ were incubated with 10⁻⁴ mg ml⁻¹ pronase C at 37°C for 1 h. The membranes were washed twice and resuspended in PBS+.

Plasma membranes were freshly prepared for each experiment. Control membranes were suspended in buffer without enzymes or fixatives.

Results

Axons originating from nasal or temporal retina differ in their growth pattern on membrane carpets consisting of alternating stripes made of plasma membranes from anterior and posterior tectum (Walter et al. 1987). Nasal axons do not distinguish between these stripes growing freely across the stripe borders whereas temporal axons grow only on the stripes made of anterior tectal membranes (Fig. 1A,B). In order to investigate the components of the plasma membranes which are responsible for this decisive behaviour of temporal axons, we have been looking for treatments of anterior and posterior membranes which, on the one hand, render them indistinguishable to temporal axons, but which, on the other hand, do not interfere with membrane properties that support axonal outgrowth. It was found that a brief heat treatment (63°C for 8 min) of both vesicle suspensions of anterior and posterior membranes changes the properties of the membranes in such a way that in a subsequent assay with a striped carpet temporal axons (Fig. 1C) grow like nasal axons, that is without any preference (Fig. 1D). They now freely cross boundaries between anterior and posterior membranes. Nasal axons continue to grow unselectively in these conditions.

To test whether this loss of selective behaviour is due to an inactivation of an attractive component in anterior membranes or a repulsive component in the posterior membranes, treated membranes were tested against untreated membranes in various combinations.

Fig. 1E shows that treatment of anterior membranes tested against untreated posterior membranes does not affect the choice of temporal axons. However, when posterior membranes are treated and tested against untreated anterior membranes a loss of temporal axon selectivity is observed (Fig. 1G). Temporal axons now cross the stripes and grow all over the membrane carpet. They even seem to have a slight preference for the treated posterior membranes.

Since treatment of posterior membranes alone is sufficient to change the preference of temporal axons so that they now grow on the formerly avoided membranes, it is likely that there is normally a repulsive substance in the posterior membranes which is selectively recognized by temporal axons and which is inactivated by brief heat treatment.

As shown in Fig. 1G temporal axons grow on both untreated anterior and treated posterior membranes. There is a slight preference for the treated posterior membranes. This could reflect a potentially graded distribution of a repulsive component with a small but non-negligible amount in the anterior tectum.

A minor effect is seen with nasal axons, which favour slightly the treated anterior membranes in Fig. 1F and the treated posterior membranes in Fig. 1H. An equivalent result was obtained when treated membranes were tested against untreated membranes of the same tectal area (Fig. 2). That nasal and temporal axons both show slight preferences for treated membranes probably indicates that heat treatment not only inactivates a repulsive factor specific to posterior membranes and recognized by temporal axons but in addition may inactivate another nonspecific repulsive component common to all tectal membranes. Alternatively, the heat treatment could activate a nonspecific attractive component.

In the experiments described, heat treatment of membranes for 8 min at 63°C has a strong influence on selective growth of temporal axons and almost no effect on the growth rate. Prolongation of the heat treatment as well as the use of higher temperature results in decreased or no outgrowth. Very strong heat inactivation like 80°C for 20 min destroys completely the ability of anterior and posterior membranes to support axonal growth. Presumably choice and outgrowth promoting components are different proteins, the repulsive posterior one being more sensitive to heat than the growth-permissive components of the plasma membrane.

Other protein-inactivating treatments like fixation or proteolytic treatment with pronase C show in general the same result. After strong inactivation (0.4 % glutaraldehyde, 30 min; 10⁻² mg ml⁻¹ pronase C, 1 h) there is almost no outgrowth and, therefore, selectivity cannot be observed. After limited inactivation, both growth and selectivity are affected. In
Fig. 1. Growth of retinal axons on membrane carpets of untreated and heat-treated (63°C, 8 min) membrane fragments. The pictures represent a typical growth pattern of retinal fibres from the temporal (left) and nasal (right) half-retina on a given membrane carpet as indicated at the right margin. Axons are growing from left to right. (A,B) Growth pattern on untreated membranes. (C,D) Growth pattern on heat-treated anterior and heat-treated posterior membrane fragments. (E,F) Growth pattern on heat-treated anterior and untreated posterior membrane fragments. (G,H) Growth pattern on heat-treated posterior and untreated anterior membrane fragments. The actual stripe array of each carpet is indicated at the right edge of each axonal pattern. a, anterior membranes; p, posterior membranes; a*, p*, heat-treated anterior or posterior membranes; a/p indicates the order of the alternating stripes of the preparation of the membrane carpets, the posterior membranes being prepared first and marked by the addition of green fluorescent beads. Bar 100 μm.
Fig. 2. The degree of preference of temporal and nasal axons for heat-treated (indicated by *) or untreated types of membranes. Experiments of various combinations of treated and untreated membranes as given in the bottom line have been evaluated. a, anterior; p, posterior temporal membranes; t, temporal; n, nasal retinal axons; *, treated membranes; O, preferred substrate. For example, first experiment: O : p = test of untreated anterior membranes against untreated posterior membranes. Both combinations with anterior membranes, as type A and as type B stripes, have been prepared. The degree of preference has been evaluated by comparison with four reference pictures (Walter et al., 1987). Temporal axons prefer to grow on anterior membranes, nasal axons do not show any preference.

contrast to heat inactivation these treatments, unfortunately, inhibit growth much more quickly than they inhibit selectivity. Thus, since selectivity of temporal axons can only be studied during growth, these treatments are less suitable to investigate whether this selectivity is based on specific attractivity of anterior, or specific repulsion of posterior, cell membranes.

Discussion

The presented work is a continuation of the preceding paper by Walter et al. (1987). It was observed that temporal retinal axons show a preference for growing on plasma membranes of anterior tectum when given a choice between anterior and posterior tectal membrane substrates. This preference results from a direct interaction between growing axons and the substrate, and is clearly not the result of a diffusible material. Furthermore, it cannot be explained by mechanical restrictions resulting from edges between the membrane stripes, or the order in which the stripes of membrane carpets were produced.

The figures presented show patterns of axon fascicles on membrane carpets formed after 2 days in culture. Each pattern is typical for nasal or temporal half-retina. The fascicles do not seem to represent the actual path of a single growth cone because axons far behind the growth cone tend to fasciculate passively during the culture period. They adhere to each other better than to the substrate. The axons are elastic and under tension (Bray, 1979). Even though the route taken by single axons is not identical with the position of the fascicle, the growth pattern reflects an average value of preference of the axons.

Two different principles could result in a preference of axons for a specific substrate. One as discussed by Letourneau (1975) is based on differential adhesion and assumes a higher concentration of adhesive substances in the preferred substrate. The other postulates repulsive components in the avoided substrate leading to a specific avoidance reaction of axons (Kapfhammer & Raper, 1987). This would be similar to contact inhibition type I between cell types (Heaysman, 1978). Posterior membranes and anterior membranes both contain growth-promoting components for temporal and nasal axons. This was shown in the preceding paper (Walter et al. 1987) by growing axons on membrane carpets of purely one type or the other.

The results obtained so far seem to be in accordance with the expectations for a substance mediating contact inhibition. It is possible to block the preference of the temporal axons with several protein-denaturing treatments of the membranes without affecting the amount or rate of outgrowth on the striped carpets. Nasal and temporal axons do not distinguish between the treated membranes. In tests with various combinations of treated versus untreated membranes it is shown that the treatment of posterior membranes is sufficient to make anterior and posterior membranes equally attractive. Therefore, the simplest explanation is that temporal axons recognize a repulsive substance in the undenatured membrane fragments of posterior tectum and that nasal axons do not.

The inference of the inhibitory nature of the posterior membranes raises an intriguing question. How is this inhibitory effect of posterior membranes compatible with the observation reported in the preceding paper that temporal axons grow equally well on anterior and on posterior membranes if these membranes are not offered in alternating stripes but rather in single stripes of either only anterior or only posterior membranes? Possibly mechanisms determining the direction of growth are not coupled to mechanisms controlling the speed of growth (see Bonhoeffer & Gierer, 1984). Alternatively, it is conceivable that contact inhibition is involved, but may be overcome after a while by habituation. In this context, the experiments of Kapfhammer (Kapfhammer, Grunewald & Raper, 1986; Kapfhammer & Raper, 1987) and J. A. Raper & E. B. Grunewald (unpublished data) may be of interest. These authors show that retinal axons retract when they contact
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sympathetic axons. This is mediated by a collapse of the growth cone structure. After collapse and retraction of the original growth cone, a new one forms and starts moving in the previous direction. Upon a second encounter with the sympathetic axon there is a good chance that the growth cone will cross over and, in fact, the third approach is almost always successful. Thus the growing axon may be losing its sensitivity. Analogously, it is conceivable but by no means proven that axons continuously exposed to posterior membranes may become habituated and lose their sensitivity towards the inhibitory component of posterior membranes, which may be achieved by a modification of the receptor (Pfenninger, 1986).

In this new test system, temporal axons show the same behaviour as they did in a previous assay system in which they had to choose between monolayers of tectal cells (Bonhoeffer & Huf, 1982). The posterior cells or plasma membranes that present the inhibitory component for temporal axons are derived from a tectal area which is never innervated by these axons (Crossland, Cowan & Rogers, 1975), as temporal axons project to the anterior tectum from the earliest stages of innervation. Among the various mechanisms proposed for the formation of the retinotectal map, guidance by chemical markers (Sperry, 1963) still seems to be the most promising. Gierer (1981) postulates, in a theoretical approach, a graded distribution of a few molecules which are specifically recognized by growing axons. These components could be either of an inhibitory or attractive nature. The present results show that axons originating in the temporal retina avoid a component of the posterior tectum that they will never innervate in vivo. Although it has not been shown for temporal axons that their specific avoidance of posterior membranes in vitro is related to the phenomenon of map formation in vivo, the results may be taken as circumstantial evidence that avoidance reactions or repellent components may play a critical role in the formation of neuronal projections.

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References


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