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# *IN VITRO* EXPERIMENTS ON AXONAL GUIDANCE AND GROWTH-CONE COLLAPSE

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## Summary

In the retinotectal projection, nasal retinal axons project to posterior tectum, while temporal axons project to the anterior part of the tectum. In *in vitro* experiments, a similar specificity can be observed: the nasal and temporal retinal axons can be guided by tectal membrane components so that, for example, temporal retinal axons, when growing on a striped substratum consisting of anterior and posterior tectal membranes, express a very strong preference for the anterior stripes. This preference is not due to attractivity of anterior membranes but rather to avoidance of posterior material, although the pure posterior membranes are a very good substratum for growth of temporal axons. The repellent guidance molecule has been identified. Interestingly, besides guidance this molecule causes another reaction: when growing temporal axons are exposed to medium containing either posterior membranes or artificial lipid vesicles containing the repellent guidance molecule, the axonal growth cones collapse. As in guidance, there is a clear regional specificity: e.g. the repellent guidance molecule derived from posterior tectum induces collapse of temporal but not of nasal axons.

Since the guiding and the collapse-inducing activity are expressed by one and the same glycoprotein molecule ($M_r 33\times10^3$, linked to the membrane by phosphatidylinositol) and since another molecule has been identified by Keynes' group which also expresses both guiding and collapse-inducing activity, one might speculate that axonal guidance and axonal collapse have something in common. Models of axonal guidance will be discussed.

## Introduction

Topographic neuronal projections are found in various functional areas of the central nervous system (Udin and Fawcett, 1988). The mechanism of the formation of these very highly ordered projections is not yet understood. The retinotectal projection of lower vertebrates and birds has often served as a system for experimental and theoretical investigations of axonal guidance and offers several experimental advantages. It consists of two structures, the retina being the source of the axons and the tectum being their target. The projection from source

Key words: growth cone, gradients, axonal guidance, growth cone collapse.

to target is very precise and topographic: in general, nasal axons project to posterior tectum and temporal axons to anterior tectum, dorsal axons to ventral tectum and, *vice versa*, ventral axons to dorsal tectum (Gaze, 1970). The projection contains many axons, about $10^6$ in the chick visual system. The source and target of the axons are anatomically nicely separated. Both have large surface areas that facilitate isolation and specific staining of target or source regions.

Early experiments on restoration of the visual system after cutting the optic nerve have suggested that both the cells of the target organ and the ingrowing axons carry on their surface molecules which serve to recognize positional or directional cues. Guidance was attributed to graded distributions of such surface molecules (Sperry, 1963; Gierer, 1981; Fraser, 1980; Bonhoeffer and Gierer, 1984). The complexity of the cell surface composition is prohibitive for a simple and straightforward biochemical or immunological search for directional or positional cues in the cell membrane.

Therefore, we have tried to develop *in vitro* systems of axonal guidance that allow the identification of guiding molecules. Two such *in vitro* systems, a guiding system and a system in which growth of axons is strongly influenced by external stimuli, will be reviewed in this presentation. The investigations to be discussed have led to the identification of a guiding molecule. It is a glycoprotein of $M_r 33\times10^3$ anchored in the cell membrane by a glycosyl–phosphatidylinositol linkage.

## The *in vitro* guidance system

Earlier experiments had suggested that retinal axons are guided to their target by positional or directional cues expressed on the surface of tectal cells (Bonhoeffer and Huf, 1980, 1982). In order to test whether growing retinal axons can distinguish between tectal membranes of different origins an *in vitro* system was developed in which growing axons were given the choice of two different substrata, anterior and posterior tectal membranes (Walter *et al.* 1987*b*). The tectal membranes were offered as a carpet consisting of alternating stripes of anterior and posterior tectal membranes. Axons growing on such carpets are confronted with a choice of the two substrata at the border between anterior and posterior membranes.

The preparation of striped carpets is depicted in Fig. 1. Cell membrane fragments of anterior or posterior origin are prepared by centrifugation (Walter *et al.* 1987*b*) and suspended in buffer. First suspension *A* (e.g. posterior membranes) is placed on a Nuclepore filter. The Nuclepore filter is supported by a silicone matrix containing a system of many parallel narrow channels which can be connected to a vacuum (Fig. 1A). When the vacuum is applied, membrane fragments are collected in parallel stripes on the Nuclepore filter. Sucking through the filter is continued until the pores are occluded, then the Nuclepore filter is placed on a porous support, suspension *B* (e.g. anterior membranes) is pipetted onto the filter and the lanes between the stripes of the membrane *A* fragments are

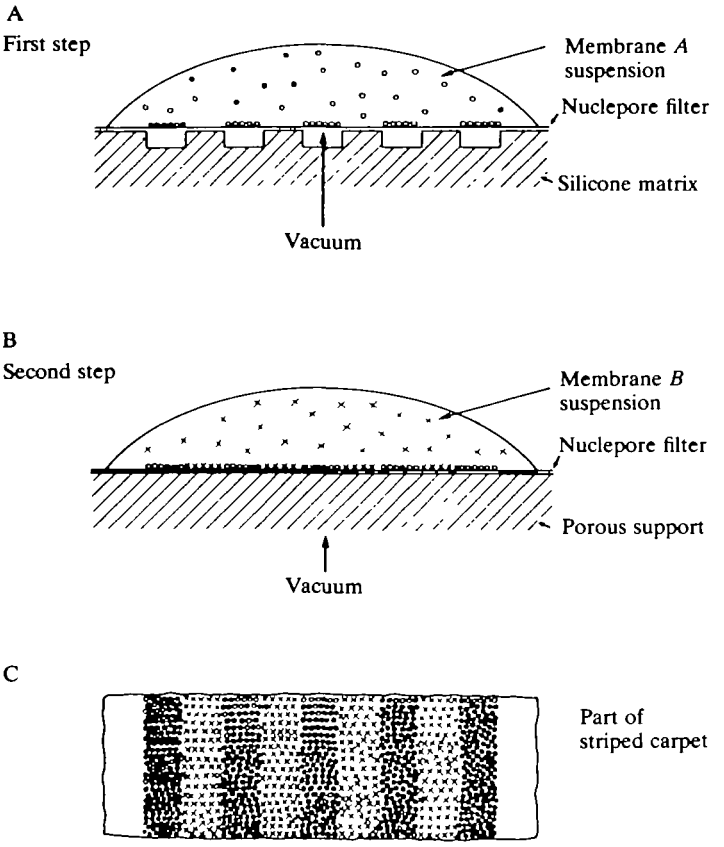


Fig. 1. Preparation of striped carpets. (A) A Nuclepore filter is placed on a silicone matrix containing many parallel channels. A suspension of membrane *A* vesicles is placed onto the filter and a vacuum is applied to the channels. Membrane *A* vesicles are collected in stripes above the channels until the filter is occluded by the membranes. (B) The filter containing the membrane *A* stripes is placed onto a porous support. Upon sucking, membrane *B* fragments are collected in the empty lanes between the membrane *A* stripes. (C) Part of a striped carpet, schematic drawing (width of the stripes 90 $\mu$m).

filled with type *B* fragments. Thus, a carpet with alternating *A* and *B* stripes is formed (Fig. 1C). When retinal explants are placed on such carpets, temporal retinal axons, which *in vivo* terminate in anterior tectum, grow exclusively on anterior membranes (Fig. 2A), i.e. they show *in vitro* the same regional specificity as *in vivo* and are clearly guided in the orientation of the stripes. Nasal axons in this experiment, however, cross the boundaries between anterior and posterior substrata quite freely (Fig. 2B); they do not show the expected preference for growth on posterior membranes (Walter *et al.* 1987*b*). It should be mentioned, however, that in a similar experiment (Y. von Boxberg, unpublished results), in which the cell membranes were prepared differently, the expected preference of nasal axons for posterior material is found. Thus, from experiments with this

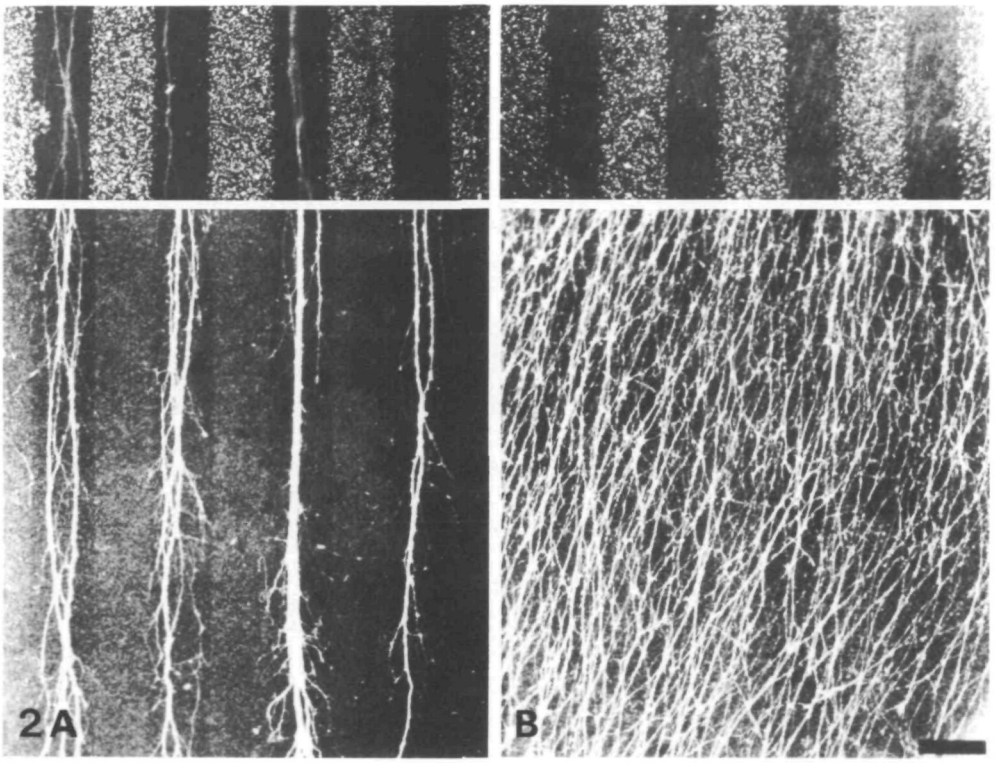


Fig. 2. Temporal (A) and nasal (B) axons growing on striped carpets. Retinal explants have been stained with rhodamine-B-isothiocyanate (RITC) and were placed on striped carpets consisting of anterior and posterior tectal membranes. (A) Temporal axons grow preferentially on anterior stripes and leave the posterior stripes free of neurites. (B) Nasal axons do not show a preference for one of the stripes. The position of posterior lanes is indicated in the upper part of the figure. Fluorescent beads served as a marker for the posterior lanes. Scale bar, 100 $\mu$m.

*in vitro* system it follows that retinal axons can distinguish between anterior and posterior tectal membranes and that nasal and temporal axons have different recognition abilities. It has further been shown that the difference between anterior and posterior tectum exists only during the time when the projection is being formed. It disappears in chick at about embryonic day 12 (E12), the end of the formation of the projection (Walter *et al.* 1987*b*) and in mouse at about postnatal day 0 (P0) (Godement and Bonhoeffer, 1989), a time when the projection is finished. In fish, where new ganglion cells are produced and connected to the visual centre throughout their life, the anterior/posterior difference is detected at all ages (Vielmetter and Stürmer, 1989).

These and other experiments are in keeping with the notion that the observed guidance phenomena may be related to *in vivo* guidance. It is of interest to note that both the guiding components as well as the molecules and mechanisms recognizing the guiding molecules seem to be conserved through evolution. For

example, in the stripe assay, temporal retinal axons of fish (Vielmetter and Stürmer, 1989) and mouse (Godement and Bonhoeffer, 1989) can be guided by chick tectal membranes.

The results of the stripe assay allow two alternative explanations of the observed guidance; growing temporal axons could be attracted by anterior membranes or, alternatively, they could be repelled by posterior membranes or posterior membranes could be inhibitory for growth of temporal axons. Several experiments indicate that anterior membranes are not attractive and that posterior membranes are either repellent or inhibitory for temporal axons. Inactivation of posterior membranes by heat (Walter *et al.* 1987*a*) or by treatment with the enzyme phosphatidylinositol-specific phospholipase *C* (PI-PLC) (Walter *et al.* 1990) or by treatment with an antiserum directed against posterior tectal membranes (E. Cox and B. Stahl, unpublished results) abolishes the repellent or inhibitory property of posterior tectal membranes. Membrane carpets consisting of alternating stripes of anterior and of inactivated posterior membranes no longer guide temporal axons; the axons cross the stripe boundaries freely.

As mentioned above, the striped outgrowth of temporal axons on carpets of alternating anterior and posterior stripes could be due to repulsion of posterior membranes or to inhibition of growth of temporal axons by posterior membranes. Interestingly, temporal axons which never grow on the posterior lanes of the striped carpets grow quite well on posterior membranes if they have no other choice. Growth rate and number of axons are similar to those achieved on an anterior substratum (Walter *et al.* 1990) (Fig. 3A,B). This implies that posterior membranes either do not cause inhibition of growth at all or, if they are inhibitory, growth cones of temporal axons can adjust or habituate so that the posterior material no longer has an inhibitory influence on their growth. There are other investigations that support the idea of repulsion and show no growth inhibition. The growth rate of temporal axons approaching anterior/posterior borders at a slight angle and then growing along the border has been determined. The growth rate seems not to be reduced while the axons are in contact with the posterior material (Müller, 1988; Walter *et al.* 1990). Only those axons approaching the boundary at large angles show a transient reduction in the growth rate. The growth cones of such axons frequently collapse (see below).

### Induction of growth-cone collapse

In 1986, Kapfhammer *et al.* observed in time-lapse studies that some growth cones collapse when they encounter heterotypic neurites *in vitro* (Kapfhammer *et al.* 1986) (Fig. 4). The process starts a few minutes after the first contact. The growth cone retracts its lamellipodia and most of the filopodia, rounds up, becomes more dense under phase contrast, loses contact with the substratum and retracts under the tension of the axon, often leaving behind a thin thread.

In order to investigate the molecular mechanisms leading to growth-cone collapse, a mechanism that could be involved in guidance by growth inhibition,

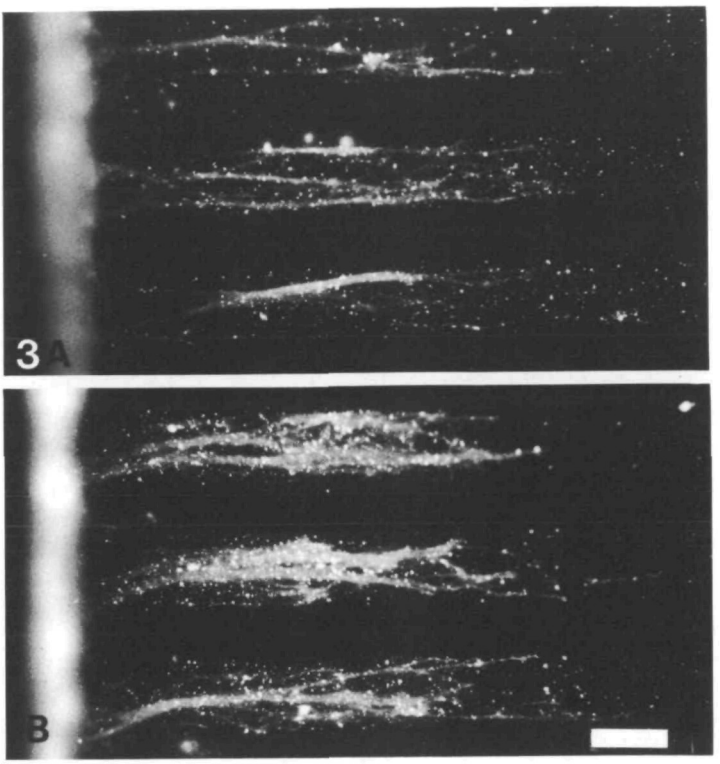


Fig. 3. Growth of temporal axons on carpets (A) consisting exclusively of posterior stripes; (B) consisting exclusively of anterior stripes. The explants have been fluorescently labelled with 3,3′-dioctadecyloxacarbocyanine perchlorate (DIO) (Honig and Hume, 1986). All cultures have been incubated for the same length of time (36 h) under standard conditions. Scale bar, 100 $\mu$m.

Raper and Kapfhammer tried to isolate and identify the component which induces the collapse. For this end they developed a simple *in vitro* assay for induction of growth-cone collapse (Raper and Kapfhammer, 1990). Isolated membrane fragments of whole brains were added to peripheral axons growing on laminin-coated coverslips. This led to a collapse which was either observed with time lapse recordings or after fixation. Cox *et al.* (1990) have applied this assay system to investigate collapse of growth cones in the retinotectal system. Interestingly, with respect to regional specificity, the observations resemble the findings in the *in vitro* guiding assay. Temporal growth cones are caused to collapse by contact with posterior tectal membranes (Fig. 5), whereas anterior tectal membranes have no strong effect on the morphology of the growth cones (Fig. 6). No visible change in growth rate and morphology is apparent if anterior membranes are added to nasal growth cones (Fig. 7). The same is true for the majority of nasal growth cones when posterior membranes are added (Fig. 8). However, unpublished time-lapse studies revealed that a minority of nasal growth cones showed a transient change in growth rate and morphology. Quantitative evaluation of some experiments is

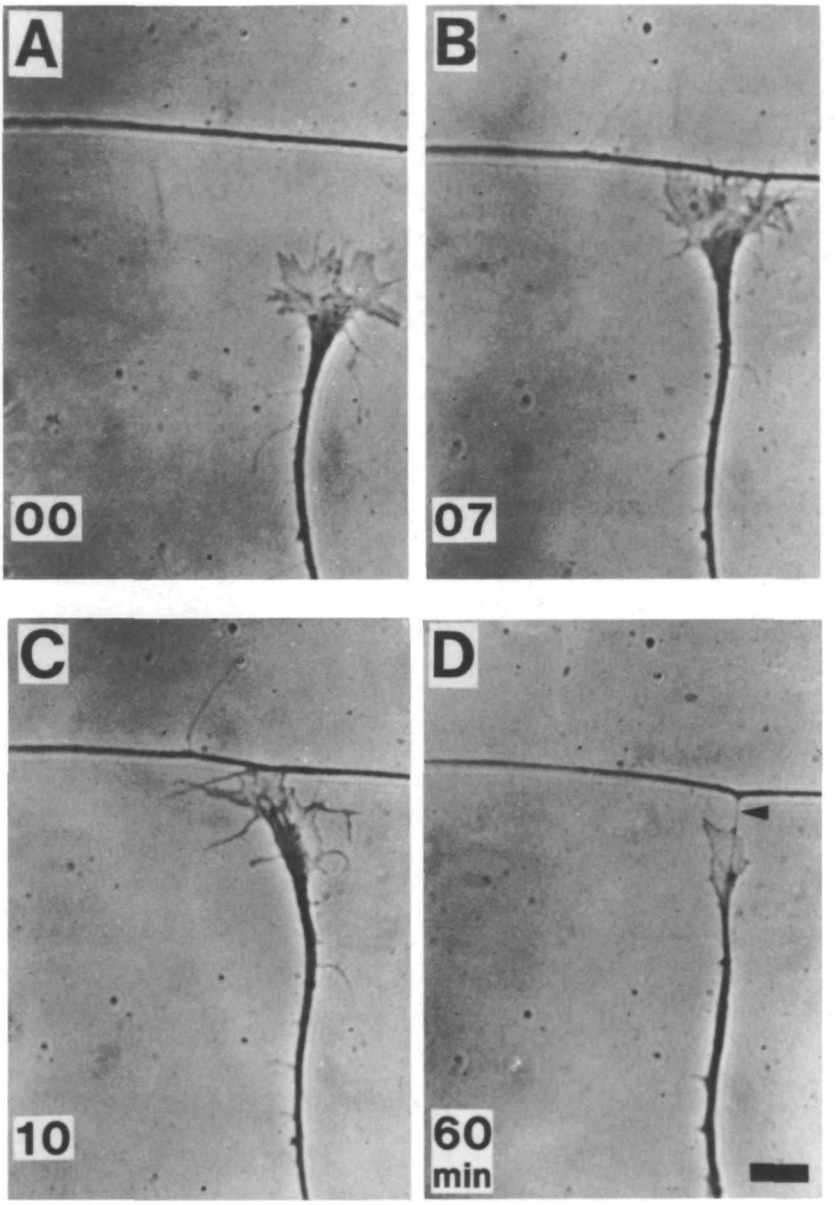


Fig. 4. The first documented growth-cone collapse (Kapfhammer and Raper, 1987*a*). Retinal growth cone encountering a sympathetic neurite. In this case growth-cone collapse is not complete (courtesy of J. Kapfhammer). Scale bar, 10 $\mu$m.

given in Table 1. Besides normal and collapsed growth cones there are always some growth cones which have an intermediate appearance (Fig. 9A,B,C). In fixed material it is not possible to decide whether these growth cones are in the process of collapsing and so they are listed as intermediate growth cones.

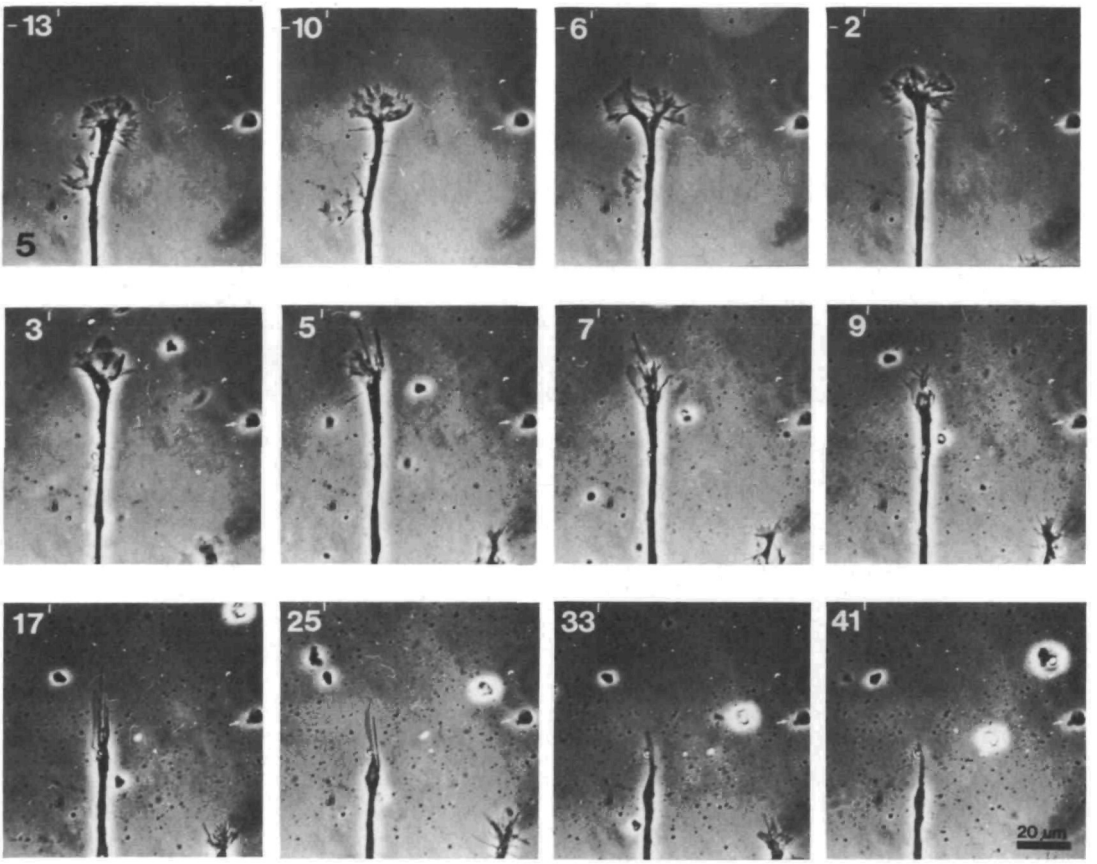


Fig. 5. Addition of posterior tectal membranes to temporal growth cones induces growth-cone collapse. Posterior tectal membranes were added to temporal axons growing on a laminin substratum. Pictures were taken at times (in minutes) indicated at the upper left corners. First indication of collapse of temporal growth cones is seen at about 5 min. Collapse is complete at 15–20 min. Note, that the number of membrane particles settled in the focal plane of the growth cone increases with time.

The experiments described show quite clearly that posterior tectal membranes can cause temporal retinal growth cones to collapse. This collapse-inducing activity can be abolished by treatment with the enzyme PI-PLC (J. Walter, B. Stahl and F. Bonhoeffer, in preparation) and with antiserum (B. Stahl, B. Müller, Y. von Boxberg, E. Cox and F. Bonhoeffer, in preparation) directed against posterior membranes (Table 2). This is analogous to the PI-PLC and antiserum sensitivity of the guiding activity of posterior membranes (Walter *et al.* 1990; E. Cox, unpublished results; B. Stahl, B. Müller, Y. von Boxberg, E. Cox and F. Bonhoeffer, in preparation). Another similarity between guiding and collapse-inducing activity came from preliminary findings concerning the adaptation or habituation phenomenon, namely that temporal axons, which avoid posterior membranes if they have the choice between anterior and posterior

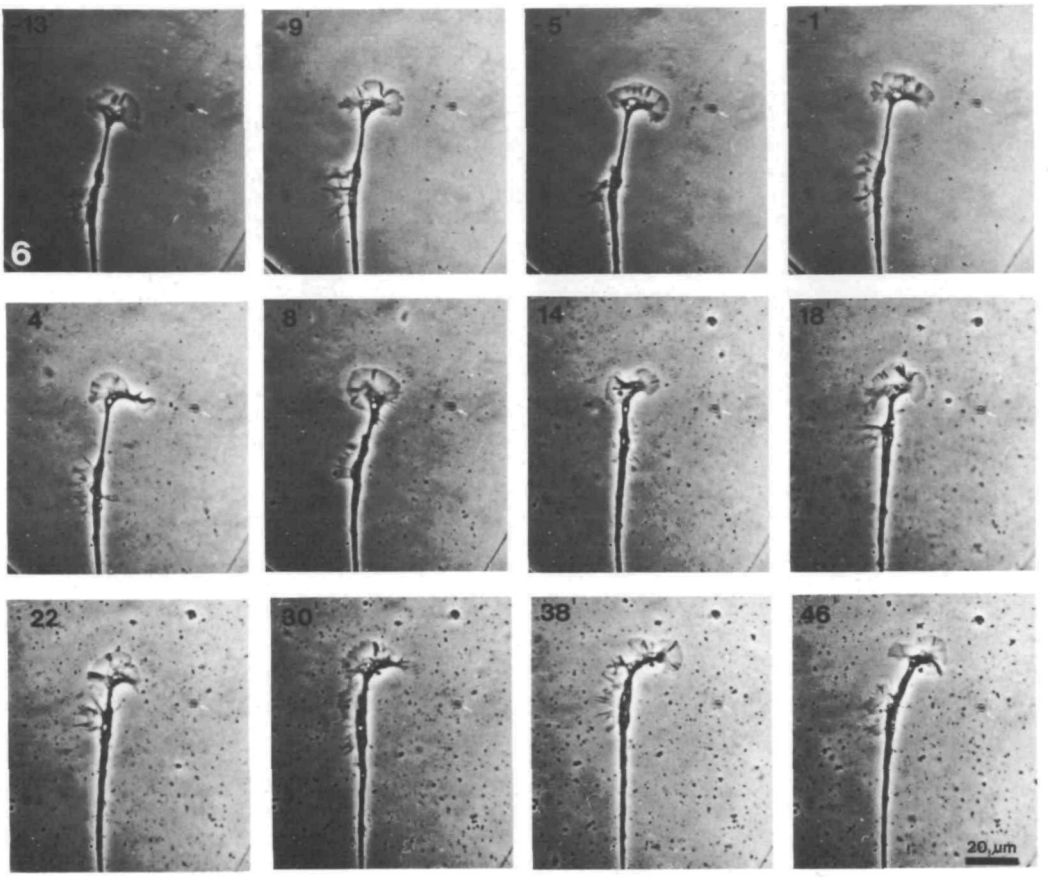


Fig. 6. Addition of anterior tectal membranes to temporal growth cones induces no collapse. The only difference compared to Fig. 5 is that the vesicles are of anterior tectal origin.

stripes, do grow on posterior membranes if there is no choice. Posterior membranes do not induce collapse of temporal growth cones when these are growing on posterior membranes (B. Müller, unpublished results). Thus, temporal growth cones can somehow become resistant to the collapse-inducing activity.

### Identification and isolation of the guiding and collapse-inducing $M_r$ $33\times10^3$ glycoprotein

The similarities between the guiding and the collapse-inducing activity suggest that the two phenomena are due to one and the same molecule. In order to find a substance responsible for one of the two processes, a protein was sought that is recognized by the antiserum mentioned above, and the concentration of which is higher in posterior than in anterior tectal membranes (B. Stahl, B. Müller,

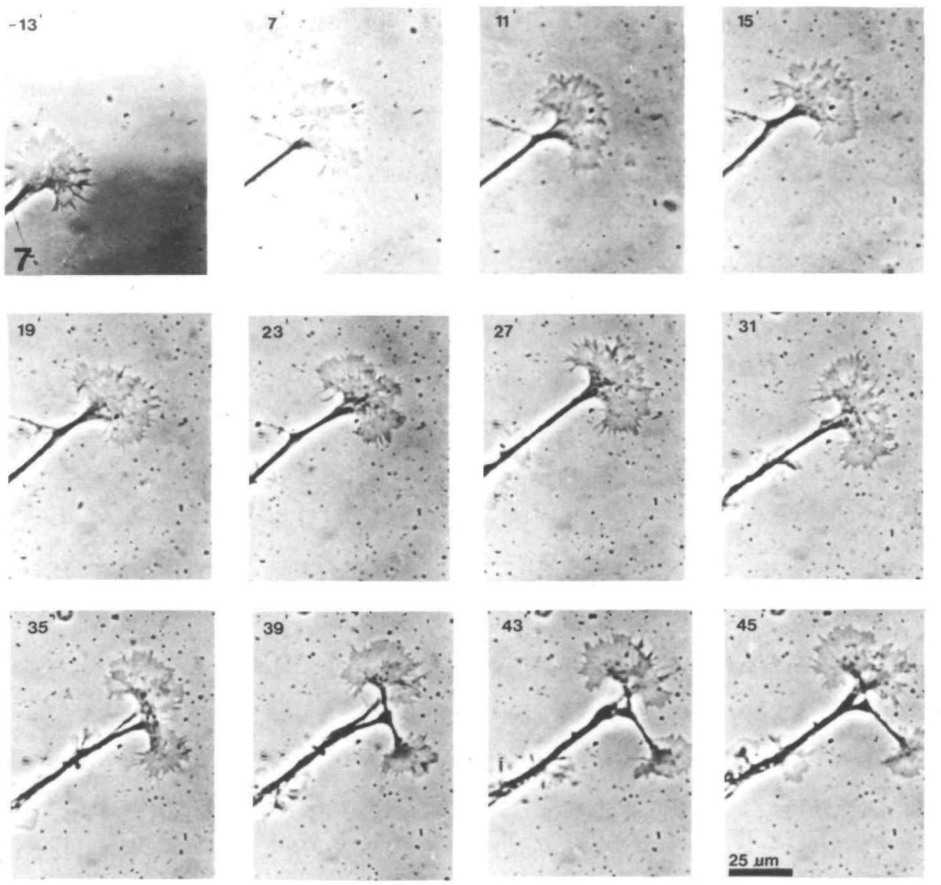


Fig. 7. Addition of anterior tectal membranes to nasal growth cones induces no collapse.

Table 1. *Effect of posterior membrane vesicles on temporal and nasal growth cones*

| | Temporal growth cones | Nasal growth cones |
|---|---|---|
| Normal | 17.7±6.3 % | 54.2±8.7 % |
| Intermediate | 13.8±0.07 % | 19.6±0.07 % |
| Collapsed | 68.5±6.2 % | 26.2±8.6 % |

Growth cones were classified into three groups: normal, intermediate and collapsed. Values are mean±S.D. ($N$=4).

Y. von Boxberg, E. Cox and F. Bonhoeffer, in preparation). One such candidate is seen in a blot of a one-dimensional gel stained with antiserum. In Fig. 10 the arrow marks a component of $M_r$ $33\times10^3$ which is expressed much more strongly on posterior than on anterior membranes. This component is enriched by a

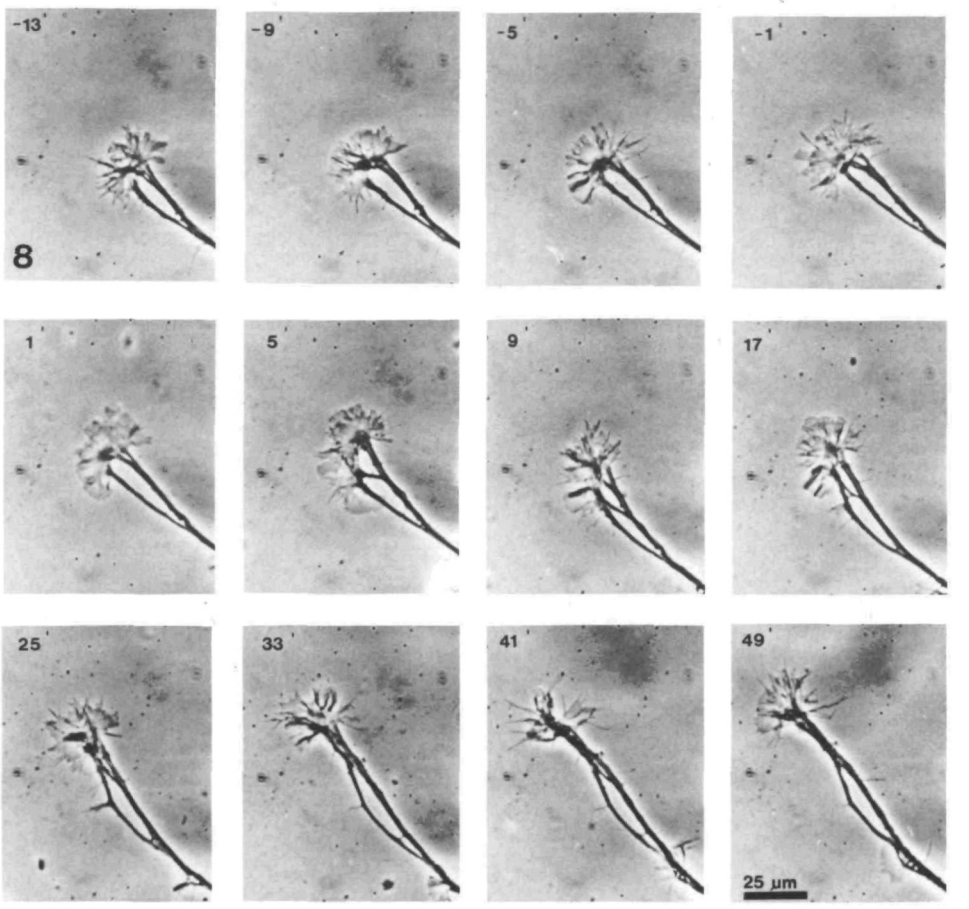


Fig. 8. Addition of posterior tectal membranes to nasal growth cones induces no collapse.

Table 2. *Inactivation of the collapse-inducing activity by PI-PLC and antiserum*

| | Percentage of temporal growth cones | | |
|---|---|---|---|
| | Normal | Intermediate | Collapsed |
| Control (+culture medium) | 73.1±6.0 | 15.8±1.3 | 11.3±4.7 |
| Posterior membranes (untreated) | 15.2±9.3 | 11.9±2.7 | 72.9±12.0 |
| PI-PLC-treated posterior membranes | 70.6±7.7 | 13.9±3.9 | 15.5±4.7 |
| Antibody-treated posterior membranes | 45.4±5.4 | 26.8±5.3 | 27.8±9.7 |

Values are mean±S.D. ($N$=5).

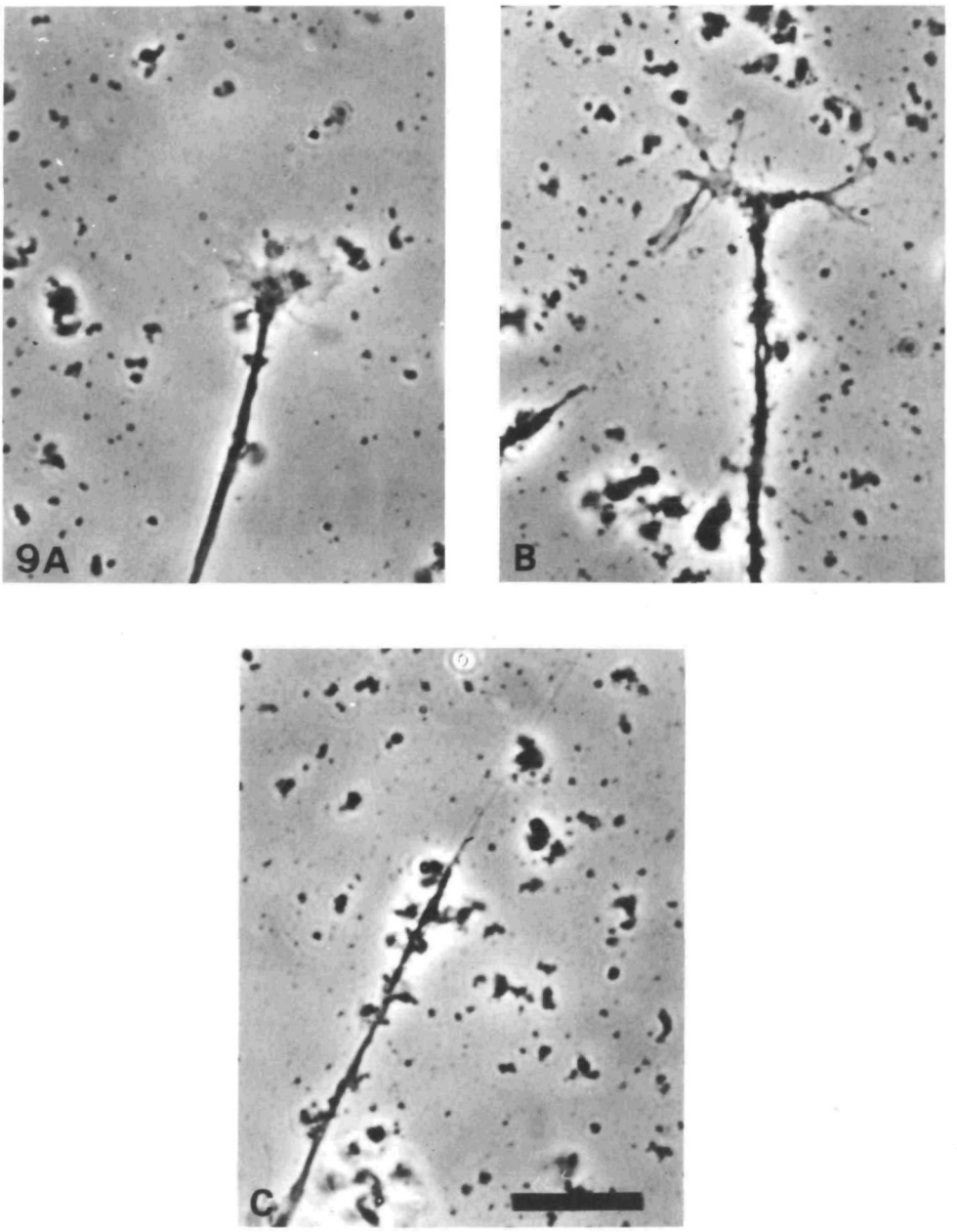


Fig. 9. Classification of growth cone morphology. (A) Normal growth cone; (B) intermediate growth cone; (C) collapsed growth cone. Scale bar, 20 $\mu$m.

purification procedure consisting of the following steps (B. Stahl, B. Müller, Y. von Boxberg, E. Cox and F. Bonhoeffer, in preparation).

(1) Embryonic brains of E9 and E10 chicks are homogenized in buffer containing urea and spermidine; after removal of nuclei, non-lysed cells and other

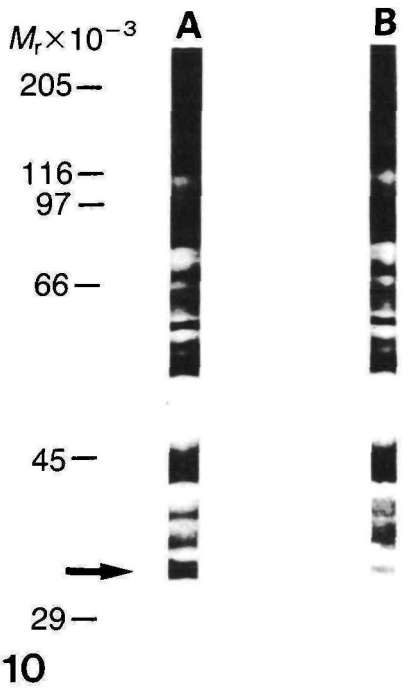


Fig. 10. Immunoblot of posterior (A) and anterior (B) membranes. The arrow marks an $M_r 33 \times 10^3$ protein which is differentially expressed in anterior and posterior tectal membranes.

debris by low-speed centrifugation, membranes are pelleted and washed. The protein composition of this crude membrane fraction is depicted in Fig. 11A.

(2) The membranes are solubilized in buffer containing urea and 3-[(3-cholamido-propyl)-dimethylammonio]-1-propane sulfonate (CHAPS) and non-solubilized material is removed by centrifugation. Solubilized membranes are fractionated in the presence of CHAPS on DEAE Sepharose. Fractions are eluted at various NaCl concentrations; the composition of a $1\,\mathrm{mol\,l^{-1}}$ NaCl fraction is shown in Fig. 11B.

(3) Solubilized phosphatidylcholine is added to the various fractions. They are freed of CHAPS and urea by dialysis. This step leads to the formation of lipid vesicles. Lipid vesicles prepared with the protein fraction eluted from DEAE Sepharose at $1\,\mathrm{mol\,l^{-1}}$ NaCl contain only three major protein bands (as shown in Fig. 11C). One of these bands, the $M_r 33 \times 10^3$ component, is a glycoprotein. It is recognized by peanut lectin (Fig. 12). Its concentration in the posterior tectum decreases with developmental age (Fig. 13), as required, and it contains a glycosyl–phosphatidylinositol anchor, as shown by its sensitivity to the enzyme PI-PLC (Fig. 14). The vesicles containing the three protein bands have been tested in the collapse and in the stripe assay. They contain both collapse-inducing and guiding activity (B. Stahl, B. Müller, Y. von Boxberg, E. Cox and F. Bonhoeffer,

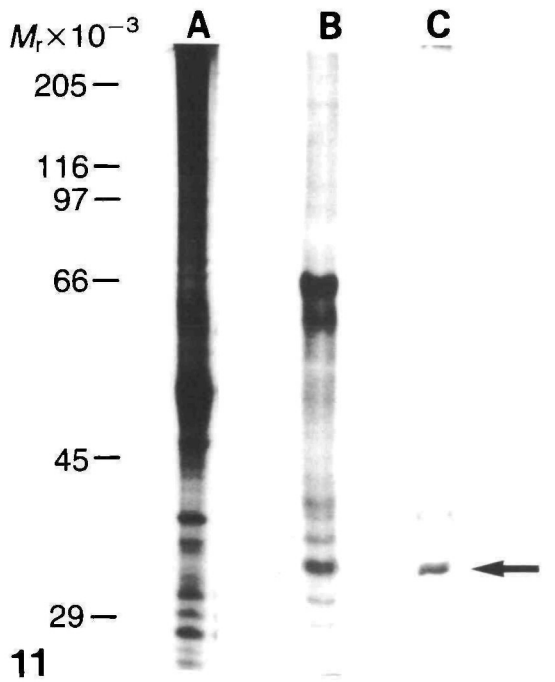


Fig. 11. Biochemical enrichment of the $M_r 33 \times 10^3$ protein. (A) Crude membrane fraction; (B) 1 mol $l^{-1}$ NaCl eluate of DEAE Sepharose column; (C) phosphatidylcholine vesicles containing the 1 mol $l^{-1}$ NaCl eluate. Cells are stained with silver. Arrow marks the $M_r 33 \times 10^3$ component.

in preparation). Comparison of the specific activities of the native posterior membranes and of the reconstituted vesicles gives an enrichment of the posterior components by a factor of at least 350 (B. Stahl, B. Müller, Y. von Boxberg, E. Cox and F. Bonhoeffer, in preparation). This is a low estimate since it is based on the determination of biological activity which may not be stable during the purification procedure.

Direct proof that the $M_r 33 \times 10^3$ glycoprotein is involved in the formation of the topographic projection is still missing. It is most likely to come from *in vivo* experiments with antibodies directed against this molecule. Such *in vivo* application during the formation of the retinotectal projection may interfere with the precision of its topography.

**Molecules with guiding and collapse-inducing activity identified in other systems**

Membrane-bound molecules which have similar inhibitory (or repellent or guiding) activities for axonal growth and which are different from the $M_r 33 \times 10^3$ protein have been found in several other systems. Caroni and Schwab (1988*a*,*b*) were the first to show that the central nervous system (CNS) contains membrane-bound components which are inhibitory for growth of sympathetic and sensory

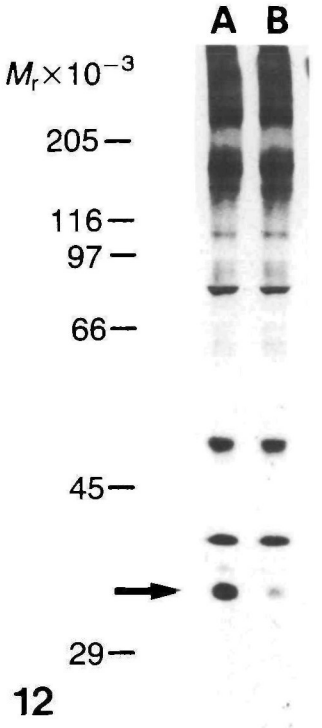


Fig. 12. Peanut lectin binds to the $M_r 33 \times 10^3$ component (arrow). (A) Posterior membranes; (B) anterior membranes.

neurites. The inhibitory components have been characterized as two proteins of $M_r 35 \times 10^3$ and $250 \times 10^3$ by monoclonal antibodies and were shown to be part of the CNS myelin. Both components are probably different from the $M_r 33 \times 10^3$ component described in this presentation, for two reasons. First, both components are myelin-associated. Myelin does not occur in the source of the $M_r 33 \times 10^3$ component, because chick tecta and chick brains are not myelinated up to embryonic day E10. Second, the monoclonal antibody IN-1 isolated by Caroni and Schwab does not inactivate the repellent activity in the stripe assay (P. Caroni and M. E. Schwab, unpublished results).

Recently, Davies *et al.* (1990) have identified two glycoproteins ($M_r 55 \times 10^3$ and $48 \times 10^3$) which are found in the posterior and not in the anterior halves of sclerotomes and which seemingly have an inhibitory or repellent activity on growth of motor and sensory axons and on neural crest migration. These glycoproteins cause growth cones of dorsal root ganglia to collapse. These results are analogous to the findings with the $M_r 33 \times 10^3$ protein.

Raper and Kapfhammer (1990) have described a collapse-inducing activity derived from chick brain. The molecular weight has not yet been determined. In contrast to the activity described here, this collapse-inducing activity is destroyed by high concentrations of urea. This may be taken as an indication that this

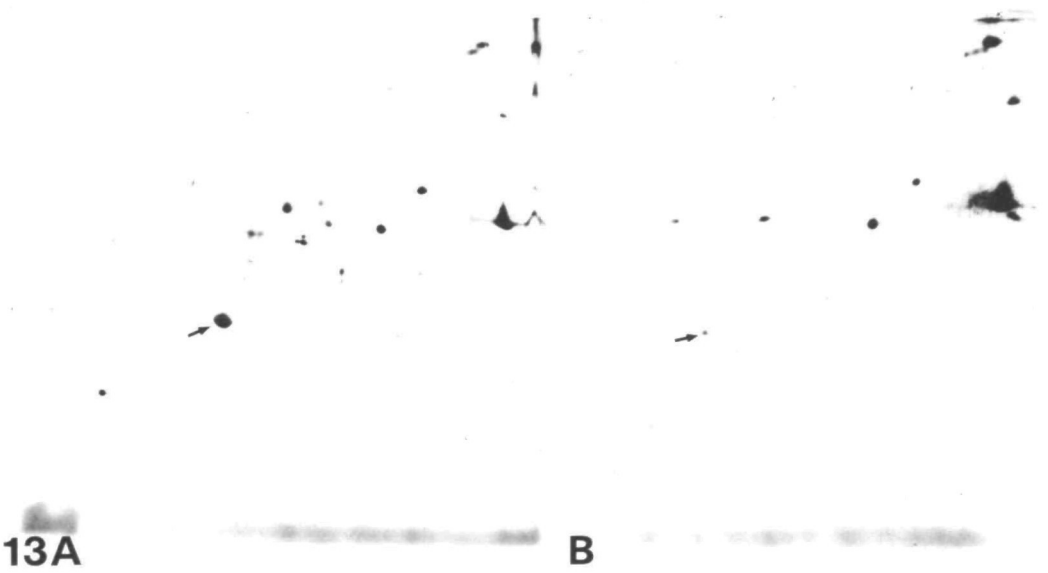


Fig. 13. Age-dependent expression of the $M_r$ $33\times10^3$ glycoprotein (arrows). Immunoblots: (A) embryonic day 9, (B) embryonic day 15.

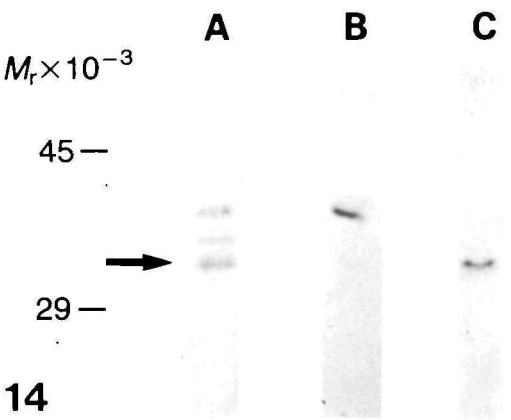


Fig. 14. PI-PLC sensitivity of the $M_r$ $33\times10^3$ protein. Immunoblots: (A) reconstituted membrane proteins before treatment with PI-PLC; (B) membrane pellet after 60 min incubation with PI-PLC; (C) supernatant after 5 min incubation with PI-PLC.

molecule is different from the $M_r$ $33\times10^3$ component. However, more experiments are needed to confirm this conjecture.

**Relationship between axonal guidance and growth-cone collapse**

The finding that one and the same molecule is involved in two different

phenomena suggests that the two phenomena are related and have a common denominator. What is the relationship between guidance and growth-cone collapse? What is the basic mechanism? The answer is not known. Two hypotheses have been discussed in a review quite recently (J. Walter, T. E. Allsopp and F. Bonhoeffer, in preparation) and will be mentioned here only very briefly.

In the model of axonal guidance suggested by Gierer (1987) the growth cone is a structure which is extremely sensitive to very slight concentration differences between its edges. The high degree of sensitivity is needed to explain guidance by an external gradient. Such a gradient normally has a spatial extension which is large compared to the dimensions of the growth cone. Therefore, concentration differences between the two margins of the growth cones are very small. In this model, the slight external gradient is converted to an internal gradient which is amplified by an autocatalytic process. The growth cone reacts only to concentration differences, i.e. to gradients. In this guidance model, collapse is interpreted as an over-reaction of the growth cone to sudden and strong changes in concentration of the guiding substance that occur when membranes are added to growing axons (J. Walter, T. E. Allsopp and F. Bonhoeffer, in preparation).

In the other model, collapse is seen as the basic phenomenon of guidance. Induced collapse and the accompanying processes, such as loss of adhesion, reorganization or destruction of the cytoskeletal structure and the resulting paralysis, are assumed to be transient and very local within the growth cone. It is suggested that these local phenomena direct the growth of the axon. Besides the local collapse-inducing or paralysing activity this model requires a habituation phenomenon in order to allow guidance of axons in the presence of high concentrations of a repellent guiding molecule (J. Walter, T. E. Allsopp and F. Bonhoeffer, in preparation).

To understand axonal guidance we will have to collect more information about the various components involved, about the receptor of the guiding signals, the role played by second messenger systems, their interaction with the cytoskeleton and its motility. Today we are still very far from understanding how axons are guided to their target.

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