In vitro assay to test differential substrate affinities of growing axons and migratory cells

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Summary. An in vitro assay is presented in which different soluble substrates are arranged in narrow alternating stripes which forces growing axons and migratory cells to choose between them. The usefulness of this assay is exemplified by offering goldfish retinal axons and glial cells of the optic nerve a variety of substrates in stripes. Given a choice between substrates of unequal growth supporting activities axons and migratory cells grow in stripes, thus expressing their preference for one of the substrates. Growth in stripes was observed 1. when a substrate with growth promoting properties was next to one which did not possess these properties, 2. when the growth promoting activity of a substrate applied to both stripes was in one stripe blocked by an antibody, 3. when two different growth promoting substrates were offered.

Key words: Choice assay – Soluble substrates – Retinal axons – Migratory cells – Growth in stripes

Introduction

Growing axons and migratory cells interact with their environment and receive signals that guide them along specific pathways. The growth cone's and cell's natural environment is complex such that the factors involved in axonal growth and guidance in vivo are hardly known. Valuable tools for analysing the mechanisms that mediate axonal navigation and directed cell migration are in vitro assays.

The purpose of this study is to present and to exemplify the application of an in vitro assay in which different substrates are arranged in narrow alternating lanes so as to force growing axons and migratory cells to choose between them. The axons and cells that recognize and respond to such substrate differences are expected to accumulate on one of the lanes and to grow in stripes.

A choice assay similar in its design has been described earlier (Walter et al. 1987a, b), in which growing retinal axons were exposed to cell surface membranes of two different regions of their target (Walter et al. 1987a, b; Vielmetter and Stuermer 1989a). In contrast to the membrane assay in the assay of the present study soluble proteins are bound to glass supports in narrow alternating stripes.

To demonstrate the ability of axons and cells to express a preference when being exposed to "striped" substrates we used goldfish retinal axons and migratory goldfish glial cells (Bastmeyer et al. 1989) and a variety of proteins as substrates.

Some of these results have been published in abstract form (Vielmetter et al. 1986).

Material and methods

Substrate stripes: To bind two different substrate proteins in parallel and alternating stripes a special silicone matrix was designed (Fig. 1). This matrix has parallel channels of 50 μm width which are separated by bars of 40 μm width. On both ends the parallel channels merge into an inlet and outlet channel, respectively. This matrix is placed onto a glass surface, such as a coverslip. The first protein solution is filled into the open channels through the inlet channel. The protein binds to the glass (incubation periods of 2 h at 37° C). Unbound protein is removed by 3 washes with phosphate buffered saline (PBS, pH 7.4). To saturate open binding sites a solution of hemoglobin [Hb] is added into the channel system and after 2 h at 37° C and 3 washes in PBS the matrix is removed and the second substrate protein applied to the coverslip. Protein 2 will bind to the lanes which have previously been protected by the bars. Again, 3 washes of PBS follow after 2 h of incubation at 37° C. In some experiments the hemoglobin applied to the first stripe was coupled with FITC, to visualize the stripes at the end of the experiment.

The protein concentration in the solution that was given into the channels was always 5 $\times$ higher than the concentration of the same protein in the solution that was applied to the whole coverslip (after the removal of the matrix). This rise in the protein concentration for the channels was necessary to compensate for the smaller volume of solution (and hence lower amount of total protein) that the channel system receives.

In 2 experiments antisera against the substrate proteins laminin and ependymin (see below) were used to demonstrate their efficacy

SILICONE MATRIX:

Fig. 1. Schematic representation of the silicone matrix B: bars, C: channel, I: inlet, O: outlet channel, T: transverse channel, G: glass-coverslip

in blocking the growth promoting properties of these proteins. The antibodies, a polyclonal antiserum against EHS laminin (kindly provided by D. Edgar) and a polyclonal antiserum against ependymin (protein and antibody provided by R. Schmidt), diluted 1:10 with PBS, were added to the channel of the matrix after laminin and ependymin, respectively, had been applied and bound to the glass over the incubation period of 2 h. In control experiments neutral rabbit serum (same source as the antibodies), diluted 1:10, was used instead of the antisera. Incubation time was 2 h at 37°C and was followed by 3 washes with PBS, before Hb was added. After the removal of the matrix, and upon subsequent application of laminin or ependymin, respectively, to the entire coverslip, neutral rabbit serum was added. Incubation times and washes with PBS were as described above.

Substrate proteins and their concentration

Substrate proteins were laminin (EHS, Gibco BRL) fibronectin (human, Serva), concanavalin A (Con A, Serva), poly-L-lysine (Plys, 150–300 kD, Sigma), hemoglobin (Hb bovine, Serva), ependymin from goldfish extracellular brain fluid (containing ependymin β and γ) prepared after Schmidt and Lapp (1987), and provided by R. Schmidt, and glycoproteins from cell surface membranes of the goldfish brain and enriched by lentil lectin affinity chromatography (LL-extract, see below).

1 mg lyophilized fibronectin was dissolved in 1 ml buffer (0.1 M NaHCO_3 and 2 M Urea) and dialysed 24 h against PBS (pH 7.4). The stock solutions of laminin (1 mg/ml in 50 mM Tris-HCl and 0.15 M NaCl (pH 7.2)) and fibronectin were diluted with PBS to a concentration of 20 $\mu\text{g}/\text{ml}$ for laminin and 50 $\mu\text{g}/\text{ml}$ for fibronectin or to 100 $\mu\text{g}/\text{ml}$ and 250 $\mu\text{g}/\text{ml}$ respectively when applied to the channels. Hemoglobin, concanavalin A and poly-L-lysine were dissolved in PBS and used in a concentration of 4 mg/ml for Hb, 5 mg/ml for Con A and 200 $\mu\text{g}/\text{ml}$ for PLys and in 5 times higher concentrations for the channels.

Round 100 μm thick glass coverslips (20 mm in diameter), cleaned in boiling HNO_3 served as substrate supports.

Extraction of glycoproteins from cell surface membranes of the goldfish brain (LL-extract)

A fraction enriched in cell surface membranes from 50 adult goldfish brains was obtained as described in Vielmetter and Stuermer (1989). To solubilize membrane proteins the membranes were homogenized in 2 ml OG-lysisbuffer: 20 mM Tris-HCl, pH 7.4, 100 mM n-octyl b-D-glucopyranoside, 150 mM NaCl, 1 mM CaCl_2 , 1 mM MgCl_2 and inhibitors (aprotinin (25 $\mu\text{g}/\text{ml}$), pepstatin (5 $\mu\text{g}/\text{ml}$) leupeptin (25 $\mu\text{g}/\text{ml}$), and 2,3-dehydro-2-deoxy-N-acetylneuraminic acid (15 $\mu\text{g}/\text{ml}$)). Homogenization was carried out in an ultrasound-bath (Transsonic 310, ELMA) for 30 min. The membrane lysate was centrifuged 10 min at 150×10^3 g and the resulting supernatant 1 h at 150×10^3 g. The supernatant was incubated with 1 ml lentil lectin-sepharose 4B (Pharmacia) at 4°C over night under agitation. The column material was washed with OG-lysisbuffer 5 times and the bound glycoprotein fraction eluted by 1 h incubation at 4°C with elution buffer: 100 mM n-octyl b-D-glucopyranoside, 1 M methyl α -D-mannoside and inhibitors (see above). The eluate (lentil lectin extract = LL-extract) was concentrated using Ultrafree centrifuge tubes (Millipore) and adjusted to a final protein concentration of 1 mg/ml. Protein concentrations were measured using Bio-Rad protein-assay. Prior to use the LL-extract was diluted with PBS to a final concentration of 20 $\mu\text{g}/\text{ml}$ and 100 $\mu\text{g}/\text{ml}$, respectively.

Retinal explants. Explants (of 300 μm width) from isolated goldfish retinæ were placed onto the substrate proteins, perpendicular to substrate lanes. The preparation of these explants and culture conditions were as described earlier (Vielmetter and Stuermer 1989a).

Axon staining. To stain the axons with Rhodamine (according to McKenna and Raper 1989) axons were fixed with 2% glutaraldehyde in PBS (2 h), washed in distilled water, dried at room temperature and incubated for 10 min with a Rhodamine solution (1 mg Rhodamine isothiocyanate (RITC) in 10 μl DMSO and 10 ml PBS). Excess RITC was removed through washes in 70% and 96% ethanol (1 min each), the preparation was dried at room temperature, embedded in Entellan (Boehringer) and photographed either under fluorescent light with the appropriate filter sets on a Zeiss axiophot or with phase contrast on a Zeiss axiovert.

Glial cells. Glial cells were obtained from explants of the regenerating goldfish optic tract as described in Bastmeyer et al. (1989).

Results

Axonal outgrowth and elongation, as well as the restriction to defined domains is dependent on the properties and spatial distribution of the substratum with which the growth cone interacts.

Growth of goldfish retinal axons on glass surfaces or glass saturated with Hb (Hb-glass) is poor. Giving axons a choice between lanes of Hb-glass and any other substrate, allows to evaluate whether the other substrate possess growth promoting properties exceeding that of Hb-glass. Provided that axons can discriminate in this choice assay substrates of different growth supportiveness the axons are expected to grow along the substrate stripe of higher growth promoting activity.

A variety of substrates, either commercially available (laminin, Con A, fibronectin) or prepared from goldfish brains (Lentil lectin (LL-) extract, ependymin) were compared to Hb-glass. When exposed to alternating lanes of these substrates and Hb-glass the axons grew in lanes. Figure 2 demonstrates axonal growth when exposed to LL-extract versus Hb-glass. The axons grew on the LL-

Fig. 2a, b. Growth pattern of goldfish retinal axons on a striped substrate, consisting of **a** LL-extract in the first stripe and Hb-glass in the second stripe. **b** Hb-glass in the first stripe and LL-extract in the second stripe. Axons grow on the LL-extract. The explant

was to the left. The first stripe can be distinguished from the second stripe by its lighter tone and because it is 10 μm wider. Calibration bar in **a** for **a** and **b**: 200 μm

Fig. 3a, b. On a substrate of alternating stripes of laminin and fibronectin the axons elongate preferentially on laminin, whether it is in the first stripe (as in **a**) or in the second stripe (as in **b**). Explant to the left. Calibration bar: 200 μm

Fig. 4a, b. Axons cross freely over the striped substrate when both stripes contain **a** laminin or **b** fibronectin. The stripes are visible in **b**. Explant to the left. Calibration bar 200 μm

extract-lanes, whether LL-extract had been applied to the first (Fig. 2a) or to the second lane (Fig. 2b). Thus, they exhibited the ability to express a preference for the substrate which promotes their growth better than Hb-glass.

A similar pattern of axonal growth, i.e. axonal growth in stripes, was also evoked by all other substrates tested. In other words, these substances which provoke axonal outgrowth and axonal growth along substrate stripes can be defined as substrates with growth promoting properties for goldfish retinal axons.

The assay system offers further the possibility to compare the growth supporting activity of two different substrates which both are preferred over Hb-glass by giving axons a choice between two of these substrates in

alternating lanes. When exposed to alternating stripes of laminin and fibronectin, the axons were found to accumulate on the laminin lanes (Fig. 3). This preference for laminin was independent of whether laminin was in the first and fibronectin in the second stripe (Fig. 3a) or vice versa (Fig. 3b). Thus laminin is defined by the axons' preference as the better substrate.

That mechanical borders may arise from the application of the proteins and restrain the axons to one of the lanes was excluded by control experiments. Instead of different, one and the same protein solution (i.e. either laminin or fibronectin) was used for the first and second stripe. Under these conditions the axons did not exhibit any tendency to grow in stripes. Instead, they crossed

Fig. 5a, b. Growth pattern of goldfish retinal axons on alternating lanes of ependymin blocked with anti-ependymin (lane 1) and unblocked ependymin (lane 2). Explant to the left. Calibration bar 200 μ m

Fig. 6. Goldfish glia cells grow on the first stripe when offered a choice between laminin added to polylysine (first stripe) and polylysine alone (second stripe). The explant from which the cells emerged is to the left hand corner. Calibration bar 200 μ m

over the substrates in all directions (Fig. 4) indicating that growth along stripes is not a result from mechanical barriers but from a distinction of substrates with different growth promoting properties. The growth permissivity of laminin turned out to exceed that of all other substrates tested in this study since pairing of the other substrates with laminin (LL-extract, ependymin, Con A) always resulted in a preferential growth on the laminin lanes.

If antibodies are available against a substrate that has been established as being growth supportive the stripe assay can readily be used to judge the efficacy of antibodies to block the growth promoting substrate properties. This was tested here in two experiments using a polyclonal serum against EHS laminin and ependymin, respectively. Exemplified in Fig. 5 is the growth of axons in stripes when exposed to ependymin (lane 2) and ependymin blocked by anti-ependymin (lane 1) (Schmidt et al., unpublished). Lane 2 ependymin was saturated with neutral rabbit serum. The axons grew on the second lane. In control experiments anti-ependymin in lane 1 was replaced by neutral rabbit serum. Here, the axons grew randomly over the substrates or showed a slight tendency to prefer lane 1, but never lane 2. A similar result was obtained with laminin and anti-laminin (not shown). These antibody tests prohibit a reversal of the order in which the substrates and the antibodies are laid down. The antibodies have to be added to the substrate in lane 1. If applied second they would bind, due to their

affinity to the substrate molecules, to the substrate molecules in lane 1 and 2.

Lastly, not only axons but also migratory cells can be challenged to express a substrate choice. Fig. 6 shows the alignment of goldfish optic tract glial cells (Bastmeyer et al. 1989) in alternating lanes. In these experiments the entire coverslip had been precoated with poly-L-lysine. After it had dried, the matrix was placed onto the coverslip and laminin was filled into the channels. Thus, the substrate in the first lane consists of laminin added to poly-L-lysine and that of the second lane is previously dried poly-L-lysine alone. The glial cells grow on the poly-L-lysine/laminin lane. Additive versus exclusive substrate lanes as those can only be created if the substrate with which the glass is precoated retains its growth promoting activity after drying. Drying prior to positioning of the matrix is required to guarantee the tight adherence of the matrix to the glass.

Discussion

We have presented a choice assay that can be used to evaluate whether growing axons or migratory cells discriminate between substrates with different growth promoting properties and whether they express a preference for one of them. This assay provides narrow alternating substrate lanes such that the growth cones are required to interact repeatedly with different substrata and to

choose between them. Accumulation on one of the two lanes indicates that the growth cone or the cell has discriminated the substrates and prefers one of them. That growth in stripes is not created by mechanical boundaries in this assay is shown by the random unrestricted growth of axons in situations in which both stripes contain identical substrates.

Growth in stripes was observed, first when a substrate with growth promoting properties was next to one which did not possess these properties (Hb-glass), second when the growth supporting activity of a substrate applied to both stripes was in one stripe blocked by an antibody, third when two different substrates were offered.

The proteins fibronectin and laminin, one of the protein pairs used here, are both growth promoting molecules for goldfish retinal axons. Therefore, growth in stripes here reflects the ability of the growth cones to detect differences of two substrate proteins and to elongate on their substrate of preference.

Axonal growth in stripes does not always and necessarily imply that the preferred substrate has a special attractiveness. Axons from the temporal chick retina exposed to either rostral or caudal membranes from the tectum were able to extend on both types of membranes equally well. If they were, however, exposed to a striped carpet of rostral and caudal tectal membranes, they accumulated on the rostral membranes since the caudal membranes have repellent properties (Walter et al. 1987a, b; Vielmetter and Stuermer 1989a). The choice reflected not a preference for rostral but an avoidance of caudal membranes (Walter et al. 1987b).

The foregoing results exemplify the usefulness of this assay in exposing goldfish retinal axons to a variety of substrates. Among the substrates were two which were isolated from the goldfish brain, ependymin and the LL-extract. Ependymins are well characterized molecules. They are known to possess an HNK1 epitope, which is typical for cell adhesion molecules (Königstorfer et al. 1989). The molecular composition of the LL-extract is not yet known. The characterization of one of its

components, a 200 kd glycoprotein, which is associated with growing (young and regenerating) retinal axons in goldfish is currently underway (Vielmetter and Stuermer 1989b). Obviously, other proteins of the natural environment of growing axons and migratory cells can be tested for neurons of any desired source. Thus, the assay may be used as a tool for the analysis of axonal and cell guidance.

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