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In vitro experiments reconstituting topographic map formation

Development of the retinotectal projection

Topographic maps are a fundamental organizational feature of the wiring of the central nervous system. They are characterised by neighbouring neurons in the projecting area being connected with neighbouring neurons in the target. Thus, the spatial order within the stimulus is recast in higher brain areas in a scaled but topologically non-perturbed form. The best-studied model system for topographic projections is the retinotectal projection, i.e. the axonal connection between the retinal ganglion cells (RGCs) in the eye and target neurons in the optic tectum in the midbrain. The retinotectal projection is orientated such that RGCs situated in the temporal part of the eye project to the anterior part of the tectum, whereas nasal RGCs terminate in the posterior part of the tectum (**Fig. 1a**). Along the perpendicular retinal axis, dorsal RGCs project to lateral neurons in the tectum and ventral RGCs terminate in the medial tectum. It is usually assumed that mechanisms governing map formation along both axes are in principle similar to each other. Thus, this review only discusses the formation of topographic mapping of the retinal temporal-nasal axis to the anterior-posterior axis of the tectum.

Analysing topography development through embryological experiments

First insights into the mechanistic principles of topography formation were deduced by Roger Sperry and colleagues mainly from analyzing behavioural consequences of surgical interference with the system. After cutting the optic nerve of fishes or amphibians, retinotectal connectivity was rigidly regenerated even when the eye was rotated, leading to grossly maladaptive animal behaviour [1]. Furthermore, Attardi and colleagues found that for example after ablation of the temporal retina, axons from the remaining nasal half terminated only in the posterior part of the tectum [2]. This means axons were bypassing free termination sites in the anterior tectum and were growing to the more distant, but correct target cells instead.

These observations led Sperry to formulate his influential chemoaffinity hypothesis. To account for the observed target selectivity, he postulated chemical markers on retinal and tectal cells which would enable the axons to match the appropriate target positions. However, he also noted that the available information stored in the genetic material of an organism might be insufficient to endow every single one of the vast number of nerve cells in the brain with a qualitatively different chemical label. Thus, he proposed that the positional and, as a matter of fact, even directional information on the target needed for topography formation could instead be conveyed by quantitative marker distributions, i.e. concentration gradients of only a few molecules [3]. Major criticism of the chemoaffinity hypothesis arose due to ablation experiments, which indicated that the system shows substantial mapping plasticity, which cannot be explained by rigid chemoaffinity. For instance, a perfect map was established even after removal of a tectal half ('map compression') or half of the retina ('map expansion') (for a review see [4]). These phenomena of mapping plasticity, however, occur months after regeneration has first reconstructed the map according to the rules of rigid chemoaffinity and might therefore involve independent mechanisms.

In vitro dissection of topography formation

The main goal of in vitro approaches is to identify a reduced equivalent of a biological system. It is often easy to criticize these approaches for being overly simplistic and prone to artefacts. However, their strength lies in a comparatively easier interpretation and, more importantly, in their ability to identify a sufficient subsystem that can perform a particular biological function (**Fig. 2**). Thus, it is not surprising that major insights into retinotectal map formation, including the identification of the involved guidance cues, were gained from a series of in vitro experiments.

An initial study aiming to reconstitute topography formation *in vitro* was published by Bonhoeffer and colleagues. They cleverly arranged two tangent glass surfaces, which were covered with cell mono-layers from two different brain areas, such that retinal axons were able to choose on which glass they would grow. Chicken RGC axons indeed showed differential affinities for tectal vs. retinal, i.e. for target vs. non-target neural cells un-

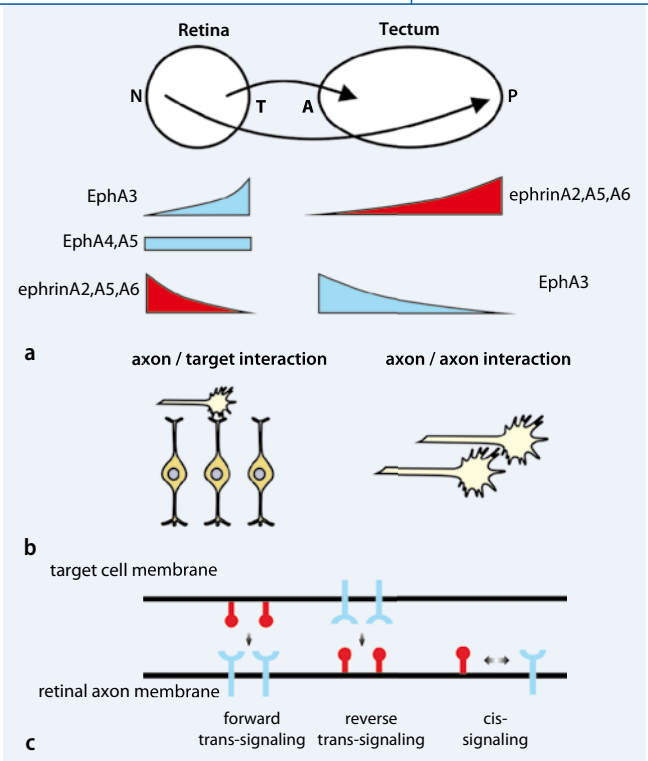

Fig. 1 ▲ Topographic mapping along the anterior/posterior axis of the chicken retinotectal system. **a** Nasal axons terminate in the posterior tectum and temporal axons in the anterior tectum. Topographic mapping is achieved by an interaction between graded EphA receptors and ephrin-As on the retinal axons and graded ephrin-A and EphA guidance signals on the tectum. **b** On its way to the topographically correct position in the tectum retinal growth cones are guided by guidance cues presented by cells of the target tissue. They are able to interact with other growth cones as well (fibre–fibre interactions), which may contribute to topographical mapping. **c** Summary of all established molecular interactions between ephrin-As and EphAs that govern topographic map formation

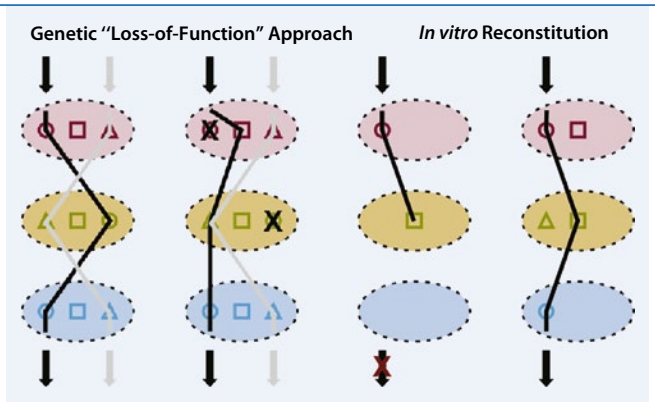

Fig. 2 ▲ Advantages of in vitro reconstitution vs. a genetic loss-of-function approach. *Circles*, *squares* and *triangles* are supposed to represent nodes of the complex molecular network underlying biological function. Input/output of the network and pathways through the network are symbolized by *arrows* and *lines*, respectively. *Coloured ovals* indicate functionally equivalent (at least partially redundant) elements. In the normal state, each pathway is preferentially associated with elements of the same shape. Two pathways are indicated in *black* and *grey*, symbolizing the pathway of interest and any other pathway, respectively. Even double loss-of-function of the black pathway's *red* and *green* circles does not yield a discernible alteration of the network output, since equivalent nodes (*red square* and *green triangle*) are deployed instead. The loss-of-function approach, designed to identify *necessary* components of the pathway, is therefore not informative in this case. The in vitro reconstitution ("reverse engineering") approach, in contrast, will yield the expected output only when a sufficient set of elements is reconstituted. The approach, therefore, is suited to identify such a *sufficient* set of functional modules for a process of interest. The individual representatives of each functional equivalence group used by the reconstitution are, however, not necessarily identical on the molecular level to those preferred by the normal state of the system (e.g. *green square* instead of *green circle*)

der these conditions [5], corroborating the chemoaffinity view of retinotectal map formation.

The most valuable finding, however, in support of chemoaffinity was the experimental demonstration of a graded activity spanning the rostral-caudal extent of the tectum [6]. For this experiment, the authors used basically the same experimental set-up as in their previous study, but this time cellular mono-layers were prepared from anterior and posterior tectum. Temporal axons grew predominantly on rostral tectal cells whereas nasal axons did not show a preference for either of these substrates. Furthermore, when preparing cell mono-layers from consecutive fifths of the tectum, temporal axons, when given the choice, always grew preferentially on the substrate of the more anterior origin. This elegant experiment provided early indications about the sensitivity of temporal axons to a tectal gradient running in the anterior to posterior direction.

Early models of topography formation suggested, in contrast to differential affinities on the target, differential affinities between retinal axons to be the organizing mechanism governing map formation (◘ **Fig. 1b**). By means of elaborate in vitro experiments, in which retinal axons were able to choose between axons of different retinal origins in Y-shaped tracks, it was found that temporal axons decided for temporal over nasal axons whereas nasal axons did not show any preference [7].

The identification of the molecules constituting the tectal anterior-posterior gradient was strongly facilitated by now classical in vitro experiments: To show that the putative guidance cue would be membrane-bound, instead of using tectal cellular mono-layers, retinal explants were cultured on isolated cell-membrane vesicles made from different parts of tectal tissue. These binary membrane substrates were presented as parallel alternating stripes orthogonal to the explant, hence the name "stripe assay". Temporal axons avoided growing on membranes prepared from the posterior third of the tectum, whereas nasal axons, again, did not show any preference [8]. The outcome of this experiment is therefore consistent with the previous mono-layer experiment. Most surprisingly, however, the anterior preference of the temporal axons could be abolished by heating or treatment with phosphatidylinositol-specific phospholipase C (PI-PLC) of the posterior, but not the anterior membranes [9, 10, 11]. This indicated the existence of a GPI-anchored, repulsive protein in the posterior membranes that would be responsible for the observed behaviour of temporal retinal axons, which is in fact "posteri-

or avoidance" rather than "anterior preference".

Based on this assay, functional candidate molecules were identified biochemically and characterised as members of the GPI-anchored ephrin-A protein family [12, 13]. Ephrin-A5 (then called RAGS) and ephrin-A2 (formerly ELF-1) as well as their receptors, the EphAs, were shown to be expressed in reciprocal gradients in the chick tectum and retina, respectively (**Fig. 1a**).

Furthermore, membranes derived from ephrin-A over-expressing cells could evoke similar avoidance behaviour of temporal axons as the one caused by posterior membranes [13, 14].

It had been known from gene expression studies in chicken and mice that, in addition to the tectal ephrin-A gradient, a counter-gradient of EphAs existed and that, in addition to the retinal EphA gradient, a counter-gradient of ephrin-As existed (**Fig. 1a**, [15, 16]). Given that members of the Eph family have the ability for bidirectional signalling [17, 18], it seemed possible that ephrin-As might also function as topographic guidance receptors on retinal axons ("reverse signalling", EphA->ephrin-A) and tectal EphAs as their ligands (**Fig. 1c**). Indeed, in vitro stripe assays with purified EphA7 protein clearly established the role of reverse signalling in topographically guiding retinal axons [19].

Moreover, the existence of both EphA and ephrin-A on one axon allows interactions of the proteins in *cis* (**Fig. 1c**). Evidence for this was first provided by a modified ephrin-A stripe assay [20]: When using low ephrin-A2 concentrations for stripe generation, only temporal axons showed a decision against the ligand stripes while nasal axons showed no preference. However, when ephrin-A5 was retro-virally over-expressed in the retina, temporal axons did not show any response to the ephrin-A2 stripes at all. Conversely, after removal of the GPI-anchored axonal ephrin-As with PI-PLC, nasal axons became sensitive to the ephrin-A2 substrate and avoided the ephrin-A2 stripes like the temporal RGC axons. The authors proposed that *cis* interactions of axonal ligand and receptor might lead to masking of the axonal receptor. Receptor molecules which are bound in *cis* would be unavailable for ligand substrate recognition in *trans*. Support for this mechanism came from a study that found evidence for different binding sites for the ligand of the EphA receptor allowing a steric discrimination of *trans* and *cis* interactions [21]. Receptor masking was suggested to convert a constant nasal-temporal level of EphA4 expression into a functional nasal<temporal gradient of EphA4 [20].

In addition to ephrin-As and EphAs, other molecules were identified through in vitro assays that may have an impact on topographic guidance. Analysis of the tectal membranes used for the stripe assay provided evidence of an additional GPI-anchored 33-kDa protein with higher concentration in posterior than anterior membranes [22]. This protein, called RGM (repulsive guidance molecule, [23]), was clearly shown to be involved in regulating neuronal differentiation, cell-survival and cephalic neural tube closure. Yet, its role in topographic map formation remains controversial [24, 25].

Very recent evidence introduced a rather unexpected type of guidance cue, the homeodomain transcription factors engrailed-1 and engrailed-2 (En1/2) [26], which are expressed in anterior<posterior gradients in the developing midbrain. It had been known for some time that their viral mis-expression resulted in topographic mapping defects such that temporal axons avoided patches of engrailed expression, whereas nasal axons terminated in them [27, 28]. Furthermore, En1/2 mis-expression led to ephrin-A up-regulation. Thus, it was argued that engrailed transcription factors might have an instructive role in setting up the ephrin-A guidance cue gradients on the target [29, 30]. Most surprisingly, however, in vitro stripe assays on the functional role of extracellular En1/2 in retinal axon guidance indicated that engrailed might increase axon sensitivity to sub-threshold ephrin-A5 concentrations by a yet unknown mechanism [31]. It is currently unclear how the interplay of engrailed, RGM and ephrin-A during retinotectal topography formation is realized, if they have distinct functions or if they are redundant actuators during map formation.

In summary, it is evident that in vitro approaches have played a major role in

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Abstract
Topographic axonal projections are a prevalent feature of brain connectivity. The retinotectal mapping of the chick is the best-studied model system of this type of neuronal connectivity. Its formation is commonly explained by interactions between graded markers of the ephrin-A/EphA family expressed on both retinal ganglion cell growth cones and on the tectal target area. Surprisingly, most insights into retinotectal development have been gathered through in vitro rather than in vivo experiments. In vitro assays not only enabled the biochemical identification of the postulated molecular markers but also helped to understand the signals conveyed by them. Thus, it was established in vitro that forward (ephrin-A->EphA) as well as reverse signalling (EphA->ephrin-A) are simultaneously needed for topographically appropriate guidance of retinal axons. However, no in vitro assay yet exists that fully reproduces topography formation. New in vitro techniques such as micro-contact printing or micro-fluidic networks may help to improve existent assays and to identify a sufficient set of functional components that reconstitutes topography formation.

Keywords
Axon guidance · Retinotopy · Explant assays · Micro-contact printing · Ephrin-A/EphA system

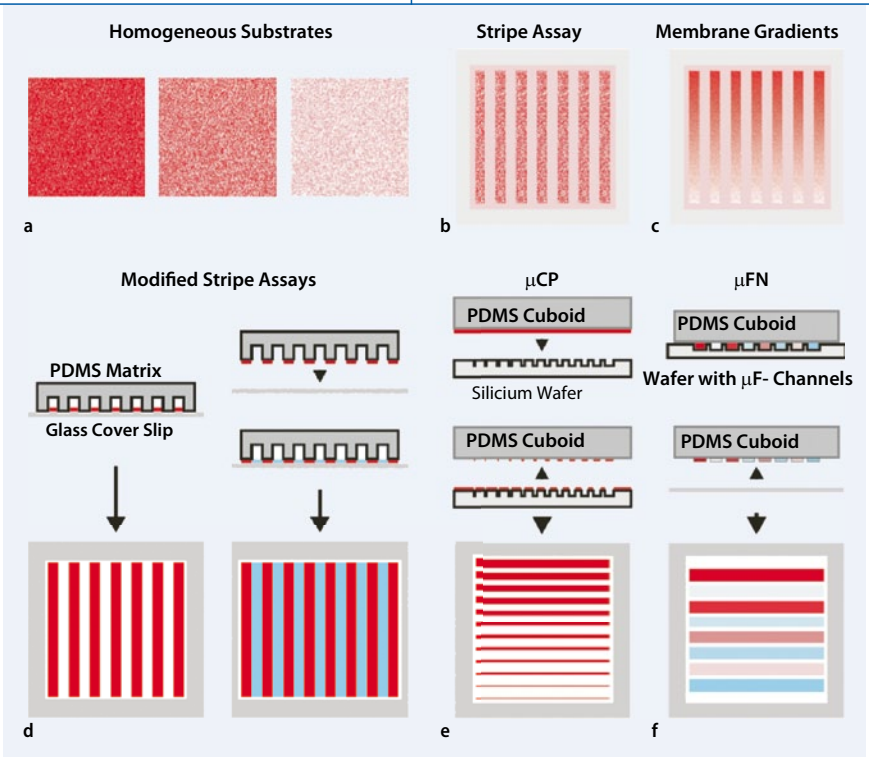

Fig. 3 ▲ Different techniques for the fabrication of in vitro guidance substrates for retinal axons. **a** Homogeneous substrates of tectal membrane vesicles or membranes generated from ephrin-A2 overexpressing cells at different concentrations (*red*) can be used for example to investigate the differential outgrowth of axons from different retinal positions [33]. **b** Binary substrate of adjacent stripes of anterior and posterior tectal membranes or membranes generated from ephrin-A over-expressing cells (*stripe assay*) can be used to analyze the decision behaviour of retinal explants [8]. **c** Graded stripe substrates of tectal membranes or membranes generated from ephrin-A over-expressing cells used to analyze the stopping behaviour of retinal axons [36, 37]. **d** Modified stripe assays using purified guidance proteins. Briefly, a silicone matrix consisting of a field of 90-μm wide channels separated by bars is placed onto a glass or petri dish surface. Protein can be applied to the surface by adsorption from the channel lumen (*left*, [9]), printing from the bars or both (*right*, [42]). **e** To produce highly precise and customized geometric distributions of a particular guidance cue, micro-contact printing of proteins (μCP) can be used [38]. In short, a silicium wafer is produced by voltage electron beam lithography in which the desired pattern is etched. Then a polydimethylsiloxane (PDMS) cuboid is homogeneously covered with protein solution and placed onto the silicium wafer. After removal, the pattern of the wafer is transferred to the PDMS cuboid, which can then be used as a stamp on glass cover slips or a Petri dish surface. After the printing process, the surface is coated with Laminin (*white colour*). The pattern depicted here is a gradient consisting of lines, which are printed with a fixed protein concentration, but vary with respect to their sizes and spacing. **f** Producing counter-gradients of ephrin-As (*red*) and EphAs (*blue*) might be possible with micro-fluidic networks (μFN) etched into a silicium wafer. This can be used to deliver protein solutions containing different concentrations of the axonal guidance molecules onto a PDMS cuboid without intermingling. In short, alternating channels of a μFN can be filled with ephrin-A and EphA and transferred to a PDMS cuboid. In a subsequent contact printing step, the protein can be transferred onto a polystyrene culture dish or glass cover slip. In this way, stepwise substrate-bound concentration counter-gradients may be fabricated

elucidating the mechanistic principles and in identifying the molecular cues of topographic guidance of retinal axons. Thus, they have laid the foundation for the cues' subsequent genetic analysis. Despite their theoretical value for identifying necessary components of a biological system, the interpretation of genetic loss-of-function studies on the retinotectal projection has been complicated by functional redundancy and robustness of the system [32].

Novel in vitro approaches for a more comprehensive understanding of topography formation

In vitro assays have been invaluable in elucidating the intricacies of retinotectal map formation. Unfortunately, they have fallen short of reconstituting complete topographic in vivo behaviour of the system so far. Therefore, new in vitro assays were developed to achieve this goal.

Graded distributions of a single guidance cue

The biggest conceptual advantage of stripe assays is that they enable the analysis of axon decision behaviour in response to a simple binary substrate (e.g. anterior vs. posterior tectal membranes, high vs. low EphA etc., see e.g. **Fig. 3b**). However, any topographically graded axon behaviour seen in vivo cannot be reproduced under such an experimental paradigm.

Looking for the elusive topographically graded axon growth, Hansen and coworkers [33] cultured retinal explants, taken from contiguous nasotemporal positions, for a fixed period of time on homogeneous cell membrane substrates (**Fig. 3a**). These substrates contained different amounts of transfected ephrin-A2 simulating different target positions. The authors reported a differential and biphasic outgrowth of retinal explants on ephrin-A2-containing membranes depending on retinal nasotemporal origin of the explant and the ephrin-A2 concentration used. At lower concentrations axons grew faster, at higher concentrations slower compared to a neutral substrate ("biphasic"). However, at the presumptive target concentration axons did not stop but also grew on the neutral substrate. The authors concluded that their results are evidence for bifunctional topographical guidance of retinal axons by ephrin-A2, i.e. ephrin-A2 being repulsive as well as attractive depending on concentration. According to the general interpretation of the chemoaffinity theory [34, 35], it is concentration *differences* in guidance cues that constitute their directional guidance effect on retinal axons. Thus, homogeneous substrates are suboptimal to study topographic guidance and the authors possibly measured general axon outgrowth rather than guidance.

Since retinal axons are guided by *gradients* of guidance cues according to the chemoaffinity hypothesis, several studies sought to produce gradients of guidance signals (**Fig. 3c, e, f**) and gathered important insights on how RGCs might interpret them. Baier and colleagues first

succeeded in fabricating sigmoid gradients of tectal membranes [36]. They reported that the observed stopping of temporal RGC axons in the gradients might be correlated with the maximal gradient slope. Subsequent work using approximately linear and exponential gradients provided contrasting evidence that temporal axons entered membrane gradients independent of their slope and showed an avoidance reaction at specific membrane concentration values [37]. At the point of this avoidance reaction, temporal axons did not stop but separated into two axon fascicles which escaped to both edges of an individual gradient stripe ("Y-shaped fascicle morphology"). The authors argued that this behaviour is in principle related to a stopping reaction of the axons. This behaviour may have been caused by limitations of the gradient fabrication technique used, which does not allow for a precise adjustment of the guidance cue distribution. Furthermore, the membrane preparation used in the above studies was an undefined growth substrate in terms of the guidance cue composition.

Micro-contact printing of proteins (μCP) can be used to micro-engineer guidance substrates with highly precise and customized geometric distributions of a particular guidance cue (**Fig. 3e**). In addition, the guidance cue concentrations used can also be adjusted specifically. Thus, von Philipsborn and colleagues used this technique to fabricate substrate-bound linear ephrin-A guidance cue gradients [38]. These gradients consisted of protein dots and lines printed with a fixed protein concentration. By gradually varying size and spacing of the dots and lines discontinuous linear gradients of defined slopes could be generated with unprecedented precision. When temporal retinal explants were placed in front of these gradients, emanating growth cones showed a distinct stop reaction but remained in a non-collapsed, motile state (**Fig. 4a, b**). This stopping was found to be predominantly concentration-dependent but also demonstrated a slight dependence on the slope of the ephrin-A gradients used: the steeper the gradient, the higher the ephrin-A concentration to which retinal axons were able to grow. Consistent with the outcome of the stripe assay, nasal axons did not show any response to the guidance cue presented as micro-contact printed gradients.

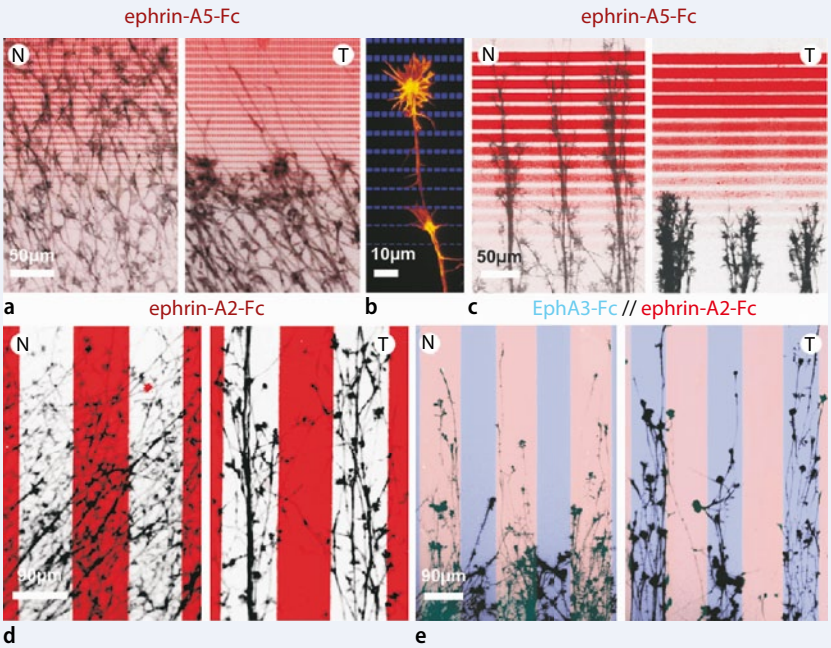

Fig. 4 ▲ Guidance of retinal axons on different in vitro substrates. **a** Retinal axons growing on an ephrin-A5 gradient fabricated with micro-contact printing. Nasal growth cones overgrow an ephrin-A5 gradient without showing any reaction. By contrast, temporal growth cones form a distinct stop zone in the ephrin-A5 gradient. Antibody-stained ephrin-A5 is shown in *red*. **b** Higher magnification of a single growth cone in an ephrin-A5 gradient as in **a**. The substrate and the growth cone are shown in *pseudo-colours*. **c** Retinal axons growing on an ephrin-A5 gradient fabricated with micro-fluidic networks. Superimposed laminin lanes direct outgrowing retinal axons in a striped pattern into the graded ephrin pattern. Temporal axons stop in an ephrin-A5 gradient, whereas nasal axons do not. This is consistent with the result obtained on the substrates fabricated with micro-contact printing suggesting that micro-structured substrates can be reproducibly used to investigate axon guidance in vitro. **d** Modified stripe assay showing retinal growth cone axon responses to ephrin-A2-Fc (displayed in *red*). Temporal axons prefer to grow on laminin containing stripes (*white*), thus avoiding ephrin-A2, whereas nasal axons do not respond. **e** Modified stripe assays with adjacent stripes of EphA3 (*blue*) and ephrin-A2 (*red*) ("double stripe" substrates). Retinal axons show topographically appropriate growth behaviour, i.e. temporal axons grow on receptor stripes (resembling receptor distribution at their in vivo target in the anterior tectum) and nasal axons grow on ligand stripes (in accordance with the distributions found at their target in the posterior tectum)

Another way of fabricating graded guidance substrates with high precision is using micro-fluidic networks (μFN) consisting of parallel channels into which protein solution can be filled ([39], **Fig. 3f**). Subsequently, the gradients can be transferred to a glass-cover slip [39, 40] and their effect on retinal axons can be analyzed. In contrast to μCP, μFN can be applied to efficiently fabricate protein patterns with constant geometry but different protein concentrations in the individual micro-fluidic channels. The resulting guidance cue distribution is therefore step-like on a micro-scale. Temporal axons stopped in μFN gradients at reproducible guidance cue concentrations, whereas nasal axons did not show any response (**Fig. 4c**). This is consistent with the results obtained with μCP gradients and stripe assay experiments, suggesting that micro-structured substrates have a promising potential to study axon guidance in vitro.

Substrates with two guidance cues

For a long time, in vitro assays were only yielding a topographically differential but not a topographically appropriate response of retinal axons. In particular, all temporal axons indiscriminately avoided a topographically inappropriate target (posterior membranes/ephrin-As) or stopped in ephrin-A gradients, whereas nasal axons usually showed no response at all. A rare indication of topographical-

ly appropriate response of retinal axons in vitro was reported by von Boxberg and colleagues [41]. They performed stripe assays for which the tectal membranes were gained by a special fractionation technique. In their experiments, temporal axons appropriately preferred anterior and nasal axons posterior membranes. However, the composition of the membrane preparations is unknown, rendering it impossible to explain this intriguing result with the activity of specific guidance cues or even underlying molecular interactions.

Although the relevance of ephrin-A and EphA counter-gradients in the tectum for the guidance process has been convincingly demonstrated, conventional in vitro assays do not expose retinal axons to ephrin-As and EphAs simultaneously (e.g. **Fig. 3d**, left). Conceivably, this might have been the reason why a topographically differential axon decision under defined in vitro conditions has been elusive for a long time. A stripe assay substrate enabling a choice for the axons between ephrin-As and EphAs was shown in preliminary experiments to successfully overcome these limitations and reconstruct topographically appropriate RGC axon behaviour for the first time under defined in vitro conditions (**Fig. 4e**). For this, Gebhardt and colleagues performed stripe assays with alternating stripes of EphA receptor and ephrin-A ligand [42]. Ephrin-A and EphAs stripes were fabricated by first printing one molecular species onto a petri dish with a silicone matrix consisting of adjacent channels and bars. Afterwards, the other protein was injected into the channels and allowed to adhere to the surface, thereby generating separated alternating stripes of both proteins (**Fig. 3d**, right). In this experiment, temporal axons grew on EphA and nasal axons on ephrin-A stripes corresponding to their in vivo target. This evidence suggests that giving retinal axons the opportunity to interact with ephrin-As and EphAs at the same time resulted in topographically appropriate behaviour of RGC axons.

The ultimate goal for any in vitro reconstitution of topographic mapping is to obtain a continuous topographically appropriate stopping of all RGC axons along the whole retinal nasal-temporal axis. This may be achieved by exposing retinal explants to continuous counter-gradients of EphA receptor and ephrin-A ligand as an in vitro substrate which closely mimics the in vivo situation. However, this is very challenging to realize from a technical point of view. First, separate functional gradients of both receptor and ligand at appropriate concentration ranges are needed. Second, these gradients must be in close spatial proximity without inactivating each other (ephrin-As and EphA bind to each other strongly, thus reducing growth cone access). µFNs could be used to deliver counter-gradient substrates onto a silicone stamp by filling alternating channels of the network with ephrin-A and EphA at different concentrations (**Fig. 3f**).

Plasticity of retinotectal mapping

As has been mentioned in this review and extensively elsewhere [4] rigid chemical matching as suggested by the chemoaffinity theory is a rather error-prone mechanism for the topographic sorting of axons. For instance, chemoaffinity is not easily reconciled with the map expansion and compression seen in retinal or tectal ablation experiments without assuming a change in the respective gradients or the gradient read-out. Yet, no evidence exists for any concentration adjustments of the guidance cues in vivo. Rigid chemoaffinity also lacks the plasticity needed to react, e.g. to gradient fluctuations or general noise that is to be expected in biological systems. Nevertheless, a precise topographic map is almost always formed in vivo. Several regulatory mechanisms have been suggested to explain mapping plasticity such as activity dependent refinement [43] or competition for synaptic targets [44]. Since adaptation to changing environmental conditions is a recurrent theme in biological systems, one can easily envisage that adaptive mechanisms in response to the guidance signal may help to overcome the obvious limitations of rigid chemoaffinity. The problem is, however, that continuous adaptation to a membrane-bound topographic guidance cue would inevitably result in a loss of the differential identity of a growth cone which is needed for recognition of the correct target position.

It has been shown convincingly that neuronal growth cones are able to adapt to diffusible guidance cues [45]. Xenopus spinal neuron growth cones underwent consecutive phases of desensitization and re-sensitization when challenged with increasing basal concentrations of the diffusible guidance cue Netrin-1. Such a continuous adaptation is reminiscent of the chemotaxis mechanism in bacteria (see e.g. [46]). However, as in bacteria, it can merely be used to steer towards or away from a source of attractant or repellent, respectively, instead of conveying a signal for stopping at a defined position in a gradient.

In vitro data for growth cone adaptation to membrane-bound topographical cues is rare and some are also subject to misconceptions: If given a choice between anterior or posterior membrane substrates (or different ephrin-A concentrations), an avoidance response of temporal RGC axons is usually observed. However, despite the repulsive effect of posterior membranes, both nasal and temporal axons have the general ability to grow on either posterior or anterior membranes when presented homogeneously [8]. This is usually considered face-value evidence for adaptation of retinal axons. We would like to point out that adaptation on homogeneous guidance cue substrates is not necessarily needed to explain this behaviour, but is already covered by the established interpretation of chemoaffinity by gradient matching: Topographic axon steering is most likely based on using directional information provided by the gradients [35, 47]. The growth cone can realize this by detecting concentration differences. Deciding for the lower of two concentrations is then loosely taken as an indication of a repulsive quality of the topographic cue. However, on homogeneous guidance cue substrates, this directional guidance signal is missing and no repulsion is to be expected even without adaptation. This experiment is therefore already consistent with the current interpretation of topographic axon guidance and based on this evidence no adaptive mechanism needs to be incorporated.

However, strong in vitro evidence of retinal growth cone adaptation elicited by a homogeneously distributed topographic cue was provided by experiments in which a temporal retinal explant was placed on a "concentration pedestal" of tectal posterior membranes in front of and underlying a membrane gradient. Retinal axons invaded the gradient and grew to correspondingly higher concentrations than without pedestal [37]. This might indicate that a growth cone could adapt to guidance cues without losing the ability to detect and process the guidance signal topographically.

Further in vitro studies with more refined growth substrates are needed to understand growth cone adaptation and how it could be reconciled with topography formation.

The exceptional precision of gradient experiments using μCP substrates could be exploited to quantify the rules of growth cone adaptation, e.g. by measuring subtle alterations of the stop reaction as a function of pre-adaptation with defined concentrations of soluble ephrin added to the culture medium.

In summary, in vitro assays have been a major tool for deciphering the molecular guidance code which is responsible for retinotectal map formation. However, a sufficient in vitro system that displays full topography map formation is still elusive. Using newly available techniques for guidance substrate generation as discussed in this review might overcome this limitation and may ultimately help to fully understand topographic mapping.

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