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Reading of concentration gradients by axonal growth cones

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Wiring up the nervous system occurs as a self-organizing process during animal development. It has long been proposed that directional growth of axons towards their targets is achieved by gradients of guiding molecules and the conceptual framework of gradient guidance was introduced more than a decade ago. Novel experimental results now allow the formulation of models incorporating more mechanistic detail. We first summarize some crucial *in vitro* and *in vivo* results concerning the development of the chick retinotectal projection. We then review two recent theoretical models based on these findings (the models of Nakamoto and colleagues, and of Honda). Neither model considers the latest observation that putative guidance ligands, in addition to their tectal expression, are expressed in a similar pattern on the retina and that a disturbance of this expression affects topography. These findings suggest that retinal axons might grow into the tectum until they have reached a ligand concentration matching that of their site of origin. We call this the imprint-matching concept of retinotectal guidance. As a framework for pinpointing logical difficulties of the mechanistic description of the guidance process and to stimulate further experiments we finally suggest two extended versions of Honda's model implementing imprint matching, which we call 'the variable set-point' and 'the gradient-sensitive adaptation' model. Strengths and weaknesses of both mechanisms are discussed.

Keywords: topography; retinotectal projection; growth cone; gradient; axon guidance; ephrins

1. INTRODUCTION

During development of the nervous system the growth cones of projecting axons have to go to very specific sites. As shown in many instances they do so by directed growth rather than by random walk and selection of the correct site. What molecular mechanisms bring them to their appropriate positions? Since slopes of concentration gradients can define directions, it was already suspected many years ago (Sperry 1943, 1963) that axons might be guided by graded distributions of some guiding molecules. At first sight, as long as one looks at the gradients and the growth cones (or migrating cells) at low magnification, this seems to be a very plausible explanation. It also seems to be a rather economical way. However, looking at the growth cones at higher magnifications reveals the first principal difficulty, which is illustrated in figure 1. The steepness of the gradient at low magnification is easily recognizable. However, the slope of the same gradient presented only at higher magnification is not easily detected because the concentration differences between the various areas of the growth cone are extremely small. Nevertheless, if the gradient is the guiding cue the growth cone would have to be able to evaluate such small concentration differences. Are growth cones really guided by gradients and if so, what is the cellular mechanism of gradient guidance? How are the tiny concentration differences between the various parts of the growth cone detected, amplified and converted to give a directional signal to the cytoskeleton. This might require an elaborate mechanism. Finding an answer to these questions is one of the goals of our research.

In principle two different strategies might solve the sensitivity problem. One way to measure the gradient and to enlarge the concentration difference would be that the growth cones, like bacteria, would determine concentrations only after having moved within the gradient for a longer distance and thus having experienced a larger external concentration change. Alternatively, a small external difference could be amplified by some internal autocatalytic processes. Anatomical observations argue against the former explanation (Fujisawa *et al.* 1981, 1982; Fujisawa 1987; Stuermer 1988*a*,*b*). The involvement of autocatalytic processes in the growth cone orientation has been suggested by Gierer (1981).

To investigate the mechanism of axon guidance in more detail we studied the process *in vitro* and tried to interpret the experimental results on the basis of relatively simple models. Pursuing this approach we experienced a number of unexpected difficulties, which will describe and discuss in this article.

2. *IN VIVO* AND *IN VITRO* OBSERVATIONS OF THE DEVELOPMENT OF THE RETINOTECTAL PROJECTION

Our main experimental system is the retinotectal projection of chickens and fishes. During the formation of the retinotectal projection, retinal axons from a certain

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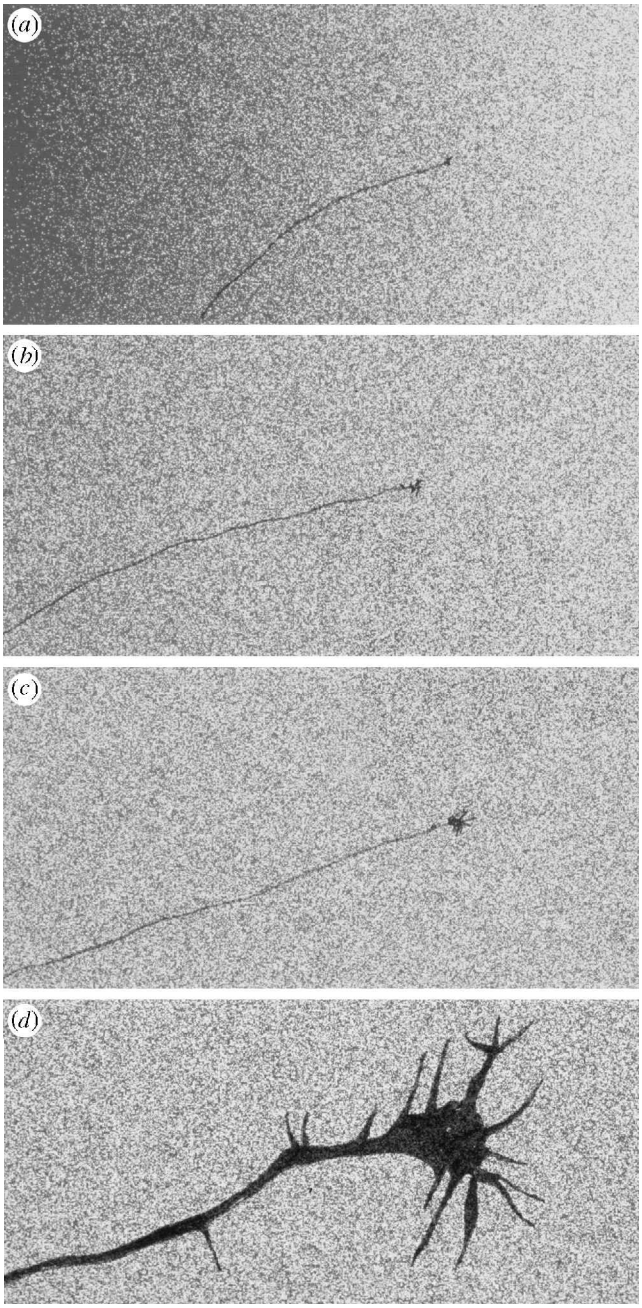

Figure 1. A growth cone in a linear gradient. Drawing of an axon growing in a graded field visualized at four different magnifications. The steepness of the gradient in relation to the size of the growth cone is the same at all four magnifications and corresponds roughly to the *in vivo* situation of a retinal growth cone (10 μm) migrating on the tectal surface (5 mm). Relative magnifications are (*a*) ×1, (*b*) ×3, (*c*) ×10, and (*d*) ×100.

position within the retina have to find their very specific target site in the target organ, the tectum opticum (figure 2). Since it seemed very likely that the guiding cues are membrane bound (a likely, but by no means proven assumption) we developed an *in vitro* assay in which growing retinal axons are offered a choice between two substrata to grow upon, a membrane preparation derived from the anterior part of the tectum and another analogous preparation derived from the posterior part. These two substrata were arranged in very narrow alternating stripes so that axons growing on these stripes are

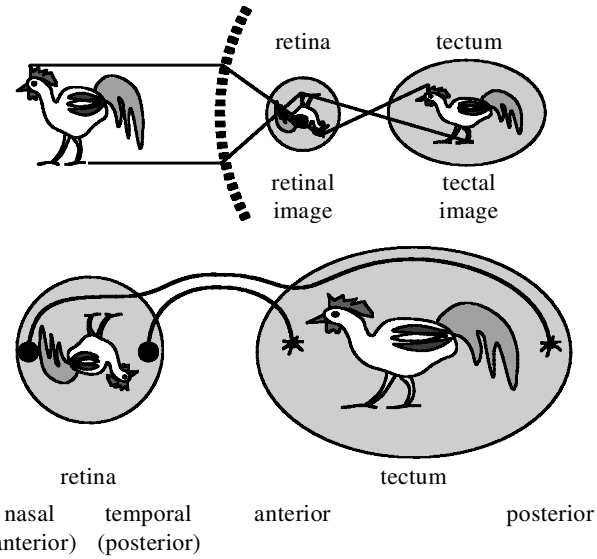

Figure 2. The chick's visual system. Very schematic view of the chick's visual system showing the projection from the retina to the tectum. The retinotectal projection is topographic, i.e. neighbouring points in the retina are connected to neighbouring sites in the target organ.

repeatedly simultaneously exposed to a choice (figure 3) of the two substrata originating from different positions of the target organ. In this assay, temporal retinal axons show a strong preference for growing on membranes derived from the anterior tectum, which is their *in vivo* target area. These axons actively avoid stripes of the posterior membranes due to a repulsive activity of the latter. Surprisingly, however, nasal axons in this assay did not distinguish between the two substrata and the transition between temporal and nasal behaviour in this respect was very abrupt (0.1 mm within the retina).

The *in vitro* assay was used to identify the repulsive membrane components. Some of these components have been cloned (Drescher *et al.* 1995). They turned out to be ligands (nowadays called 'ephrins') of receptor tyrosine kinases. Other potential candidates, like the repulsive guidance molecule (Müller *et al.* 1996), are unfortunately still resisting attempts to clone them. Both types of components act repulsively on temporal axons *in vitro* and are capable of guiding axons in the *in vitro* stripe assay into lanes of anterior tectal membranes. They could be the molecules which are responsible for the formation of the topographic retinotectal projection along the anterior–posterior axis because they occur concomitantly with the development of this projection, they have a graded distribution in the tectum, and at least some of these components have been conserved during evolution (reviewed by Tessier-Lavigne & Goodman 1996). Nevertheless, the direct experimental proof that a smooth ephrin gradient could guide axons *in vitro* is still lacking. This has also not yet been shown for the posterior membranes. However, it is quite obvious that the repulsive activity at sharp boundaries of ephrins or posterior membranes has a guiding influence on temporal axons. At present we are trying to design *in vitro* experiments with artificial gradients of ephrins with the aim of showing that the gradients influence the direction of axonal growth. Our first results indicate that in these gradient assays, as in the stripe

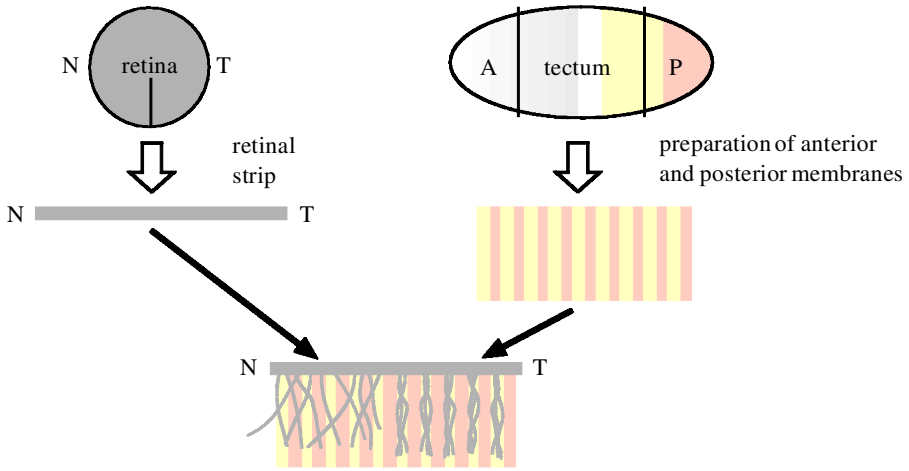

Figure 3. Stripe assay: guidance of retinal axons *in vitro*. The details of the experimental system are described in the materials and methods section of Walter *et al.* (1987*a*,*b*). In essence a strip of embryonic chick retina extending from nasal (N) to temporal (T) retina is explanted on a carpet of tectal membranes. The tectal cell membranes are derived from the anterior (A) and the posterior (P) tectum and are arranged in alternating narrow (100 μm) stripes. The retinal explant is incubated at 37 °C in 4% CO_2 on the striped carpet for about two days. The retinal explant sends out temporal and nasal axons. When temporal retinal axons reach the border between A and P lanes they show a clear preference for growing on A. On the other hand, nasal axons show in this assay no preference and cross the A–P borders freely as shown in the lower part of the figure.

assay, retinal axons do not show a graded but a binary response with all temporal axons stopping at a certain position and all nasal axons being non-responsive.

It is not clear what makes the axons stop when they have reached their *in vivo* target. There are at least two conceivable mechanisms: first the axons might follow the guiding gradient until they become exposed to an antagonistic gradient which makes the axon stop. This would require two antagonistic gradients on the target organ of which for the retinotectal system only one has been discovered so far. Alternatively, the gradient might serve not only as a directional marker on the basis of its slope but also as a positional marker depending on the absolute concentration values. Theoretical analysis (Gierer 1987) shows that guidance to the target in each dimension can be achieved either by two antagonistic gradients or by one graded cue with two antagonistic evaluations. In the latter case, there must be internal processing in the growth cone, for example, attraction at low concentration levels of the gradient and inhibition at high levels, leading to an optimal position in between; the final position depends on interactive parameters of the searching growth cone. In these versions of gradient models target position is dependent on absolute concentrations of guiding molecules, not on slope. Thus the gradient would give two commands to the growth cone, for example, (i) grow uphill, and (ii) stop at the concentration c. The latter view has recently been corroborated by experiments of Rosentreter *et al.* (1998). They showed in *in vitro* gradient assays, that temporal axons react to the cue at a defined concentration within the gradient irrespective of gradient slope. However, if the absolute concentration of the ligand is raised, axons always climb up the gradient for the same increment, as if they had adapted to the elevated basal level of the guidance cue.

Before discussing conceivable guidance models we would like to summarize some of the most recent findings concerning the *in vivo* distribution of the ephrins and their receptors in the retinotectal system. Experiments by Drescher *et al.* (1995) and Cheng *et al.* (1995) had shown some time ago that the ephrins A5 and A2 have a graded distribution within the tectum with a maximum at the posterior pole. The distributions of some of the corresponding Eph receptors (Eph A4 and Eph A5) are not graded. However, one of them (Eph A3) has a graded distribution with a maximum at the temporal side which projects to the anterior pole of the tectum. Interestingly it was recently found that the ligands ephrin A2 and A5 are not only expressed in a graded fashion on the target organ but also on the projecting retinal axons (Hornberger *et al.* 1999) (figure 4). These authors have given good evidence that the presence or absence of these axonal ligands determines their temporal or nasal behaviour *in vitro* and *in vivo*.

3. MODEL SYSTEMS BASED ON THE COMPLEMENTARY EXPRESSION OF RECEPTOR AND LIGAND

(a) *The mass action model for topographic mapping: reading only positional information*

The conceptual features of axonal guidance by gradients were discussed some years ago by Gierer (1987). Based on the new observations, made by Nakamoto *et al.* (1996), that Eph receptors and the corresponding ligands are expressed in complementary gradients in retina and tectum these authors have suggested a mechanism for the formation of the retinotopic projection. According to this model, a growth cone reads an input signal proportional to the graded ligand concentration of the tectum, compares this signal with an internal threshold value and stops growing when the difference is zero. The strength of this input signal I depends on the concentration of the receptor–ligand complexes. The threshold value is called the 'standard value' (S) and is the same for all growth cones. The concentration of receptors [R] on the growth

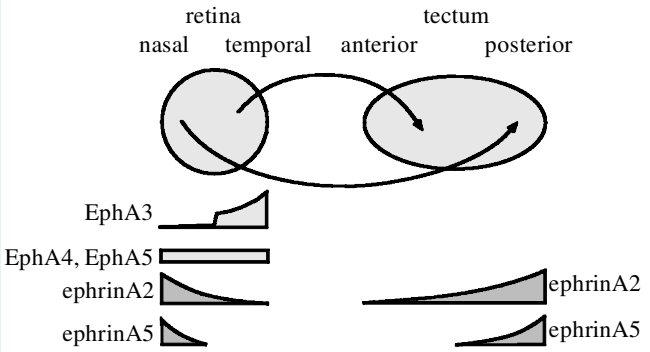

Figure 4. Expression of ephrin A ligands in the tectum and on retinal ganglion cell axons. Projection scheme of retinal axons and a summary of the expression pattern of Eph A family members in the retina and the tectum. The Eph A receptors Eph A4 and Eph A5 are uniformly expressed in the retina, whereas Eph A3 is expressed in the temporal retina in a gradient, and there is little or no expression in the nasal retina. Ephrin A2 and ephrin A5 are expressed in retinal ganglion cells including their axons in a high-nasal-to-low-temporal gradient, whereby the expression domain of ephrin A5 is restricted more to the nasal retina than that of ephrin A2. Both ligands are expressed in the tectum in a high-posterior-to-low-anterior gradient. The expression domain of ephrin A5 is restricted more to the posterior half of the tectum than that of ephrin A2. The projection of temporal axons onto the anterior tectum and the projection of nasal axons onto the posterior tectum are indicated.

cone depends on the position of its cell body in the retina. The ligand concentration $[L]$ within the tectum increases from the anterior to the posterior pole. On the basis of this model the strength of the signal I is proportional to the concentration of the receptor $[R]$ and the ligand $[L]$ according to the law of mass action: $I = [RL] = K[R][L]$, assuming that the number of receptor–ligand complexes is low in comparison to the number of free receptors and ligands. Every growth cone migrates until the signal strength I reaches the standard value S. Because all growth cones have the same S but different $[R]$, they will stop at different values of $[L]$ in the tectum. For example, if the hypothetical value for $S = 1200$ and $K = 1$, then a growth cone with $[R] = 20$ will grow to a position with $[L] = 60$, a growth cone with $[R] = 30$ to $[L] = 40$ and so on. In other words, to get the same strength I, growth cones with low receptor concentrations (from the nasal part of the retina) will grow until they reach a part of the tectum with high ligand concentration (posterior part) whereas growth cones with a high receptor concentration (from the temporal part of the retina) will already stop at positions with low concentration of ligands (anterior part of the tectum). Thus the mass action model offers an explanation of how growth cones might recognize their target position within the gradient. Importantly, the model does not explain how growth cones are guided to this target position.

(b) *The servomechanism model: reading both directional and positional information*

In principle, gradients carry two different kinds of information: a positional information based on the local concentration of ligands, and a directional information based on the slope of the concentration gradient. Honda (1998) developed an extended mass action model which describes how growth cones make use of both the directional and the positional information of a gradient to find their targets.

The key concept in Honda's model is a servomechanism (Honda 1998). As in the mass action model the target position of a growth cone is defined by the requirement that the input strength I has to reach the intrinsic standard value S. In addition, however, the model makes use of the directional information provided by the gradient by introducing a novel parameter, namely the difference of the local input strength I and the standard value S. The smaller this difference, the smaller is the distance of the growth cone to its final position. To navigate in two spatial dimensions, a growth cone needs two independent sets of complementary gradients and two independent S-values, one set for each dimension. The following description is only for one dimension (x); an analogous formalism is used for the other dimension (y). Let $[R_A]$ be the receptor concentration on a growth cone with its cell body at the position A in the retina and $[L_x]$ the ligand concentration at the position x in the tectum with S being the constant standard value. Then $I_x = K[R_A][L_x]$ represents the signal strength measured by a growth cone at position x in the tectum and $D_x = S - I_x$ is the difference between the standard value S and the local input strength I_x. In order to determine the direction of growth the growth cone has to

(i) calculate the local difference signal $D_x = S - I_x$;
(ii) sense the ligand concentration at a new position in a + or − direction;
(iii) calculate the difference signal at the new position $D_{probed} = S - I_{probed}$;
(iv) compare the difference signals D_x and D_{probed} at the local and the probed position;
(v) calculate an output corresponding to the probability for accepting the new versus staying at the current position.

The probability considerations are introduced in order to account for fluctuations in the input strength I. The probability P of the growth cone to remain at the current position is $P = p(D_x)/(p(D_x) + p(D_{probed}))$. The probabilities $p(D_x)$ and $p(D_{probed})$ depend on the distance of the local position from the final position and have a maximum where $D = 0$. If the difference signal at the probed position D_{probed} is smaller than that at the current position D_x, the probability of staying at the current position is lower than that of moving to the probed position, and vice versa. After having approached their final position, the site where the difference value $D = 0$, forward and backward migration of the growth cone will have the same probability. Thus net growth will be zero. This could be interpreted to mean that growth cones do not stop moving but are getting trapped at their final region.

The difference signal D of local and probed positions on a homogeneous substrate do not depend on the direction of growth and therefore the probability of a growth cone to accept the new position is 50% everywhere. Thus it would not migrate at all. However, in fact growth cones do also migrate on homogeneous substrates. In the servomechanism model this problem has been solved by

assuming that the tendency of probing the forward direction (+) is higher than the tendency of probing the backward direction (−). This results in a net forward movement, i.e. in growth of the axon. According to diverse experimental observations (e.g. Bray 1979) the forward tendency is a plausible assumption.

The described algorithm is independent of whether the probed position is lying within the range of a filopodium or far away, for example, at the axon shaft. Therefore there is no *a priori* need to amplify small concentration differences measured within various parts of a growth cone. In simulations the model appears to be rather tolerant of noisy input signals and the probability for a growth cone to get trapped in local minima is low.

(c) *In the servomechanism model one and the same guidance molecule can be either an attractive or a repulsive cue for the growth cone*

Depending on the relationship between local position and the intrinsic standard value S of the growth cone the effect of a ligand gradient can be either attractive or repulsive (figure 6). This is in contrast to the mass action model. Here the authors of that model postulate that the ligand has to have a repellent effect on the growth cone (Nakamoto *et al.* 1996). However, the idea that the same molecule could act repulsively or attractively fits well with results of Poo and co-workers (Song *et al.* 1997; Ming *et al.* 1997) in an analogous system. They demonstrated that the effect of certain guidance molecules on growth cones depends on the cAMP–cGMP concentration within the neuron itself. A decrease of one of these second messengers converts attraction by the source of the molecules into a repulsion and vice versa, i.e. the effect of the guidance molecules depends on the internal state of the growth cone.

4. COMPARISON OF THE SERVOMECHANISM MODEL WITH *IN VIVO* AND *IN VITRO* RESULTS

(a) *The model fits well with certain aspects of the* in vivo *situation*

Sperry (1943) demonstrated for the first time that axons read positional information from their target area. Regeneration studies of the optic nerve in newts (Fujisawa *et al.* 1982) and in fishes (Stuermer 1988*a*,*b*) have provided clear evidence that both normally and ectopically ingrowing retinal axons show directed (non-random) growth towards the target zone. Similarly, in the chick, an initially loose projection is sharpened by the interstitial branch formation followed by elimination of specific axonal backbranches and, to a lesser extent, by abrupt course corrections of the growth cone towards the appropriate target zone (Thanos & Bonhoeffer 1987; Nakamura & O'Leary 1989). Retinal axons of some zebrafish pathfinding mutants enter the tectum from an ectopic position but grow directly to their correct termination zones (Trowe *et al.* 1996). In the mouse, the strategy seems to be different. There the majority of the growth cones overshoots, the retinotopic projection is established by the formation of interstitial branches and elimination of the major growth cones (Roskies & O'Leary 1994). However, despite of all interspecies differences, the *in vivo* situations demonstrate that growth cones find their target position independently of their entry point into the tectum. This is a general feature of gradient models of axonal targeting and is thus a strong indication that guidance by gradients is involved somehow. Indeed, simulations with the servomechanism model showed that all virtual growth cones migrate towards their target zone regardless of the position of the starting point in the graded field. Then they get trapped, staying active and mobile in their specific termination area.

(b) *Some originally unexpected results of the stripe assay become easy to explain*

As mentioned in § 2, the stripe assay was developed to understand the mechanisms of the retinotopic projection and to identify guidance molecules. Some results of this assay were rather unexpected. The initial expectation was a graded response in the behaviour of the axonal population. The extreme nasal growth cones should show a clear growth decision for the posterior stripes and the extreme temporal ones for the anterior stripes. Growth cones originating from areas between these two poles should show a graded transition between these two extremes. However, in the stripe assay experiments only two different behaviours were observed: all temporal axons irrespective of their temporal position grow on stripes made up of anterior membranes, whereas all nasal axons show no preference and grow equally well on stripes of both, anterior and posterior membranes. Also unexpected was a sharp transition between the behaviour of these two populations (Walter *et al.* 1987*a*).

One possible explanation for this outcome of the stripe assay experiments could be that the *in vitro* system is somehow incomplete, i.e. a guidance factor for nasal axons is lost or diluted during the membrane purification procedure. Another explanation, also not satisfying, speculates that the function of a graded distribution of guidance molecules would only be to define the posterior part of the tectum in a step-like function. However, experiments with the stripe assay showed that temporal axons can distinguish between stripes made of membranes from neighbouring tectal areas, independent of the absolute positions in the tectum, indicating a graded distribution of the cue (Bonhoeffer & Huf 1982).

The servomechanism model offers an interesting alternative explanation. Figure 5*b* shows the result of a simulation of the stripe assay on a carpet of alternating lanes of high and low ligand concentrations. On the left side of the figure are the starting points of axons. Their guidance receptor concentration increases from the lower to the upper lanes. This is equivalent to an explant strip reaching from the nasal to the temporal part of the retina. From axons with low receptor concentration to those with high receptor concentration there are three distinguishable axonal behaviours: (i) decision for the stripes with a high ligand concentration (lower part of figure 5*a*,*b*); (ii) no decision at all (middle part of figure 5*a*,*b*); and (iii) decision for stripes with a low ligand concentration (upper part of figure 5*a*,*b*). The transition between these behaviours is sharp. The position of these transitions depends on the ligand concentration of the stripes relative to the intrinsic standard value S of the axons and could be changed in such a way that only two axonal behaviours remain. Under these assumptions the

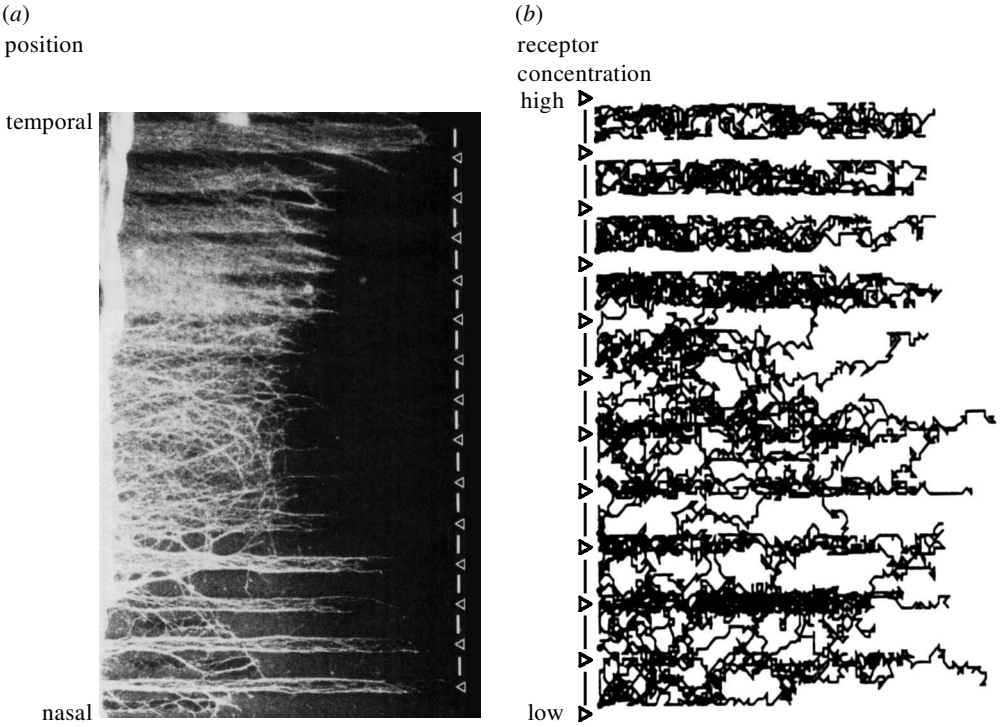

Figure 5. Von Boxberg's guidance experiments and Honda's simulation. (*a*) A temporal to nasal retinal explant was placed on a membrane carpet, which consists of alternating lanes of anterior and posterior tectal membranes. In contrast to the standard stripe assay the tectal membranes for this assay have been prepared by a different mild purification procedure, involving iso-electric focusing (Von Boxberg *et al.* 1993). The position of the anterior lanes is indicated by bars. Temporal axons grow in membranes derived from the anterior tectal pole, nasal axons show a preference for growing on membranes from the posterior pole indicated by triangles. Given the choice, nasal and temporal axons prefer to grow on membranes of their own target position. (*b*) Corresponding simulation of axonal growth and guidance on the basis of the Honda model (Honda 1998). The figure shows the trajectories of virtual growth cones starting on the left side of the figure growing into a field consisting of alternating stripes with two different ligand concentrations. The lanes with low ligand concentrations are marked with vertical bars (anterior membranes L_a), the lanes with high ligand concentrations with triangles (posterior membranes L_p). The receptor concentration of the growth cones increases linearly from the lower (nasal axons) to the upper (temporal axons) stripes. There are three different behaviours distinguishable: decision for stripes with anterior membranes (vertical bars) in the upper part; no decision in the middle and lower part; and a preference for stripes with posterior membranes (triangles) in a region within the lower third. The behaviour of the growth cones (preference or no preference) depend on the ligand concentrations, the standard value S and the receptor concentration of the growth cone. Depending on these factors a simulation could show all three, two or only one of the described decisions. The following parameters have been used for the simulation (in arbitrary units): $L_a = 40$, $L_p = 50$, $S = 2500$, the receptor concentration ranges from 0 to 110.

outcome of the simulation looks like the usual experimental result: no decision for the nasal axons (low receptor concentration) and decision against one type of the stripes for the temporal axons (high receptor concentration). According to this model, it should be possible to see one, two or all three different behaviours in one assay depending on the relative ligand concentration of the two sorts of stripes. Modifying the standard purification procedure, Von Boxberg *et al.* (1993) were indeed able to show a stripe assay where temporal axons grow on anterior stripes and nasal axons on posterior stripes (figure 5*a*). The experiments contain as internal controls the normal decision of temporal axons. This makes them trustworthy and convincing despite their not being very reproducible. One possible explanation for the lack of reproducibility was originally that the activity of a factor which influences nasal axons is labile and is thus only sometimes preserved.

The servomechanism model offers a better explanation for the preference of nasal axons for the posterior stripes: the novel purification scheme might change the concentration of active ligands in the membrane preparations derived from anterior and posterior tectal tissue and the preference of nasal axons for posterior membranes could critically dependent on their concentration difference in the stripes. This notion can possibly be verified by further experiments, for example, by changing the relative ligand concentration in the stripes by specific antibodies.

(c) *A graded response* in vivo *is not in contradiction to a step-like transition between nasal and temporal behaviour in the stripe assay*

Surprisingly the results of the simulations show clearly that a stepwise transition from nasal to temporal behaviour of the axons in the stripe assay can be produced even if the very same axons display a graded response in a gradient field. They show further that the very same factor can have a repellent or an attractive effect on an axon population (figure 6). The character of the effect depends exclusively on the relationship between the signal strength I at the current position of the growth cone and

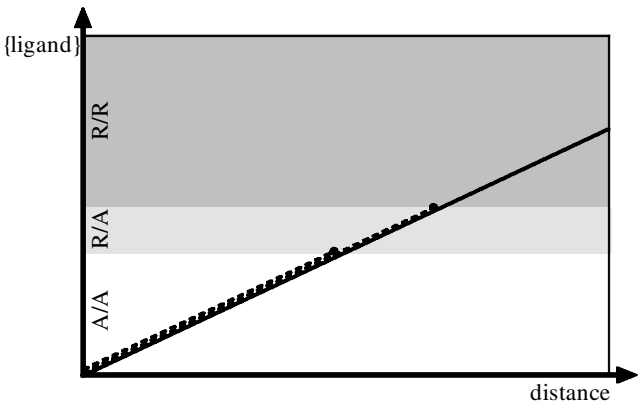

Figure 6. Repulsive and attractive concentration ranges. The graph shows a linear ligand gradient and the trajectories of two migrating growth cones within this gradient (dashed lines). They stop at two different ligand concentrations. The effect of the ligands on the growth cones (attractive or repulsive) is dependent on their target positions. The whole concentration range (white area) up to the first stop-point is attractive (A/A) for both growth cones. The concentration range (light grey area) between the two stop-points has a repellent (R) effect on one growth cone and an attractive (A) effect on the other growth cone. Any concentration (dark grey area) above the second stop-point has a repellent (R/R) effect for both growth cones. Thus, the molecule itself has neither an attractive nor a repulsive effect.

the internal standard value *S*. The explanation for the uniform behaviour of a group of different axons within a certain concentration range is simple: if the probability for preference for one sort of stripe is high enough, then a large majority of axons grow onto that stripe. This has the appearance of a decision. An even higher probability does not change the apparent behaviour of the axons because most of them have already made their decision.

(d) *A step-like behaviour in the gradient assay remains difficult to explain*

In order to investigate axons growing on graded substrates, we have developed an *in vitro* assay (Rosentreter *et al*. 1998) in which the substrate is offered as a linear concentration gradient of guidance molecules. This has been done in both striped and non-striped gradients. These assays allow growing retinal axons to grow into linearly increasing concentrations of posterior membranes or membranes derived from cells transfected with guidance molecules (ephrin A5, A2). Temporal axons enter the gradient and grow unaffectedly until they reach a certain threshold concentration. In the unstriped version of the assay, temporal axons stop growing, but the growth cones stay mobile and active, as the model predicts. In the striped version of the assay, temporal axons try to avoid higher concentrations within the gradient and escape to lower concentrations at the borders of the (non-ideal) stripes. The nasal axons enter the gradient and keep growing in both versions of the assay.

Like the stripe assay, the gradient assay reveals temporal and nasal behaviours but no intermediate behaviour (Rosentreter *et al*. 1998). All temporal axons are shorter than the nasal axons. The expectation was that axons growing into increasing concentrations of guidance molecules would react in a graded manner, i.e. that individual axons depending on their receptor composition would stop at different positions in the gradient and would therefore have different lengths. Honda (1998) showed in computer simulations of the gradient assay that the appearance of the result depends very much on the shape of the concentration distribution of the ligand. Simulations with linear gradients show the expected graded distribution of axonal stop positions. Simulations with sigmoidal ligand gradients show a more or less step-like distribution of axonal lengths (Honda 1998). It would be interesting to find out if simulations of the gradient assay with a nonlinear receptor distribution and linear ligand gradients result in a similar step-like nasal–temporal switch.

Although there is admittedly a discrepancy between the simulations and the real experiments, the results certainly show that, depending on the exact parameters of the gradient, the graded response of the axons might sometimes be hard to see.

5. MODELS BASED ON THE IMPRINT-MATCHING CONCEPT OF RETINOTECTAL GUIDANCE

(a) *Novel experimental results suggest an imprint-matching concept of retinotectal guidance*

As presented up to now, Honda's model relies on the complementary expression of guidance receptors and ligands on the projecting area and the target organ as well as on a set-point value, which is the same for all retinal ganglion cells. Novel experimental results by Hornberger *et al*. (1999) and Dütting *et al*. (1999), however, indicate an additional function of the guidance ligands. These showed that the ligands are expressed not only on the tectum, but also on retinal ganglion cells. The expression pattern on the retina resembles that on the target organ. It corresponds to a gradient with low concentrations on the temporal and high concentrations on the nasal side. When Hornberger *et al*. (1999) enzymatically removed the ligand from the retinal ganglion cells, nasal axons became responsive to the guidance cue, i.e. they all behaved like temporal axons in the *in vitro* stripe assay. Furthermore, when they overexpressed the ligand in retina, the overexpressing axons were non-responsive to the guidance cue, i.e. they behaved like nasal axons in the stripe assay. These results indicate that the retinal ligand is crucially involved in the determination of target destination. The resemblance of the ligand expression patterns on retina and tectum indicates that retinal axons might grow into the tectum until they reach a ligand concentration that corresponds to that of their retinal site of origin. We would like to call this the imprint-matching concept of retinotectal guidance.

(b) *Implementation of the imprint-matching concept by a set-point variation mechanism*

If the above-mentioned results (§5(a)) are to be incorporated into a theoretical model of the guidance mechanism the first puzzling issue to be solved is the problem why the densely intermingled axons expressing both, ligand and receptor, do not disturb each other during the pathfinding and guidance process. It is therefore important to postulate that ligands on one cell bind exclusively to receptors of the same cell (*cis*-interaction)

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

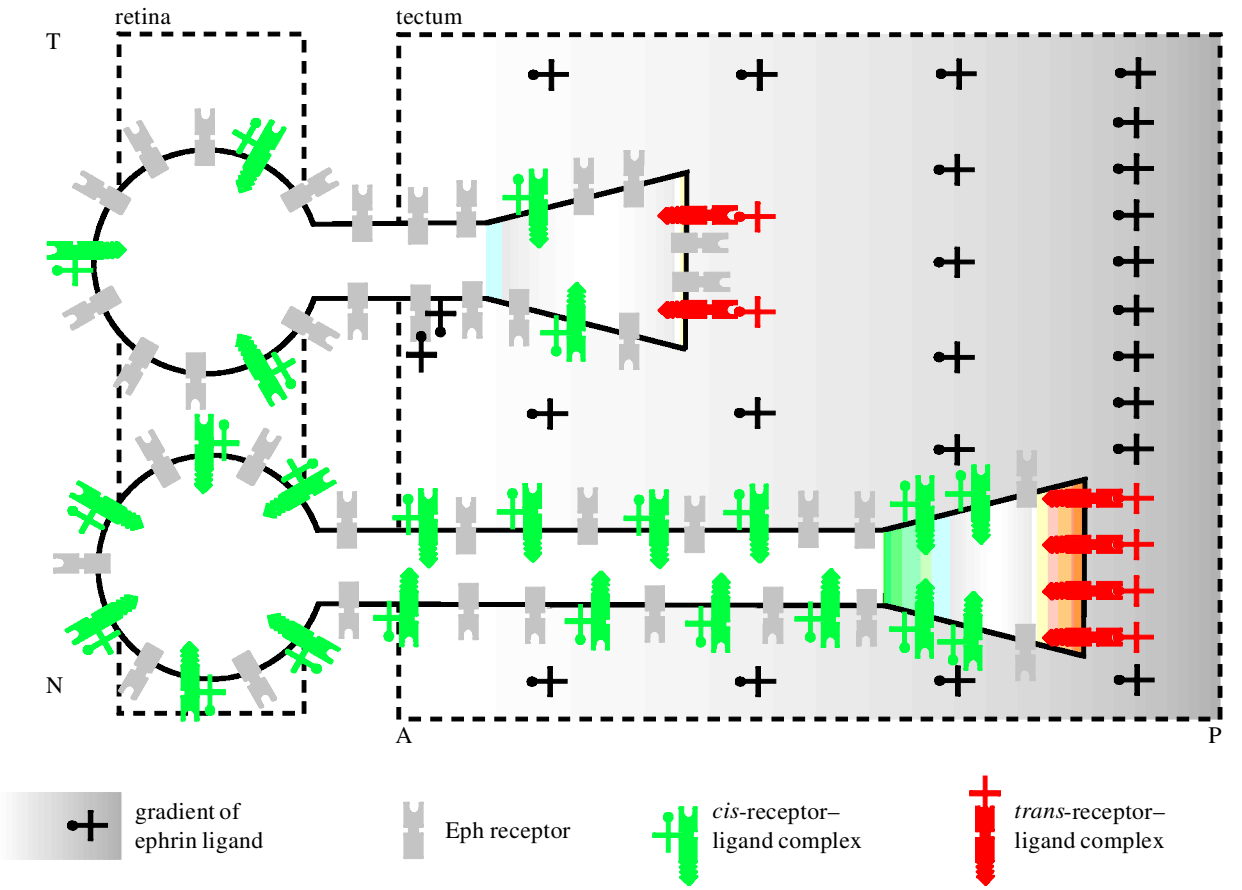

Figure 7. The set-point variation mechanism. A possible mechanistic implementation of the set-point variation mechanism. Two retinal ganglion cells are shown, whose somata (depicted as circles) reside at different positions on the retina, one more temporal (T) and one more nasal (N). Their axons are guided to correspondingly different positions of the tectum (A, anterior; P, posterior) by growth cones symbolized as trapezoids. In the most-parsimonious mechanistic implementation all retinal ganglion cells express roughly the same concentration of a guidance receptor (e.g. an Eph receptor tyrosine kinase). The first salient feature of the suggested mechanism is that matching concentration gradients of the same ligand (e.g. an ephrin ligand), which by itself is neither attractive nor repulsive, are used both to determine target destination of the projecting neurons according to their retinal position and to provide the guiding cue to the growth cone on the tectal target. The ligand is therefore expressed by retinal ganglion cells (temporal low, nasal high) as well as by cells of the tectal target (anterior low, posterior high; represented by the density of ligand symbols and grey gradient shading). The second salient feature of the model is to suggest two independent modes of receptor–ligand interaction resulting in two different pathways of signal transduction. When challenged with a ligand presented on the same cell (*cis*-interaction, green colour) the receptor generates one signal, the set-point signal, when challenged with a ligand in *trans* (red colour) an antagonistic signal is produced. The set-point signal, which provides the position-specific imprint, is encoded by the concentration of ligand–receptor *cis*-complexes. Topography is achieved because retinal growth cones search for that ligand concentration on the tectum that matches their own imprinted ligand concentration. The guidance problem therefore amounts to a match-to-sample task. The growth cone stops when the difference between both signals (symbolized within the growth cone by different shades of green and red in proportion to signal strength) becomes zero (white). As on each cell the same receptor governs both the set-point and the antagonistic signal the model is basically independent of receptor concentration as long as saturation is not reached. The graded distribution observed for some candidate receptors (e.g. Eph A3) might actually be used for an optimization of signal strength (see § 5(b)). The actual signal integration might be compartmentalized to the growth cone as indicated in this figure. Alternatively, the whole neurite might be involved depending on whether the axon participates in gradient sensing or not. The spatial separation of the signals within the growth cone is only for the sake of clarity of the graphical presentation. The subpopulation of receptors already bound to a ligand in *cis* might or might not bind an additional ligand in *trans*. If it does, the produced signals have to be strictly additive, i.e. the ternary complex has to produce both signals at once or the additional *trans*-ligand must silence all signalling through the respective receptor. Ligands already engaged in a *cis*-interaction are assumed to be blocked for additional *trans*-binding, e.g. by steric inhibition.

and not to those of neighbouring cells, which are in turn engaged in interactions with their respective *cis*-receptors. The exclusivity of these interactions might simply be due to steric hindrance. Clearly separate from the *cis*-interaction is the interaction of receptors with ligands on the target organ (*trans*-interaction).

The imprint-matching concept can now be incorporated into Honda's model by assuming the novel *cis*-interaction to adjust the set-point S. We call this the set-point variation mechanism.

As described above, the servomechanism uses the difference parameter D between the input signal I and the standard value S to calculate a directional and a positional information: $D = S - I = S - K[R][L_T]$, L_T being the ligand concentration in the tectum. S need not be a constant, it might be dependent on the concentration of

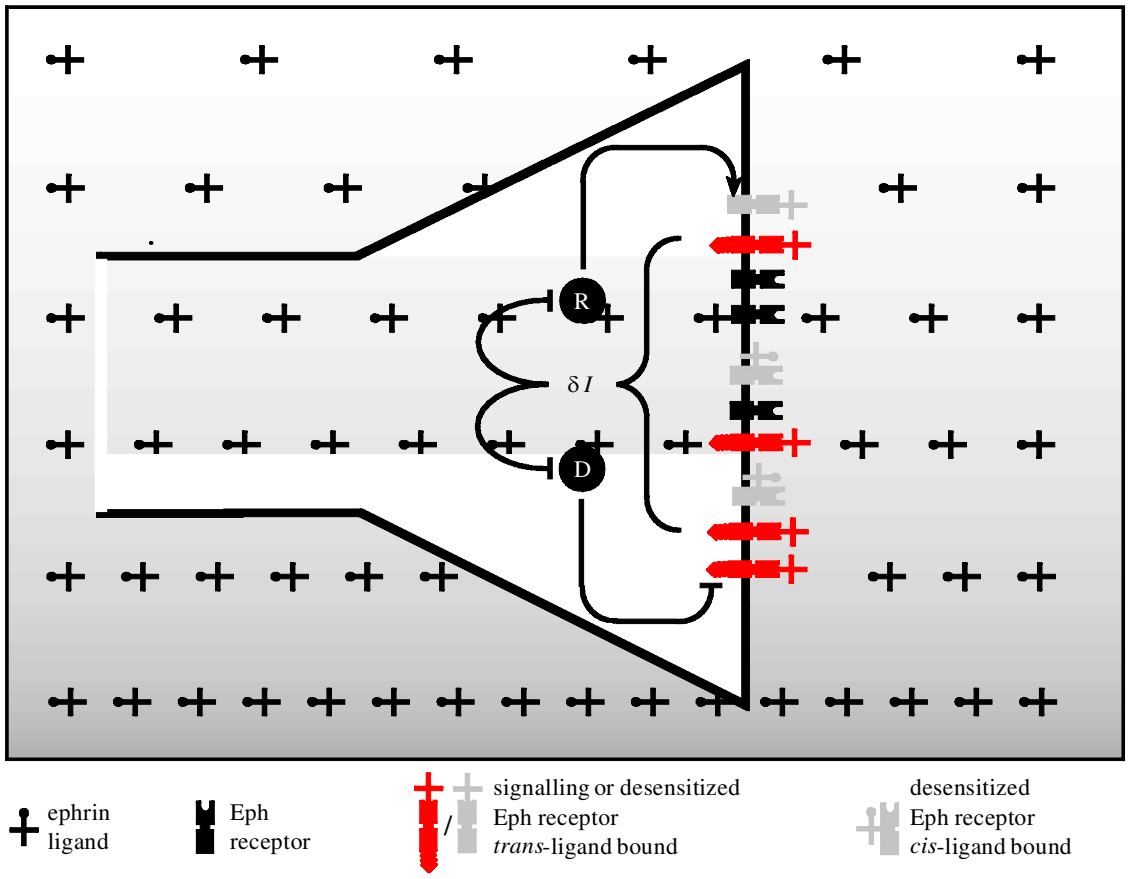

Figure 8. The gradient-sensitive adaptation mechanism. A growth cone is shown endowed with the necessary elements for gradient-sensitive adaptation. As for the set-point variation mechanism receptor–ligand *cis*- and *trans*-interactions are modelled to be strictly exclusive; now, however, they are assumed to produce the same input signal. δI is meant to symbolize a differential sensor for spatial inhomogeneity of the input signal. The autocatalytic reaction suggested to amplify the small external concentration differences across a growth cone into a large internal signal is well suited for this purpose. The output of the sensor should be inhibitory for a default receptor desensitization–resensitization machinery consisting of a desensitizing (D) and a resensitizing enzyme (R). As in standard receptor adaptation biochemistry the substrates of these enzymes are the ligand-activated receptor for the desensitizing enzyme and the desensitized but ligand-depleted receptor for the resensitizing enzyme. Desensitized receptors are shown in grey. If such a growth cone is migrating within a concentration gradient of a guidance cue, which is represented by the density of ligand symbols and grey gradient shading, the spatial differential sensor will switch off the desensitization apparatus. This allows the receptors which were in a sensitized state at the beginning of the gradient to act as proportional sensors for the input signal, thereby providing the machinery for concentration increment reading. The growth cone will stop when the input signal encoding the concentration increment reaches the constant set-point value S. Receptor symbols are confined to the leading edge of the growth cone only for the sake of clarity.

the ligand and the receptor on the growth cone, e.g. on the product of $[R][L_R]$, where L_R is the ligand concentration of the retinal growth cone. The retinal signal $[R][L_R]$ would therefore be due to receptor–ligand *cis*-interaction and the tectal signal $[R][L_T]$ to receptor–ligand *trans*-interaction. It should be noted that in the set-point variation mechanism *cis*- and *trans*-interactions lead to two distinguishable signals (set-point and reading signals). The difference signal D would therefore be $D = K_1[R][L_R] - K_2[R][L_T]$. In the simplest case, that $K_1 = K_2 = 1$, the difference D is $D = [R]([L_R] - [L_T])$.

Growth cones have reached their final position when the difference signal $D = 0$, i.e. when the input signal derived from the tectal ligand concentration matches the signal derived from the retinal ligand concentration (figure 7). The final position would only be dependent on the ligand concentrations L_R and L_T. Thus in order to stop at different termination zones growth cones must have their own specific ligand concentration L_R determining the set-point. The final position ($D = 0$) would not be changed as long as the difference between the ligand concentration in the growth cone (L_R) and in the tectum (L_T) stays constant. The function of the receptor in this model is not to define a target position as in the servomechanism model but just to detect the signal. The complementary expression of the tectal ligands and of the retinal receptors would have the function to adjust the signal strength of the input signal, i.e. low ligand concentrations are amplified by high receptor concentrations and vice versa.

A strength of this model is the relative independence of the pathfinding process of absolute receptor and the ligand concentrations. This corresponds to a gain of robustness over the servomechanism model in which the absolute value R, L and S have a strong influence on the target position.

The second messenger for the *cis*-activation of the receptor might for example be cAMP or cGMP. An increase in the intracellular cAMP level would shift the

set-point signal (*cis*-signal) and therefore the final position of a growth cone towards a region with higher ligand concentration in the tectum, i.e. the attractive region in figure 6 would be enlarged. Decreasing the cAMP level would shift the final position towards lower concentration within the tectum, i.e. the repulsive region in figure 6 would be enlarged. This fits well with results of Poo and co-workers (Song *et al.* 1997; Ming *et al.* 1997) who demonstrated that an increase in cAMP or cGMP leads to an attractive response and a decrease to a repulsive response of growth cones to guidance molecules.

A weakness of the set-point variation model is its inability to deal with adaptation phenomena observed in some other experimental situations.

(c) In vitro *experiments indicate the existence of adaptation processes in the growth cone*

It is known from the stripe assay that temporal axons grow well even on high concentrations of posterior membranes if they do not have a choice between different substrates. Albeit seeming like adaptation this behaviour could still be explained without the assumption of receptor desensitization. As described above, the servomechanism explains growth on homogeneous substrates if there is an inherent tendency of growth cones to grow forward.

However, there is some evidence for receptor adaptation in the gradient assay (Rosentreter *et al.* 1998). Temporal axons stop at a certain ligand concentration regardless of the steepness of the gradient. If the explant is placed on a constant plateau of the guidance cue, which is continuous with the gradient, i.e. if the whole experiment is carried out at an increased absolute concentration of the guidance cue, then the axons stop at a correspondingly higher absolute, but at the same relative position within the gradient. It therefore seems as if they would try to reach a certain concentration increment. This observation suggests that the guidance machinery has adapted to the basal concentration. If only the explant is put onto a pedestal of the guidance cue but the gradient is left unchanged, the axons start their ingrowth into the gradient from a zero basal level of the guidance cue. In this case there is no difference to the standard situation, in which the explant also resides on the zero level. This result indicates that the adaptation process seen in the first experiment does not take place at the level of the cell soma but most likely in the growth cone itself. Adaptation within the growth cone is not included in the set-point variation mechanism as described above.

(d) *Incorporating the imprint-matching concept via gradient-sensitive adaptation*

Adaptation of the receptors has the inherent disadvantage of turning the receptors from proportional sensors into differential sensors. Within a gradient the growth cone would thus lose its ability to detect a concentration increment, which is needed to locate the final target. The key feature of our assumption is therefore that the adaptation mechanism has to be switched off within the gradient. Therefore two elements are needed to implement gradient-sensitive adaptation (figure 8): a downstream differential sensor for spatial inhomogeneity of the intracellular guidance signal and an adaptation machinery acting on the guidance receptors which is inhibited by the output of that sensor. A differential sensor for spatial inhomogeneity is already a vital part of the servomechanism model. It is the apparatus determining the difference between the input strength at the current and a probed position for biasing the probability of an orientation change. Needless to say that when incorporating adaptation into that model the time constants have to be adjusted in such a way that no adaptation can take place in the interval between the measurements at the current and the probed position. Otherwise the spatial differential sensor would not work any more. Another option for the required differential sensor for spatial inhomogeneity would be the above-mentioned autocatalytic reaction (§ 1) that might amplify the small external concentration differences of the gradient in the vicinity of a growth cone into a large internal signal. The adaptation mechanism might consist of a pair of a desensitizing and a resensitizing enzyme, comparable to CheR and CheB in the bacterial chemotaxis apparatus. The cognate substrate of the desensitizing enzyme is the ligand-activated receptor and that of the resensitizing enzyme would be the desensitized but ligand-depleted receptor.

To prevent ligand-expressing axons from seeing each other, again a *cis*- and a *trans*-receptor–ligand interaction have to be distinguished. In contrast to the set-point variation model, however, in this case both are suggested to lead to the same intracellular signal.

With these assumptions the gradient-sensitive adaptation mechanism, like the set-point variation model, is suited to explain the results of the above-mentioned (§ 5(a)) retinal ligand-overexpression and -removal experiments by Hornberger *et al.* (1999) by desensitization–resensitization of the receptors interacting with the *cis*-ligand. Imprint matching in this model is due to the fact that the *cis*-ligands induce a desensitization of their bound receptors, thereby determining the concentration of remaining sensitive receptors. In addition the model is also able to provide an explanation of the *in vitro* gradient experiments by Rosentreter *et al.* (1998) described in § 5(c). As long as the growth cone migrates on a homogeneous substrate the differential sensor for the spatial inhomogeneity of the guidance signal would be silent, thereby permitting desensitization of the signalling receptors. As soon as the growth cone reaches the gradient the sensor would become active, thereby preventing any further adaptation. Finally the gradient-sensitive adaptation mechanism also provides an explanation for the preliminary result that axons growing down the gradient *in vitro* seemingly do not stop at all. As the differential sensor for the spatial inhomogeneity of the input signal is suggested to reside in the growth cone and not in the cell body, cells of an explant placed onto the gradient itself would not be prevented from adapting to the local concentration of the guidance cue. Growth cones emerging from the explant would therefore be pre-adapted and they would stay so, as their adaptation mechanism is silenced within a gradient. They would therefore be insensitive for decreasing concentrations of the guidance cue, which are met during downhill growth.

It should be mentioned that gradient-sensitive adaptation instead of being incorporated into the servomechanism model might also be added to the set-point

variation model. In this case the adaptation machinery has to be strictly specific for the *trans*-signal and has to leave the *cis*-signal unaltered. Although there is no logical need to fuse both models, the advantage would be a gain of robustness introduced by the set-point variation mechanism.

There is one important weakness of the gradient-sensitive adaptation mechanism. In contrast to the set-point variation model it fails to provide an explanation for the experiments by Von Boxberg *et al.* (1993). Adaptation would interfere with the preference of nasal axons for posterior membranes observed in the special stripe assay described in §4(b). The alternating lanes in the stripe assay consist of homogeneously distributed membrane vesicles with low (anterior stripes) and high (posterior stripes) ligand concentrations. According to the model, growth on homogeneous substrates permits the adaptation mechanism to act. Because nasal growth cones which grow on posterior stripes adapt to high ligand concentrations they cannot recognize the lower concentration of the ligands in anterior stripes. This makes the borderline invisible for nasal axons and therefore they would not be trapped in posterior lanes, as they are in Von Boxberg's experiments. This argumentation would not be valid, if the inhibition of adaptation following a signal from the spatial differential sensor is prolonged or if the onset of adaptation is delayed. For that purpose the time constants of these processes must be within the range of the time between two border contacts of the growth cone. Both assumptions lead to no or only partial adaptation of the receptors which might prevent growth cones from crossing the borders of the lanes.

(e) *The presented models can be distinguished experimentally*

The major feature of the set-point variation model is its independence of the receptor concentration. The model therefore predicts that receptor overexpression should show no effect as long as the receptor can read both the *trans*- and the *cis*-signal. A conclusion of the gradient-sensitive adaptation mechanism is that a plateau of sufficient length within a gradient would lead to adaptation and would therefore allow the axons to continue growth beyond their proper target. Both of these experiments seem to be feasible. Both models fall short of explaining the complexity of the experimental evidence and therefore both will be wrong in detail, but the experimental tests might provide hints as to which of them deserves further elaboration.

6. CONCLUSION

In this paper we first reviewed the results of some conceptually important *in vivo* and *in vitro* experiments addressing the establishment of topographic projections in the developing nervous system. Although similar conclusions can be drawn from other systems we focused on the chick retinotectal projection as an experimental paradigm. Whereas the conceptional framework of axon guidance by gradients of guidance molecules has long been established (Gierer 1987), novel experimental results allow the formulation of models adopting more mechanistic detail.

As a first of these evidence-based models we discussed the mass action model by Nakamoto *et al.* (1996). This model implements the observation that certain putative guidance molecules and their receptors are expressed as complementary gradients in the respective projecting and target areas. In Nakamoto's description an input signal is generated in the randomly searching growth cone by the interaction of receptor and ligand according to mass action kinetics. The projection target is reached when the input signal reaches a set-point value, which is independent of retinal position. As the receptor concentration varies with retinal position axons from different retinal sites of origin terminate at different tectal positions. The model makes use of the positional information of a gradient, but it fails to describe the observed directional growth of axons. This feature is taken into account by a more sophisticated elaboration of the mass action model presented by Honda (1998). In his description the growth cone at every position on its way towards the target determines the difference of the input signal and a constant endogenous set-point value. The result of that calculation is compared with the result of a corresponding calculation at a new position probed by the growth cone. The outcome of the comparison is fed into a servomechanism to bias the probability of changing the direction of growth. The model not only implements the directionality of axon guidance. By virtue of its comparative nature it also fits experimental observations gained in Poo's laboratory (Ming *et al.* 1997; Song *et al.* 1997) that at least some guiding molecules are not inherently attractive or repulsive, but that their attractive or repulsive behaviour is a mere consequence of internal computation of the growth cone. These features provide explanations for some of the most puzzling results gained with retinotectal axons in the *in vitro* stripe assay used to analyse their decision behaviour (non-decision of nasal axons and step-like transition between nasal and temporal behaviours).

These published models do not consider the most recent observation that the guiding ligand is expressed not only as a gradient on the tectal target but also in a matching gradient on the retina (Hornberger *et al.* 1999) and that a disturbance of the retinal expression affects the establishment of topography. These results suggest a novel concept, which we call the imprint-matching concept. It states that retinal axons might grow into the tectum until they encounter a concentration of the guidance cue corresponding to that at their respective site of origin.

In the final part we presented two extended versions of Honda's model, which implement the imprint-matching concept and which we call the set-point variation and the gradient-sensitive adaptation model. In the set-point variation model the retinal ligand is used to calculate the set-point signal according to mass action kinetics thereby providing a position-specific imprint to the projecting neurons. The searching growth cone attempts to exactly balance this set-point signal by the input signal derived from the tectal gradient. Complete balancing is achieved when the ligand concentration at the position of the growth cone just matches the ligand concentration at the axons site of origin. Axon guidance in the set-point variation model is therefore a match-to-sample task. Due to the fact that the model crucially relies on a comparison of concentrations of the same ligand on the projecting and

target area, which are measured by the same receptor, it is independent of the absolute concentration of the ligand and the concentration of the receptor as long as saturation is not reached. These features lend superior robustness to the model. To enable the growth cone to distinguish between retinal set-point and tectal input signals we postulate two independent signal-transduction pathways through the guidance receptor depending on whether the ligand is presented *cis* (on the same cell, as in the retina) or *trans* (on other cells, as on the tectum).

The set-point variation model fails to explain adaptation processes in the growth cone. The gradient-sensitive adaptation mechanism is introduced in order to implement the *in vitro* observation that growth cones seem to read concentration increments, thereby indicating an adaptation to constant basal levels of the guidance cue. Besides strictly exclusive *cis*- and *trans*-interactions (leading, however, to the same intracellular signal) the gradient-sensitive adaptation mechanism requires a differential sensor for spatial inhomogeneity of the guidance signal within the growth cone. The output of that sensor is suggested to be inhibitory for a receptor adaptation machinery. Thereby the mechanism leads to receptor desensitization on homogeneous substrates but prevents adaptation in the gradient allowing the growth cone to read absolute positional information. Gradient-sensitive adaptation can be incorporated into either Honda's model or the imprint-matching model, the first alternative being more parsimonious, the second being more robust. The two alternatives can be distinguished experimentally.

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