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Opinion

Can Molecular Gradients Wire the Brain?

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Concentration gradients are believed to play a key role in guiding axons to their appropriate targets during neural development. However, there are fundamental physical constraints on gradient detection, and these strongly limit the fidelity with which axons can respond to gradient cues. I discuss these constraints and argue they suggest that many axon guidance events *in vivo* cannot be explained solely in terms of gradient-based mechanisms. Rather, precise wiring requires the collaboration of gradients with other types of guidance cues. Since we know relatively little about how this might work, I argue that our understanding of how the brain becomes wired up during development is still at an early stage.

Trends

Concentration gradients have been implicated in many axon guidance events *in vivo*. However, axonal chemotaxis is constrained by fundamental physical limits on gradient sensing.

These limits arise from unavoidable stochastic variations in the number of ligand molecules present, the number of receptors bound at each moment, and the limited number of molecules in downstream signalling pathways.

The size of these stochastic variations can be calculated theoretically. These calculations suggest that gradients alone are unlikely to provide a directional signal reliable enough to explain the trajectories taken by axons *in vivo*.

Therefore, gradient cues likely collaborate with other types of guidance cues in ways that have yet to be determined.

Brain Wiring by Chemotaxis?

For the brain to function correctly it must be wired correctly, and many neurodevelopmental disorders are likely to be the result of wiring defects [1–4]. How does this precise wiring occur? A key hypothesis in the field, first presented by Cajal, is that axons are often directed by chemotaxis, that is, following concentration gradients of molecular cues [5–7]. These molecular cues can be either soluble or substrate-bound. This is an attractive idea: chemotaxis is important in many biological processes [8,9] and appears to be an effective way of providing directional information over the often long distances that axons must traverse *in vivo*. However, here I suggest reasons to doubt that the directional information provided by concentration gradients (either attractive or repulsive) is sufficiently reliable, by itself, to robustly direct precise brain wiring.

Evidence for Gradients in Axon Guidance

The past two decades have seen major breakthroughs in terms of identifying important families of molecular guidance cues for axons [10,11]. Support for the importance of gradients of these cues in axon guidance comes from several sources. First, often these cues seem to be present in the developing nervous system as appropriately positioned concentration gradients [12–14]. Second, *in vitro* assays show that gradients of these cues can impart directional information to axon growth [15–21]. And third, genetic manipulations of cues believed to act via gradients *in vivo* can cause axonal miswiring (e.g., [22–25], reviewed in [11,26]).

However, much of the (extremely successful) focus of axon guidance research in the modern era has been on identifying the molecules involved rather than on quantitative considerations of gradient-based guidance (for an early exception, see [5]). Showing an effect of a gradient on axons *in vitro* or that disrupting a gradient *in vivo* causes miswiring are essentially binary outcomes. By themselves these results are insufficient to quantitatively explain the precise paths that axons normally take *in vivo*. Indeed, it remains to be demonstrated that any nontrivial axon trajectory *in vivo* (such as a stereotyped turn) can be quantitatively explained by purely gradient guidance. Instead I suggest that, for the parameters relevant to axon guidance events, the fidelity of purely gradient-based directional sensing is, unavoidably, very low, and probably insufficient by itself to provide sufficiently reliable guidance information for robust *in vivo* wiring.

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There are two further problems with the gradient guidance hypothesis that, owing to length limitations, I am unfortunately unable to consider here. Specifically, (i) how a shallow external gradient can be converted into an internal gradient of cytoskeletal resources sufficient to induce a turn [27] and (ii) whether or not appropriate protein gradients (especially of soluble factors) actually exist *in vivo*. Both of these problems with the gradient guidance hypothesis could be addressed in the future by the acquisition of new data.

Fundamental Physical Constraints on Chemotaxis

Three Sources of Noise

To appreciate how physics constrains axon guidance, it is necessary to understand the basic ligand sensing constraints faced by any physical system. To detect a change in concentration across its spatial extent, any sensing device such as a neuronal growth cone must overcome three sources of noise (Figure 1) [28–34]. Firstly, thermal motion of ligand molecules means that the number in any small volume around the sensor is constantly fluctuating. Thus, even an ideal sensing device that reliably counted every molecule would sometimes produce errors in deciding in which direction the concentration is changing across its spatial extent. This is also true for ligands bound to the substrate or cell membranes: the only difference here is the rate of diffusion [35,36]. Secondly, in reality ligand molecules can only be counted through the indirect and noisy process of chance encounters with a limited number of receptors on the sensing device. This 'receptor noise' further degrades the already corrupted 'true' signal. Thirdly, these receptor binding events are then converted into decisions to move one way or another by an intracellular signalling cascade that suffers from the same problems: the noise inherent in limited numbers of molecules and chance encounters.

The Size of Thermal Noise

Here I will focus on the first two problems and for now discuss the more general problem of concentration measurement (gradient sensing being a specific case). A large body of theoretical work has examined exactly how much noise is introduced into a concentration measurement by

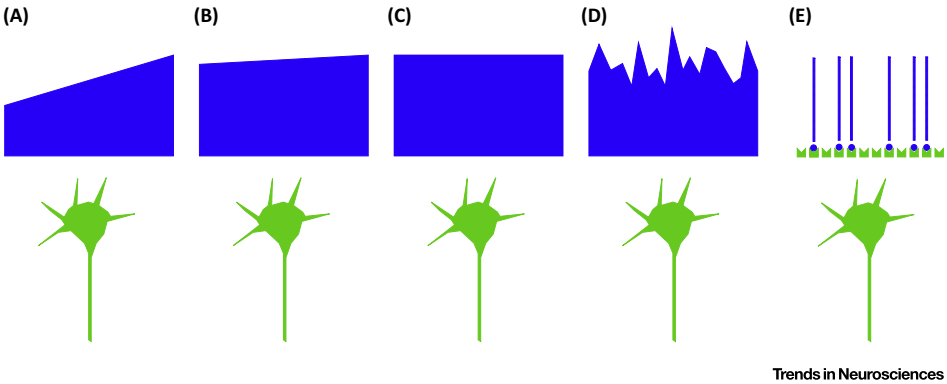

Figure 1. Noise in Gradient Sensing. (A) A typical depiction of a growth cone encountering a gradient. Here the fractional change in concentration across the growth cone width is 100%, that is, a doubling from one side to the other. However, a gradient of this steepness could only act over a very short range: assuming a useful concentration range of a factor of a thousand around K_d [40], and a growth cone width of 10 microns, this range is only $\log_2 1000 \times 10\ \mu m \approx 100\ \mu m$. (B) The gradient steepness (10%) encountered in the growth cone turning assay. (C) The gradient steepness (1%) typical of microfluidics assays and some *in vivo* gradients [14]. In this picture, it is impossible to see the concentration difference by eye. (D) In reality, the gradient is corrupted by unavoidable thermal fluctuations in the number of ligand molecules present. The relative size of these depends on the absolute concentration. (E) The information available to the growth cone about the ligand gradient is actually only a pattern of receptor binding that varies stochastically. For clarity, only 12 receptors are shown, of which half are bound (consistent with an absolute concentration close to K_d). For a 1% gradient and a concentration at the left side of the growth cone of K_d, the probability of a receptor at the far left being bound is $C/(C + K_d) = 0.5$, while the probability of a receptor at the far right being bound is 0.502. Even for a 10% gradient this probability rises only to 0.52.

thermal fluctuations and receptor binding noise [28–34]. Very roughly, thermal fluctuations introduce a source of Poisson noise to the measurement.

Thus, the size of the noise relative to the signal is $1/\sqrt{n}$, where n is the average number of molecules present in a small volume around the sensing device [28]. To bind some receptors but not reach saturation, a desirable ligand concentration is roughly *K*d, the dissociation constant for the receptor–ligand interaction. The *K*d values for the receptor–ligand interactions involved in axon guidance are often around 1 nM. In this case, there are only approximately 1000 molecules present in a small volume around the growth cone, and the unavoidable thermal noise in a concentration measurement is therefore approximately 3% relative to the signal.

Time Averaging

The above calculations refer to making a single count of the number of molecules present. Clearly, noise can be reduced by averaging several measurements, and making M measurements reduces the noise by a factor of $\sqrt{M}$ [28]. Critically however, these must be independent measurements. That is, they need to be spaced far enough apart in time that the previously counted molecules have had a chance to diffuse away, allowing new molecules to bind. In a classic calculation, Berg and Purcell [28] equated this delay time with the time it takes on average for a molecule to diffuse away distance *a*, the size of the sensing device. From this they refined the estimate of $1/\sqrt{n}$ to $1/\sqrt{aDcT}$, where D is the diffusion constant of the ligand, T is the total measuring time available, and $c = n/a^3$. Thus, for an averaging time of 100 s and a diffusion constant of 10^{-6} cm^2/s [37] the noise is now reduced to 0.3%. Notable here is the dependence on the diffusion constant: the slower a ligand diffuses the longer one has to wait to make independent measurements.

Bound versus Soluble Ligands

Concentration measurement relies on ligand molecules encountering receptors. Ligands that are bound to the extracellular matrix or the surfaces of cells diffuse approximately two to three orders of magnitude slower than soluble molecules diffusing through the extracellular space [35,36], reducing the encounter rate and requiring more time averaging to achieve the same level of accuracy. An extreme case is a gradient of completely immobilised molecules created by 'freezing' a soluble gradient: this would still be noisy (freezing a particular random thermal fluctuation), and now time averaging would not reduce thermal noise, since exactly the same molecules are sampled over time at each position. Thus, the direct coupling of receptor binding to the motility apparatus that might be possible for bound ligands, and potential issues of avidity, do not help overcome thermal noise. It is unclear what physical process could achieve precisely correct placement of individual ligand molecules to make an immobilized gradient noise-free. Even if possible, this would do nothing to reduce receptor binding noise (see later).

Downstream Processing Cannot Overcome This Noise

The thermal limit on counting noise is a fundamental physical constraint on the input signal to a sensing device. No amount of clever downstream signalling in the sensing device can improve upon this limit: the best it can do is avoid introducing further noise. This is a particular case of the 'data processing inequality': information can only be lost when passed through a noisy information channel [38]. For instance, local-excitation global-inhibition models are very effective at explaining signal amplification in gradient sensing [39], but they cannot beat the fundamental physical limits on the information content of the input signal.

Receptor Binding Noise

The second noise source is due to the stochastic binding and unbinding of ligand molecules to a limited number of receptors on the sensing device. Binding probability is determined by external concentration, and the number of receptors that are bound at each moment fluctuates. This

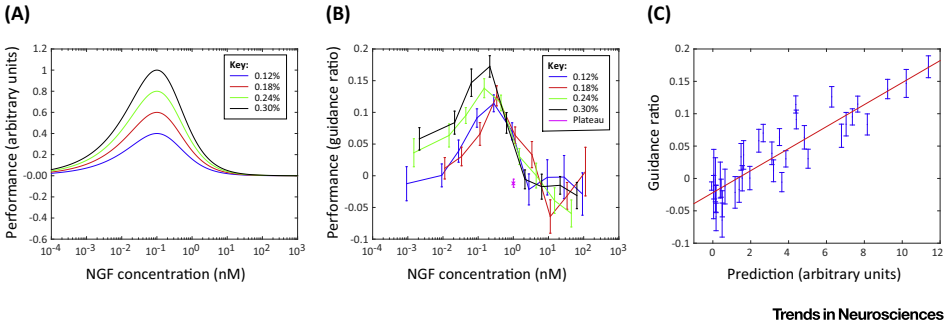

Figure 2. Chemotactic Sensitivity and Receptor Binding Noise. (A) Prediction of variation in chemotactic performance with gradient steepness and concentration from the Bayesian model of receptor binding noise of [40]. (B) Actual variation in chemotactic performance with gradient steepness and concentration for dorsal root ganglion explants grown in precisely controlled gradients in collagen gels. (C) Actual variation plotted against predicted variation. Abbreviation: NGF, nerve growth factor. Figures redrawn from [40].

time-varying signal is the only information available to the sensing device about external concentration. Even for high ligand concentrations, a limited number of receptors means receptor binding noise can be a significant constraint [30,31]. The contribution of this noise relative to thermal noise has been considered through a series of theoretical calculations [28–34]. However, here we consider an alternative approach [40], which calculated the optimal performance of a gradient sensing device in the face of receptor binding noise, ignoring thermal noise (i.e., in reality performance can only be worse). Assuming mass action kinetics, the probability P of making a correct binary (left/right) decision regarding gradient direction for an optimal sensing device making a 'snapshot' measurement was shown to be:

$$P = \frac{1}{2} + \sqrt{\frac{N}{24\pi}}\mu\sqrt{\frac{\gamma}{(1+\gamma)^3}} \quad (1)$$

where N is the number of receptors, μ is the gradient steepness (fractional change in concentration over the width of the sensing device, which for a growth cone we take to be 10 μm), and γ is the concentration relative to the dissociation constant K_d. This prediction is shown in Figure 2A. The original derivation of Equation 1 assumed shallow gradients, that is, small μ, so that the value of P fluctuates only slightly from 1/2. However, subsequent numerical simulations [41] showed that this formula is also applicable for steeper gradients, with P simply saturating at 1. This prediction was tested using the asymmetrical outgrowth response of dorsal root ganglion explants to shallow gradients of nerve growth factor over long periods in collagen gels [40] (Figure 2B). Although it was not possible to compare absolute levels of chemotactic performance between theory and experiment, examining the variation in performance with the gradient parameters μ and γ showed a good match (Figure 2C). The variation of axonal sensitivity with gradient parameters in experiments using the relatively steep gradients of the growth cone turning assay was also well predicted by Equation 1 [42]. Thus, while resting on a number of simplifying assumptions and potentially subject to modification in the future, given its fit with experimental data, Equation 1 is the most accurate quantitative expression currently available for predicting the ability of axons to detect molecular gradients.

Relevance of Physical Limits on Gradient Sensing to Axon Turning

The collagen gel experiments mentioned earlier revealed an exquisite sensitivity of axons to shallow gradients. However, in that experimental design there was the potential to average the gradient signal over very long periods of time, probably over distances longer than one growth cone diameter (effectively making μ much larger) [43]. In typical guidance scenarios, *in vivo* axons must make precisely directed turns as they grow, probably based on information only

from the growth cone, and do not have the luxury of waiting to average a gradient signal over many hours before deciding whether to turn right or left (although their entire trajectory may involve many hours of growth, at each position in the trajectory they must make a decision regarding the local direction of the gradient in a much shorter time). To derive the quantitative predictions of Equation 1 for the reliability of turning at each individual moment *in vivo* requires estimating the values of N, μ, γ, and any improvements that could be obtained by time averaging.

There have been virtually no direct measurements of the number of receptors on a growth cone. However, it was estimated in [44] that ≈900 nerve growth factor molecules were bound to the growth cone of a dorsal root ganglion axon when all nerve growth factor receptors were occupied, suggesting that 1000 is a reasonable order of magnitude to assume for N. Few gradients have been quantified at the protein level *in vivo*. Measurements of Netrin [13] and Shh [14] suggest very steep gradients of ≈20–70% within ≈50 μm of the midline of the developing spinal cord, but much shallower gradients (≈1%, i.e., $\mu = 0.01$) beyond. Absolute concentrations are unknown, but the most generous estimate according to Equation 1 is $\gamma = 1/2$, which is where the expression $\frac{\gamma}{(1+\gamma)^3}$ is maximised (i.e., a concentration of $K_d/2$). Little is known about the on- and off-rates of ligand–receptor interactions for most axon guidance molecules. However, what is known is not encouraging, implying waiting times between independent measurements of at least several minutes [44]. Since growth cones typically move one diameter within approximately 15 min, this implies very limited opportunities to average receptor binding statistics before it is necessary to make a new decision about the gradient direction. We therefore assume no time averaging occurs on this timescale. Substituting these values into Equation 1 yields values for P ranging from 1 (perfect sensing) for $\mu = 0.7$, to 0.51 for $\mu = 0.01$. Under ideal conditions growth cones should therefore be able to reliably follow the gradient close to the midline (ignoring other sources of noise), but make the right decision only 51% of the time further from the midline (compared to chance performance of 50% for this simplified binary version of the problem). Note that increasing either the number of receptors or the averaging time by a factor of 10 only each increase this estimate by a factor of $\sqrt{10}\approx 3$, i.e., 53%. In the simple 1D version of the problem, given enough steps every growth cone will make enough correct decisions to reach its target. However, the number of steps required for a particular growth cone to ensure this happens can be very large, and some growth cones will travel long distances in the wrong direction before they eventually return. If they are moving in two dimensions it is very unlikely they would reach their target in any reasonable time.

Hitting the Sweet Spot

However, it is important to note that chemotactic performance drops off rapidly away from concentrations around K_d, according to the expression $\sqrt{\frac{\gamma}{(1+\gamma)^3}}$. At concentrations much higher than K_d effectively all the receptors are bound all the time, and at concentrations much lower than K_d effectively none of the receptors are bound: in both cases P becomes too small to provide effective guidance. There is thus a relatively narrow 'sweet spot' in concentration around K_d, beyond which the ability to sense a gradient is severely degraded. This is a major problem for steep gradients, because when the concentration is changing rapidly with distance, the distance over which the growth cone can remain in the 'sweet spot' is small. For instance, consider a steepness of 20% ($\mu = 0.2$) starting from $\gamma = 1$. The concentration increase in the gradient over x growth cone diameters is then 1.2^x. Within 200 microns (20 growth cone diameters) the concentration is then $\gamma = 40$, causing a severe drop in performance.

Adaptation

Could this problem be solved by adaptation of the growth cone to the gradient? Growth cones *in vitro* sometimes take time to recover their sensitivities to small step changes in concentration

[45,46]. However, for adaptation to make an impact on the fundamental physical constraints discussed earlier, ligand concentration changes would require changing the K_d of the receptor–ligand interaction to return to the sweet spot, or increasing the number of receptors (reducing the variability in receptor binding measurements). There is no evidence for either of these phenomena in growth cones. The adaptation processes that have been measured in growth cones instead represent adaptation of downstream signalling pathways. This may be important for not saturating these pathways, but optimising downstream pathways merely ensures optimal performance given the noisy input: it does not improve input signal quality. Furthermore, models can quantitatively explain the measured fidelity of axonal gradient sensing in a collagen gel assay without invoking adaptation [47].

Multiple Gradients

Another potential means for improving chemotactic performance is integrating guidance information from multiple gradient cues [48,49]. For instance, the combination of shallow gradients of Shh and Netrin-1 produce a guidance effect on commissural axons *in vitro* when the same gradient of either cue alone is insufficient [14]. How much this can improve performance in principle can be analysed theoretically in the same manner as the calculations earlier. In particular, Mortimer *et al.* [50] generalised Equation 1, assuming that the different sets of receptors act independently and that the growth cone knows the gradients are correlated (i.e., the best possible case). This leads to an equation that depends on (in the case of two gradients) ligand concentrations, gradient steepnesses, and receptor numbers. However, the increase in P is only relatively slight; for the specific case simulated by Mortimer *et al.* (Figure 6 in [50]), the increase in the deviation from 1/2 is only approximately 50%. Thus, if P were, say,

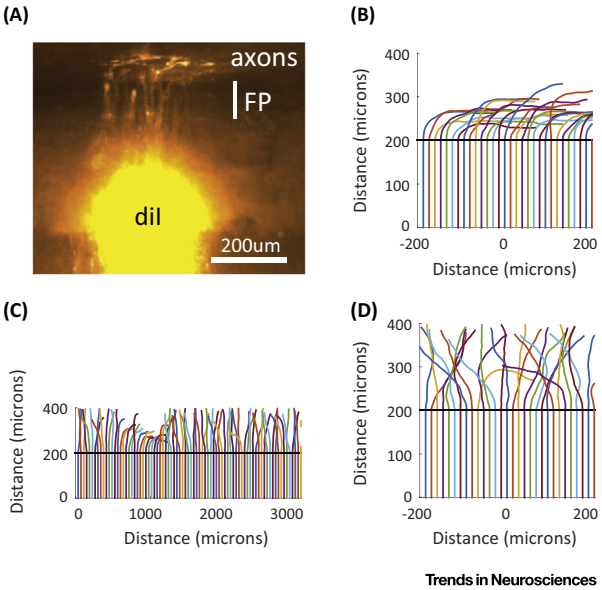

Figure 3. Mismatch between Axon Behaviour *In Vivo* and in a Simple Model. (A) Rat commissural axons (injected with 1,1′-dioctadecyl-3,3,3′3′-tetramethylindocarbocyanine perchlorate) make a sharp turn anteriorly after crossing the floor plate (FP), hypothesised to be due to the axons encountering an anterior–posterior gradient of Wnt4 (modified from [70]). (B–D) Computational simulations based on the model of Mortimer *et al.* [40,43]. The axons (arbitrary colours) grow upwards, and the gradient is encountered once the axons reach the black line at 200 μm. (B) For a 10% gradient the fidelity of turning approaches that observed in panel (A). (C) However, *in vivo* such turning occurs along the ≈3 mm length of the spinal cord. To maintain a 10% gradient over this distance requires a concentration difference of a factor of $1.1^{300} \approx 10^{12}$ between the two ends, and guidance is only effective for concentrations varying by a factor of approximately 1000 centred on K_d [40], in this simulation assumed to occur at a distance of approximately 1000 microns. (D) By contrast, a gradient of 1% requires only a $1.01^{300} \approx$ 20-fold difference in concentration over 3 mm, and is the order of magnitude of the steepness directly measured for the Wnt4 gradient [70]. However, this steepness is insufficient to induce reliable turning.

Box 1. Analysis of I*n Vivo* Data

A good test case for the above considerations is the 90 degree anterior–posterior turn made by mammalian commissural axons after they cross the midline in the developing spinal cord. The turn is sharp, stereotyped, and the encounter with the gradient is hypothesised to be tightly spatially localised (see Figure 3A in main text). Elegant work has shown that this occurs highly reliably and is dependent on an anterior–posterior gradient of Wnt4 [70]. Using Equation 1, one can perform simple simulations of how such a gradient would affect axon trajectories. In particular, here we assumes that at each time step (order 1 min) each axon grows 1 micron and, once the gradient has been encountered, turns 3 degrees. The probability of turning in the correct versus incorrect direction is given by Equation 1. As before, we assumes the number of receptors is 1000.

For these parameters, a gradient of 10% is roughly sufficient to produce the type of turn seen by the axons (see Figure 3B in main text). However, crucially, *in vivo* this turning occurs over the entire length of the developing spinal cord (approximately 3 mm). To maintain a steepness of 10% over 3 mm implies a total concentration change of a factor of $1.1^{3000/10} = 10^{12}$, which is clearly biologically implausible. Furthermore, only a relatively small length of the cord would be within the 'sweet spot', so that even if this high steepness could somehow be maintained it would not be effective over most of the cord because the absolute concentration would be either too high or too low (see Figure 3C in main text). Lyuksyutova *et al.* [70] performed a direct quantification of the Wnt4 mRNA gradient *in vivo*, and found a change in concentration of a factor of about 3 over about 3 mm. This is equivalent to a steepness over 10 microns of approximately 0.3%, which is too shallow to produce the observed fidelity of turning (see Figure 3D in main text). Of course the actual protein gradient is unknown, but from these calculations/simulations it is clear that it would be difficult, even in principle, for a single, stable gradient to explain the apparently consistent and reliable turning of axons in this system.

One possible complication is that commissural axons may be helped in their turn by encountering other axons already running longitudinally along the spinal cord. However, while this could provide a mechanism for making a sharp turn, it does not help explain their reliable choice of the direction in which to turn. There is also a gradient of Shh helping guide these axons [71]; however, as discussed in the main text the improvement offered by optimally combining two gradient cues is relatively slight. Another possible complication is that commissural axons do not cross the floor plate simultaneously along the length of the spinal cord. One could therefore imagine a dynamic Wnt4 expression pattern that is constantly reshaping the gradient, so that there is always a region of high steepness at just the point in the spinal cord where axons cross at that particular moment. However, the gradient snapshot measured by Lyuksyutova *et al.* [70] was very smooth, with no abrupt changes in steepness. In addition, there would be formidable regulatory challenges involved in appropriately matching the time of arrival of this steepness peak progressively along the spinal cord. Furthermore, if this concentration wave took the form of a bump, on the other side of the bump the gradient would be pointing in the wrong direction. Thus, while the Wnt4 gradient is clearly an important component of guidance in this system, alone it is unlikely to provide a sufficiently directional cue.

While this is a particularly illustrative example, similar considerations apply to many situations where gradients have been hypothesised to control *in vivo* guidance. Turns *in vivo* are often more gradual than the case described here, consistent with a shallower gradient cue. However, this does not relieve the problems of gradient-based explanations: these considerations of intrinsic noise imply an even larger variance in axon responses would be expected for a gradual turn in response to a shallow gradient as compared with a sharp turn in response to a steep gradient.

0.5 + 0.1 = 0.6 for one gradient, it would be approximately 0.5 + 0.15 = 0.65 for two gradients. Adding additional gradients certainly helps, but the improvement in performance is incremental rather than transformational. Combinations of gradients are still subject to the same fundamental physical limits on concentration measurement.

Thus, noise constraints imply that gradient guidance is unreliable, has a limited spatial range, and (as seen in Equation 1) varies strongly in fidelity as an axon traverses a range of concentrations. Box 1 analyses a specific *in vivo* example (see also Figure 3), and Box 2 discusses the fidelity of turning observed in *in vitro* assays.

Other Mechanisms Are Likely To Be Involved

Wiring the nervous system requires axons reach precisely defined locations. Robust guidance cues are thus essential. As discussed earlier, converging evidence from both theoretical and *in vitro* studies suggests that gradient guidance by itself is not sufficiently robust to fully explain axon trajectories *in vivo*.

Current data certainly suggest that gradient cues play an important role (see earlier). However I suggest gradient cues may often be sufficiently robust only when combined with other types of

Box 2. Analysis of *In Vitro* Data

In vivo there may be many guidance cues working in concert with gradient cues. It is only *in vitro* that the effects of gradients on axon growth can be measured in the absence of other guidance cues. Even if such assays are not a good model of *in vivo* conditions, they nonetheless provide a measurement of the unitary effect of gradient cues on axon growth. Interestingly, however, in these assays axonal growth is generally fairly variable. It is usually only via averaging that a gradient-induced bias emerges (the size of which is often very small). The commonly used growth cone turning assay [72] produces gradients at the growth cone of a steepness of approximately 10% [73]. Here, average turning angles over 1 h are consistently about 10–20 degrees, and typically some proportion of axons either do not turn or turn in the wrong direction [72,74,75]. Similar results are produced by the Dunn chamber assay [76]. Recently, several microfluidics-based assays have been introduced that offer benefits over the growth cone turning assay, including higher throughput, more stable gradients, and assay durations potentially lasting days (better matching the timescale of *in vivo* guidance events) [68,77]. However, average turning angles in these assays are also generally small. For instance, in a recent study, after 1 day's growth in a 1–2% gradient in a microfluidics chamber average axon turning was around 15 degrees [14].

An alternative approach is the 3D collagen gel assay, where neurite outgrowth from explants is examined in response to a gradient either secreted by a piece of target tissue or a block of transfected cells [17,18], or printed onto the surface of the gel [40,78]. Here, the principal mode of response is usually not axon turning but, rather, more outgrowth up the gradient than down the gradient [43]. This is another form of chemotactic response [17,43], and when the gradient is precisely controlled the response is well predicted by theory [40]. However, as before, the degree of gradient-induced bias is very modest.

Most *in vitro* studies average over the responses of a nonuniform set of neurons, which would be expected to impart variability in the results that would not be present if a pure population was used. However, even though many different types of neurons have been used in these assays, average turning angles are consistently small, suggesting that the variability in the response is at least largely due to the intrinsic variability in gradient sensing, rather than variability in the axon population being studied. Furthermore, if a weak response was due to averaging a mixture of responsive and nonresponsive neurons, one would expect to see a bimodal distribution of turning angles. However, such results have not been reported.

Therefore, although the potential for further optimisation of turning responses in these assays cannot be ruled out, data from current *in vitro* assays are consistent with the idea that growth cones are not very reliable sensors of gradient cues. Indeed, there are no *in vivo* guidance events for which the trajectories of axons have been quantitatively reproduced by *in vitro* gradient assays. The turns produced by gradients *in vitro* are generally too small, and the variability of responses *in vitro* is much higher than that observed *in vivo*.

guidance cues. Precedent for this idea comes from the relatively well-understood system of retinotectal mapping [51]. Here, the problem is to understand how axons from neighbouring points in the retina are guided to neighbouring points in the tectum. Sperry's chemospecificity hypothesis [52] proposed this occurred via the matching of graded positional cues present in each structure. This hypothesis provided a productive framework for experimental work, and the identity of the gradients that could potentially implement this mechanism was eventually discovered [12]. However, even before this discovery, it had become clear that gradients by themselves are not sufficient to fully explain the experimental data [53]. This conclusion has since been amplified by genetic manipulations of gradient cues. In particular, gradient guidance is now generally understood to be just one component of a set of mechanisms, including competition [23] and axon–axon interactions [54,55], which all conspire to produce appropriate guidance.

One intriguing possibility is that axons may work together to collectively produce a greater sensitivity to gradients than would be possible individually. For instance, recent work has suggested that cell–cell communication could improve gradient sensing in cell migration [56,57]. While the relevance of this to axons remains to be determined, it is important to note many axon guidance events *in vivo* rely on a small number of pioneer axons, which must path-find relatively independently. In some systems, there is also evidence for 'corridors', permissive zones that help to channel axons in particular directions [58]. By reducing the range of options available, such corridors could potentially make gradients more reliable.

Besides chemical signals, it is also possible that mechanical cues play a currently underappreciated role [59], and the interaction between chemical and physical cues may be important [60]. Some other types of cues include guidepost cells [6] and galvanotropism [61]. However,

perhaps owing to the difficulty of thoroughly characterising the *in vivo* environment of growing axons molecularly, mechanically, and electrically, little is known about the presence of non-gradient cues and their interactions with gradient cues *in vivo*.

Concluding Remarks

In the past two decades, dramatic progress has been made in our understanding of the molecules involved in axon guidance, and some important principles have been established. However, to adapt a quote from Winston Churchill, I suggest that our current understanding of axon guidance is not the end, nor even the beginning of the end, but it is, perhaps, the end of the beginning. In particular, I suggest that our understanding of axon guidance is still in its infancy, and that much remains to be discovered about how axons are steered to their targets *in vivo* (see Outstanding Questions). These new discoveries will be essential for understanding how gene mutations reorganise brain wiring, and how to optimally promote appropriate rewiring of the brain after injury.

It should be clear that mathematical modelling has an important role to play in these investigations. Models have a unique ability to test the adequacy of particular explanations, and refine the assumptions required for particular mechanisms to produce the outcomes observed experimentally [62,63]. An example of the impact of such models is in understanding retinotectal mapping. From initially assessing general principles [64,65], computational models of retinotectal development have now moved on to being able to explain large and diverse sets of data [66,67]. However, no such models exist for most other *in vivo* guidance systems, and computational models of axon trajectories have generally been applied only to *in vitro* data [68,69]. Thus, much hard work, and doubtless many surprises, lie ahead.

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References

1. Geschwind, D.H. and Levitt, P. (2007) Autism spectrum disorders: developmental disconnection syndromes. *Curr. Opin. Neurobiol.* 17, 103–111
2. Yaron, A. and Zheng, B. (2007) Navigating their way to the clinic: emerging roles for axon guidance molecules in neurological disorders and injury. *Dev. Neurobiol.* 67, 1216–1231
3. Lin, L. *et al.* (2009) Axon guidance and synaptic maintenance: preclinical markers for neurodegenerative disease and therapeutics. *Trends Neurosci.* 32, 142–149
4. Stoeckli, E.T. (2012) What does the developing brain tell us about neural diseases? *Eur. J. Neurosci.* 35, 1811–1817
5. Tessier-Lavigne, M. and Placzek, M. (1991) Target attraction – are developing axons guided by chemotropism? *Trends Neurosci.* 14, 303–310
6. Tessier-Lavigne, M. and Goodman, C.S. (1996) The molecular biology of axon guidance. *Science* 274, 1123–1133
7. Mortimer, D. *et al.* (2008) Growth cone chemotaxis. *Trends Neurosci.* 31, 90–98
8. Swaney, K.F. *et al.* (2004) Eukaryotic chemotaxis: a network of signaling pathways controls motility, directional sensing, and polarity. *Annu. Rev. Biophys.* 39, 265–289
9. Sourjik, V. and Wingreen, N.S. (2012) Responding to chemical gradients: bacterial chemotaxis. *Curr. Opin. Cell Biol.* 24, 262268
10. Dickson, B.J. (2002) Molecular mechanisms of axon guidance. *Science* 298, 1959–1964
11. Kolodkin, A.L. and Tessier-Lavigne, M. (2011) Mechanisms and molecules of neuronal wiring: a primer. *Cold Spring Harb. Perspect. Biol.* 3, a001727
12. Flanagan, J.G. and Vanderhaeghen, P. (1998) The ephrins and Eph receptors in neural development. *Annu. Rev. Neurosci.* 21, 309–345
13. Kennedy, T.E. *et al.* (2006) Axon guidance by diffusible chemoattractants: a gradient of netrin protein in the developing spinal cord. *J. Neurosci.* 26, 8866–8874
14. Sloan, T.F. *et al.* (2015) Integration of shallow gradients of Shh and Netrin-1 guides commissural axons. *PLoS Biol.* 13, e1002119
15. Letourneau, P.C. (1978) Chemotactic response of nerve fiber elongation to nerve growth factor. *Dev. Biol.* 66, 183–196
16. Gundersen, R.W. and Barrett, J.N. (1979) Neuronal chemotaxis: chick dorsal-root axons turn toward high concentrations of nerve growth factor. *Science* 206, 1079–1080
17. Lumsden, A.G.S. and Davies, A.M. (1983) Earliest sensory nerve fibres are guided to peripheral targets by attractants other than nerve growth factor. *Nature* 306, 786–788
18. Tessier-Lavigne, M. *et al.* (1988) Chemotropic guidance of developing axons in the mammalian central nervous system. *Nature* 336, 775–778
19. Ming, G-I. *et al.* (1997) cAMP-dependent growth cone guidance by netrin-1. *Neuron* 19, 1225–1235
20. Campbell, D.S. *et al.* (2001) Semaphorin 3A elicits stage-dependent collapse, turning, and branching in Xenopus retinal growth cones. *J. Neurosci.* 21, 8538–8547
21. Weinl, C. *et al.* (2003) On the turning of *Xenopus* retinal axons induced by ephrin-A5. *Development* 130, 1635–1643
?>22. Serafini, T. *et al.* (1996) Netrin-1 is required for commissural axon guidance in the developing vertebrate nervous system. *Cell* 87, 1001–1014
23. Feldheim, D.A. *et al.* (2000) Genetic analysis of ephrin-A2 and ephrin-A5 shows their requirement in multiple aspects of retinocollicular mapping. *Neuron* 25, 563–574
24. Bagri, A. *et al.* (2002) Slit proteins prevent midline crossing and determine the dorsoventral position of major axonal pathways in the mammalian forebrain. *Neuron* 33, 233–248

Outstanding Questions

To go beyond simple back-of-envelope calculations regarding the physical constraints on axon guidance by gradients, quantitative measurements of a number of parameters are needed. These include the number of guidance receptors expressed by growth cones, the on- and off-rates for the ligand–receptor interactions involved in axon guidance, and the spatial variation in absolute (not merely relative) concentrations of ligands *in vivo*.

Many reports in the literature present pictures only of strong guidance, de-emphasising the full range of axon behaviour that actually occurs. To understand how precise brain wiring can arise, it is critical to understand both the extent and underlying causes of response variability, and how the brain manages this variability.

It is important to understand how the fidelity of gradient guidance could potentially be improved by cooperative effects, and the collaboration of gradients with other types of guidance cues.

Our understanding of physical phenomena is based on mathematical laws. Similarly, our understanding of axon guidance will be incomplete until we have mathematical models that can predict axon trajectories (and their variability) with quantitative accuracy. In particular, to understand the role of mutations in guidance ligands and receptors in causing neural disorders, it is critical to understand quantitatively how axon trajectories are changed by altering the distribution of guidance cues present.

25. Charron, F. *et al.* (2003) The morphogen sonic hedgehog is an axonal chemoattractant that collaborates with netrin-1 in midline axon guidance. *Cell* 113, 11–23
26. Chedotal, A. and Richards, L.J. (2010) Wiring the brain: the biology of neuronal guidance. *Cold Spring Harb. Perspect. Biol.* 2, a001917
27. Song, H. and Poo, M-M. (2001) Cell biology of neuronal navigation. *Nat. Cell Biol.* 3, E81–E88
28. Berg, H.C. and Purcell, E.M. (1977) Physics of chemoreception. *Biophys. J.* 20, 193–219
29. DeLisi, C. *et al.* (1982) A theory of measurement error and its implications for spatial and temporal gradient sensing during chemotaxis. *Cell Biophys.* 4, 211–229
30. Tranquillo, R.T. (1990) Theories and models of gradient perception. In *Biology of the Chemotactic Response* (Armitage, J. and Lackie, J.M., eds), pp. 35–75, Cambridge University Press
31. Bialek, W. and Setayeshgar, S. (2005) Physical limits to biochemical signaling. *Proc. Natl. Acad. Sci. U.S.A.* 102, 10040–10045
32. Shibata, T. and Fujimoto, K. (2005) Noisy signal amplification in ultrasensitive signal transduction. *Proc. Natl. Acad. Sci. U.S.A.* 102, 331–336
33. Kaizu, K. *et al.* (2014) The Berg–Purcell limit revisited. *Biophys. J.* 106, 976–985
34. Bicknell, B.A. *et al.* (2015) The limits of chemosensation vary across dimensions. *Nat. Commun.* 6, 7468
35. Poo, M-M. (1982) Rapid lateral diffusion of functional ACh receptors in embryonic muscle cell membrane. *Nature* 295, 332–334
36. Wiegel, F.W. (1983) Diffusion and the physics of chemoreception. *Phys. Rep.* 95, 283–319
37. Goodhill, G.J. and Urbach, J.S. (1999) Theoretical analysis of gradient detection by growth cones. *J. Neurobiol.* 41, 230–241
38. Kinney, J.B. and Atwal, G.S. (2014) Equitability, mutual information, and the maximal information coefficient. *Proc. Natl. Acad. Sci. U.S.A.* 111, 3354–3359
39. Xiong, Y. *et al.* (2010) Cells navigate with a local-excitation, global-inhibition-biased excitable network. *Proc. Natl. Acad. Sci. U.S.A.* 107, 17079–17086
40. Mortimer, D. *et al.* (2009) A Bayesian model predicts the response of axons to molecular gradients. *Proc. Natl. Acad. Sci. U.S.A.* 106, 10296–10301
41. Yuan, J. *et al.* (2013) Optimality and saturation in axonal chemotaxis. *Neural Comput.* 25, 833–853
42. Goodhill, G.J. *et al.* (2015) The dynamics of growth cone morphology. *BMC Biol.* 13, 10
43. Mortimer, D. *et al.* (2010) Axon guidance by growth-rate modulation. *Proc. Natl. Acad. Sci. U.S.A.* 107, 5202–5207
44. Tani, T. *et al.* (2005) Trafficking of a ligand–receptor complex on the growth cones as an essential step for the uptake of nerve growth factor at the distal end of the axon: a single-molecule analysis. *J. Neurosci.* 25, 2181–2191
45. Ming, G.L. *et al.* (2002) Adaptation in the chemotactic guidance of nerve growth cones. *Nature* 417, 411–418
46. Piper, M. *et al.* (2005) Endocytosis-dependent desensitization and protein synthesis-dependent resensitization in retinal growth cone adaptation. *Nat. Neurosci.* 8, 179–186
47. Xu, J. *et al.* (2005) Adaptation is not required to explain the long-term response of axons to molecular gradients. *Development* 132, 4545–4552
48. Dudanova, I. and Klein, R. (2013) Integration of guidance cues: parallel signaling and crosstalk. *Trends Neurosci.* 36, 295–304
49. Garel, S. and López-Bendito, G. (2014) Inputs from the thalamocortical system on axon pathfinding mechanisms. *Curr. Opin. Neurobiol.* 27, 143–150
50. Mortimer, D. *et al.* (2011) Bayes-optimal chemotaxis. *Neural Comput.* 23, 336–373
51. Cang, J. and Feldheim, D.A. (2013) Developmental mechanisms of topographic map formation and alignment. *Annu. Rev. Neurosci.* 36, 51–77
52. Sperry, R.W. (1963) Chemoaffinity in the orderly growth of nerve fiber patterns and connections. *Proc. Natl. Acad. Sci. U.S.A.* 50, 703–710
53. Goodhill, G.J. and Richards, L.J. (1999) Retinotectal maps: molecules, models, and misplaced data. *Trends Neurosci.* 22, 529–534
54. Reber, M. *et al.* (2004) A relative signalling model for the formation of a topographic neural map. *Nature* 431, 847–853
55. Suetterlin, P. and Drescher, U. (2014) Target-independent ephrina/EphA-mediated axon–axon repulsion as a novel element in retinocollicular mapping. *Neuron* 84, 740–752
56. Ellison, D. *et al.* (2015) Cell–cell communication enhances the capacity of cell ensembles to sense shallow gradients during morphogenesis. *arXiv* 1508.04692
57. Varennes, J. and Mugler, A. (2015) Sense and sensitivity: physical limits to multicellular sensing and drug response. *arXiv* 1512.00496
58. Lopez-Bendito, G. *et al.* (2006) Tangential neuronal migration controls axon guidance: a role for neuregulin-1 in thalamocortical axon navigation. *Cell* 125, 127–142
59. Franze, K. (2013) The mechanical control of nervous system development. *Development* 140, 3069–3077
60. Moore, S.W. *et al.* (2012) Netrin-1 attracts axons through FAK-dependent mechanotransduction. *J. Neurosci.* 32, 11574–11585
61. Yamashita, M. (2013) Electric axon guidance in embryonic retina: galvanotropism revisited. *Biochem. Biophys. Res. Commun.* 431, 280–283
62. Mogilner, A. *et al.* (2006) Quantitative modeling in cell biology: what is it good for? *Dev. Cell* 11, 279–287
63. Goodhill, G.J. (2007) Contributions of theoretical modelling to the understanding of neural map development. *Neuron* 56, 301–311
64. Prestige, M.C. and Willshaw, D.J. (1975) On a role for competition in the formation of patterned neural connexions. *Proc. R. Soc. Lond. B: Biol. Sci.* 190, 77–98
65. Gierer, A. (1987) Directional cues for growing axons forming the retinotectal projection. *Development* 101, 479–489
66. Goodhill, G.J. and Xu, J. (2005) The development of retinotectal maps: a review of models based on molecular gradients. *Network* 16, 5–34
67. Hjorth, J.J. *et al.* (2015) Quantitative assessment of computational models for retinotopic map formation. *Dev. Neurobiol.* 75, 641–666
68. Nguyen, H. *et al.* (2016) A mathematical model explains saturating axon guidance responses to molecular gradients. *Elife* 5, e12248
69. Maskery, S. and Shinbrot, T. (2005) Deterministic and stochastic elements of axonal guidance. *Annu. Rev. Biomed. Eng.* 7, 187–221
70. Lyuksyutova, A.I. *et al.* (2003) Anterior–posterior guidance of commissural axons by Wnt-frizzled signaling. *Science* 302, 1984–1988
71. Yam, P.T. *et al.* (2012) 14-3-3 proteins regulate a cell-intrinsic switch from sonic hedgehog-mediated commissural axon attraction to repulsion after midline crossing. *Neuron* 76, 735–749
72. Lohof, A.M. *et al.* (1992) Asymmetric modulation of cytosolic cAMP activity induces growth cone turning. *J. Neurosci.* 12, 1253–1261
73. Pujic, Z. *et al.* (2008) Analysis of the growth cone turning assay for studying axon guidance. *J. Neurosci. Meth.* 170, 220–228
74. Song, H.J. *et al.* (1997) cAMP-induced switching in turning direction of nerve growth cones. *Nature* 388, 275–279
75. Forbes, E.M. *et al.* (2012) Calcium and cAMP levels interact to determine attraction versus repulsion in axon guidance. *Neuron* 74, 490–503
76. Yam, P.T. *et al.* (2009) Sonic Hedgehog guides axons through a noncanonical, src-family-kinase-dependent signaling pathway. *Neuron* 14, 349–362
77. Dupin, I. *et al.* (2013) Investigating axonal guidance with microdevice-based approaches. *J. Neurosci.* 33, 17647–17655
78. Rosoff, W.J. *et al.* (2004) A new chemotaxis assay shows the extreme sensitivity of axons to molecular gradients. *Nat. Neurosci.* 7, 678–682