Theoretical analysis of guidance cue synergy by simulations at multiple spatial scales

Tyler Sloan

Introduction

During embryonic development, axons must grow long distances, often making complex trajectories towards their target areas of the CNS. The developing axon is guided by its motile tip, the growth cone, which is distinct in its structure and function. As the axon extends, the growth cone senses its local environment, propelling itself forward in response to environmental cues. It has a dynamic and constantly changing shape, as was noted soon after its discovery soon after its discovery when it was observed to behave similar to other migrating cell types[1]. The analogy between the growth cone and other migrating cell types has since allowed the axon guidance field to advance more rapidly, by drawing from observations and theory from the field of cell migration.

It was proposed over a century ago that the growth cone is guided from a distance by gradients of guidance molecules[2], but this was not formally proven until the 1980's[3,4]. It is now accepted that the growth cone can respond to concentration gradients of specific molecules, such that they reorient the axon in the direction of the higher concentration; molecules with this property have thus been termed guidance cues. Since the isolation of the first guidance cues[5,6], the field of axon guidance has moved forward with a thirst for mechanism, thanks partly to the demand of the pharmaceutical industry for potential drug targets and a generation of biochemists focused on revealing the signaling pathways involved in the detection and interpretation of guidance cues within the growth cone. The years of

effort have resulted in many discovered guidance cues and signaling pathways, and there certainly remain others yet to be discovered. As the list of *bona fide* guidance cues grew, it became clear that the growth cone is often exposed to multiple cues simultaneously, and that somehow the molecular machinery must be integrating the different sources of information. The field has more recently begun to look in detail at how guidance cues collaborate, and where their signaling pathways converge within the growth cone[7].

Conceptual problem

While there are several detailed molecular pathways at play, the events leading up to a successful guidance decision by the growth cone can be generalized: molecules of guidance cue are secreted by cells in one region of the CNS and diffuses away from its source such that there are always more molecules closer to the source than further away; forming a concentration gradient. While the the concentration gradient at the macroscopic level can be described deterministically[8], at the molecular level it is best described by a stochastic random walk[9]. Guidance molecules occasionally come into contact with protein receptors on the surface of growth cones that bind to the guidance cue with high affinity. These receptor/guidance cue binding events relay signals inside the growth cone, which activate other molecules in a signaling cascade that ultimately controls the direction a growth cone will advance. It is not well understood, however, how the growth

cones signaling machinery is capable of integrating the independent signaling pathways, and where the signaling pathways converge.

This process becomes even more interesting with the condition that the growth cone must necessarily be able to determine which side senses a higher guidance cue concentration. That's to say that some computation within the growth cone must occur, and protein networks that interpret the signals must be sensitively tuned enough for the growth cone to detect subtle differences in the number of molecules from one side to the other, despite being fundamentally constrained by the binary nature of ligand-receptor binding, and statistical noise resulting from molecular uncertainty[10]. Precisely how the growth cone makes this computation is a question that is not solely within the purview of biochemistry, but can only be truly understood computationally; knowing all the different signaling molecules and interactions isn't sufficient to fully explain how the growth cone interprets the gradient direction.

With all the effort that has gone into understanding the molecular mechanism, few attempts have been made to address the problem of axon guidance at a theoretical level[11]. The most successful explanations have come from simulations which actually modeled *in vitro* experiments in a series of papers from Goodhill and colleagues[12,13,14], who have developed formal equations for the guidance of a growth cone. For this paper, I will explore some of the model parameters in depth, and run simulations

where I extend this model to predict the growth cone response to multiple guidance cues. I will then use an alternative model at the scale of the molecular interactions to confirm the robustness of the predictions.

Gradient parameters explained

Before discussing the models in depth, we must first examine the different parameters of a concentration gradient to be discussed in the following sections. The macroscopic description of a concentration gradient can be described deterministically by differential equations relating to the diffusivity of the molecule and the position within the gradient[8] (Figure 1A), however at the microscopic scale, the gradient is composed of a finite number of molecular random walks[9] (Figure 1B). It is thus entirely stochastic and uncertain, where we can only consider the relative probability that a molecule is present at a certain position. This is an important distinction to make, since the models I will explore in this paper each occur at different spatial scales of the gradient description, and the stochastic nature of diffusion influences them differently.

There are some parameters of the gradient that remain important, regardless of which spatial scale we are interested in. The most intuitive of these parameters is the concentration of the guidance cue; how many molecules are present in a given volume. Because we are interested in how well a difference in concentration or number of molecules can be computed, the next logical measure of the gradient is

the slope - the difference in concentration across a given distance. We will consider a fixed distance: the width of a prototypical growth cone. Therefore the absolute change in concentration, ΔC, across a growth cone is proportional to the difference in the number of molecules at one side of the growth cone versus the other. I will be considering gradients of a linear profile (Figure 1C-D), where the ΔC at every position is the same. The more interesting parameter that I will explore in both models is the relative (fractional) difference in concentration across a growth cone, which I define as the gradient steepness. This is a dimensionless measure (ΔC/C), which is highest at the low concentration and vice versa within a linear gradient (Figure 1D).

Model description and exploration

This report consists of two models, both will attempt to address the main conceptual problem, although each will attempt to describe at a different scale. Model 1 will consider the spatial scale of the individual axon, and will involve comparing simulation results of many axons challenged with various gradient parameters. These simulations will use a formula previously derived mathematically using Bayesian inference[13]. Model 2 is at the scale of the protein interactions. For a single model growth cone, I have simulated the diffusion of guidance molecules, their interaction with receptors on the cell surface, and the resulting signal which is relayed within the growth cone. Because this involves complex interactions between moving particles whose positions are relevant, I have used a multi-agent model to simulate the emergent asymmetry. I will compare the behavior of the models, and observe how each model responds to the addition of a second, overlapping guidance cue gradient of the same concentration and steepness, which gives independent, corroborating information.

Model 1: Axon turning simulation model

As discussed previously, a growth cone must infer the direction of the gradient from receptor/guidance cue binding events, which is a largely stochastic process, constrained by noise. For this reason, Goodhill and colleagues have published a series of papers promoting a model using Bayesian inference to estimate the

direction of the gradient[12-14] . They derived an expression wherein the signal to noise ratio (SNR) computed by the growth cone is a function of the concentration of the guidance cue, the steepness of the concentration gradient, and the orientation of the growth cone with respect to the gradient (Figure 2A). In their model, the growth cone moves forward at each time step, leaving the axon behind it as it advances. At each time, the growth cone calculates the SNR, which biases the direction the axon turns in the following way. After calculating the SNR, we sample from a Gaussian distribution with a mean equal to the calculated SNR and a standard deviation of 1 (Figure 2B). If the resulting sample is positive, the axon turns rightward an angle of $\pi/30$. If the result is negative, the axon turns leftward the same amount. In this model, axon is constantly turning in a random direction, however the direction is biased by the SNR. When a growth cone computes a strong SNR, there is a very high likelihood, though no guarantee, that the turn will be toward the gradient (Figure 2C-E).

Model 1 exploration

Using their model, I first explored the influence that parameters of the gradient has on the behavior of a large number of axons. I did this with simulations of 5000 axons per condition, and tested a logarithmic range of gradient concentration and steepness. I explored the behavior of the model by observing how a range of concentration and steepness would influence the calculated SNR and the output of simulated axons themselves (Figure 3). The influence of the steepness and

concentration components of the model on the resulting SNR are trivial given the equation in figure 2A, however it is worthwhile to compare their influence with the behavioral output of the model. For both parameters, there are subtle noteworthy differences. First, when the concentration is increased, the output of the model reaches its plateau at a lower concentration than the calculated SNR (Figures 2A-B). This could be explained by the fact that the behavior of the model output is effectively the sum of 200 iterations where the SNR is calculated. As a result, the mid-range concentrations which yield a low SNR have the potential to influence the axon direction over many iterations, as the growth cone has more time to correct its trajectory. A similar difference is seen between the calculated SNR as a function of gradient steepness, and the model output, although by a slightly different mechanism. The linear relationship defined in the SNR calculation is not strictly maintained in the model output (Figure 3C-D). At a steepness of 1, the behavior of the model plateaus, whereas the SNR continues to rise linearly (the simulation results above 1 are not shown, as I focus on the lower range for this report). This is because there is a maximum behavioral output of the model that occurs when all the simulated axons have properly reoriented towards the gradient. When all axons are already facing the correct direction, there is no further increase in the output that can be achieved by further increasing the steepness.

Model 1 extension – combined gradients

To extend the axon turning model to address this question of how multiple guidance cues could offer an advantage to axons navigating in response to multiple, overlapping concentration gradients, I begun with the assumption that each guidance cue has the ability to influence the calculated SNR independently. This is biologically consistent, as axon guidance responses *in vitro* can be measured when a single guidance cue is presented. Further, I explored the simplest model of combining signals, wherein the SNR from either cue is summed by the growth cone. This is both biologically relevant and plausible, as it has been proposed that some molecules could act as nodes of integration where multiple guidance cues eventually converge on the same intracellular signaling molecules[7]. For simplicity I've explored the model where both gradients are of identical concentration and steepness, which is reasonable given that overlapping gradients are likely to share a common source (i.e. floor-plate). It is worth noting that simply adding the signals together in this way is referred to as the suboptimal combination strategy by Mortimer and colleagues[14], as they have derived a more elaborate optimal combination rule which avoids the noise that impedes the model performance at high concentrations of both cues. Since I will be only considering concentrations below the dissociation constant (concentration = 1), I have only explored here the suboptimal combination rule, where the SNR calculated from one cue is summed with the SNR calculated from a second cue (Figure 4A). If we consider the case where the gradients are of equal concentration and steepness, then the expression simplifies to 2*SNR for the combined gradient condition.

Despite the simplicity of this approach, the model offers some interesting predictions. As would be anticipated, when the steepness of both cues is low, the the behavior of either condition is equally poor (Figure 4B, left panel). However, at a moderate steepness value, there is a point where we begin to see performance in the 2 cue model while the single cue model is performing effectively at random (Figure 4B middle panels). As the steepness continues to rise, there is a point where both models are performing exceptionally, however we can still see the 2 cue model outperforming the single cue model by looking at the trajectories. Quantifying the model output over a logarithmically defined range of concentration and steepness makes two important predictions. First, at low concentration, there is a point where a single gradient is insufficient to attract axons, however a combined gradient is sufficient, as evidenced by the 2 cue condition rising and saturating at a lower concentration than a single cue (Figure 4C). Furthermore, when the steepness is varied (and the concentration is sufficient, $\gamma=1$), a similar influence is observed upon addition of 2 cues. The model response to two gradients is shifted relative to the single cue gradient, thus there are steepness values at which a single cue is unable to attract axons, but 2 cues can (Figure 4D). These two model outcomes predict different phenomena, both equally interesting. In one case, having a second cue can help the growth cone sense the direction when the amount of guidance cue itself is limited. Effectively doubling the amount of guidance cue can help the number of signaling events reach the critical amount necessary for robustly

interpreting the direction of the gradient. On the other hand, when the concentration itself is not limiting, instead the steepness is limiting, having overlapping sources of information can help a growth cone make the proper decision. Therefore, the combined turning model predicts that the linear combination of guidance cues in the growth cone can lead to either concentration-limited or steepness-limited synergy.

Model 2: Agent based model of protein-protein interactions

I then wanted to determine whether the predictions made using the turning model (model 1) are robust to different spatial scales, or in contrast may result from oversimplification during their derivation. I implemented the 'local excitation, global inhibition' chemotaxis model[15,16] in Netlogo[17], where individual interacting units ('turtles') have their behavior and the consequences of their interactions programmed. This type of model is used typically when the interactions between individuals achieve a level of complexity due to inter-dependencies with each others' state and location. The model is initiated as follows. A linear concentration gradient of the guidance cue is created, where each molecule follows a random walk as time progresses, wherein it moves forward one position in a random heading. I then allowed enough iterations to ensure the gradient is continuous and linear. I then initialized the cell by adding 'receptors', which create the perimeter of a circle, representing the cell (Figure 5B). The receptors are able to diffuse freely laterally, while being constrained to the cell radius, which also constitutes a boundary where

molecules diffusing within the cell are unable to escape. Next, three species of intracellular signaling molecules are initialized, which are free to diffuse only within the cell. These consist of excitors, inhibitors, and response regulators. For each, the logic of their interactions are outlined below (and in Figure 5C). The model is then run for several iterations until the intracellular species have had a chance to diffuse throughout the area of the cell to avoid a lag in the response of the model due to start-up artifacts.

When the simulation is started, the guidance cue molecules diffuse and occasionally interact with a receptor at the cell surface. When this occurs, the receptor is changed into an active state (S). As the receptor diffuses at the perimeter of the cell, it will occasionally come into contact with excitors and inhibitors within the cell. If an excitor comes into contact with an active receptor, the excitor itself becomes temporarily in an active state (E). If an inhibitor comes into contact with an active receptor, it too will becomes temporarily in an active state (I). The response regulator has no such interaction with the receptors. However, if a response regulator comes into contact with an active excitor, it becomes in an active state (R). On the contrary, if an active response regulator comes into contact with an active inhibitor, it is returned to an inactive state. Thus, the response regulator is positively regulated by activated excitors, while being negatively regulated by activated inhibitors. These interactions are summarized in the schematic diagram (Figure 5C). As a slight deviation for the formalized model[15], I have added a

background level of inhibition resulting from a proportion of the inhibitors beginning the simulation in an active state. This was the best way I found of achieving a truly global inhibition throughout the cell within such short simulations. Another alteration from the formalism in Figure 5C is that the inhibitors and response regulators also diffuse throughout the cell – which I feel to more biologically plausible.

In this 'local excitation, global inhibition' model of chemotaxis, it is thought that the response regulator is ultimately responsible for conveying the direction of the external gradient. For our purposes, this is appropriate because it is known that the distribution of signaling molecules within the growth cone (such as Src-family kinases) can reflect the direction of the external concentration gradient[18]. In this model, when the growth cone is able to determine the direction of the gradient, an accumulation of active response regulators is seen preferentially at the side of the cell that experiences the higher concentration. In keeping with this role of the response regulator, I used the asymmetry of its distribution as the measure of the cells inference of gradient direction. I calculate the log ratio of the number of active response regulators in the proximal $1/3^{rd}$ to the distal $1/3^{rd}$ of the cell. This ratio is then used to compare the behavior between models. It is an assumption of my measurement and model that the asymmetry of the response regulator is directly proportional to the ability of the growth cone to determine the direction of the gradient, and thus is analogous to the SNR calculated in model 1.

Comparison of stochastic elements of models at different spatial scales

Both model 1 and model 2 occur at spatial scales which differ in at least an order of magnitude, as a result the models also differ largely in what constitute their deterministic and stochastic components. The axon turning model computes the SNR in a deterministic fashion, such that a growth cone experiencing the same concentration and steepness with the same orientation will always obtain the same calculated SNR value. Despite the derivations using stochastic differential equations[14], in the simplified equation the SNR calculation is deterministic. The stochastic component of the model is the likelihood that the growth cone uses the SNR effectively. Since the direction of the turn depends on a sample from a Gaussian distribution centered at the SNR, there remains the possibility that an axon can turn the incorrect direction, despite having a strongly positive SNR. In contrast, model 2 can be considered as entirely stochastic. Although the concentration gradient is initially set according to a defined density, each molecule of the gradient follows a random walk, and its position is thus entirely uncertain. Thus, the overall profile of the gradient is probabilistic, instead of entirely determined, as in model 1. The two models are similar in how the probability of a receptor being bound is proportional to the concentration, however in model 2, the probability of a receptor being bound also depends on the probability that the two molecules happen to occupy the same spatial location, which I believe to be a more realistic physical constraint. This adds another layer of randomness into the model. Although I consider the calculation of

the SNR in model 1 to be analogous to the computation of difference in the number of active response regulators in model 2, the SNR in model 1 is calculated as a deterministic function of concentration and steepness, whereas the response regulator asymmetry in model 2 depends on where the active receptors tend to occur at the surface of the cell, and the reaction-diffusion dynamics within the cell. More specifically, the probability of a response regulator being active depends on the relative probability of it coming into contact with an active excitor versus an active inhibitor. Likewise, the probability of either of these events depends themselves on the probability that one of these came into contact with an active receptor. Thus, the measurement of asymmetry of active response regulators across the width of a growth cone would be expected to display much more variation between runs. In order to ensure that predictions generated from model 1 hold true at the spatial scale of within a growth cone, where statistical uncertainty reigns, I then compared how model 2 describes the observed phenomena.

Model 2 exploration

I evaluated the output of the model as the ability of the signaling interactions to generate an asymmetric distribution of active response regulators (R's) between sides of the cell up-gradient (proximal) versus down-gradient (distal) (Figure 5D). I calculated the SNR as the log-ratio of the number of active response regulators in the proximal third versus the distal third of the growth cone. I considered the asymmetry in a window of 100 iterations following the onset of cell signaling events

in the gradient. I compared the magnitude of asymmetry as a function of time for various concentrations of guidance cue (Figure 5E). Consistent with model 1, there was an increase in the SNR as the concentration of the gradient was increased (Figure 5F), demonstrating that concentration is a crucial parameter. However, I wasn't able to see the same dependence on the steepness of the gradient, which most likely results from the geometry of the gradient, and that my manipulation of the steepness involved also manipulating the concentration, and thus was not an ideal comparison. This criticism aside, I was able to generate a multi-agent model, based on a published system of differential equations, that allowed me to simulate the ability of a theoretical growth cone to sense concentration differences robustly.

Model 2 extension to multiple gradients

The goal, however, was to examine the influence of combined gradients, and to observe whether the predictions from model 1 were still valid at a more stochastic spatial scale. I modified the model such that the guidance cue, receptor, inhibitor and excitor agents each have a function duplicate, such that each guidance cue signals specifically through its unique pathway. To be most consistent with the extension of model 1, wherein the SNR was computed additively, the excitors and inhibitors of each parallel pathway all converged their influence on the same response regulators (Figure 6A). I then extended the formal description of the rate of activation of the response regulator (Figure 5C) to the case with an arbitrary number of guidance cues and signaling pathways, N (Figure 6A).

To test whether the combined gradient augments the response when the guidance cue concentration is low, I compared the single cue and double cue gradient models for increasing concentration values (Figure 6B). When the concentration is at its lowest value (defined as 1x), the single cue gradient is insufficient to generate a response, whereas the 2 cue gradient generates asymmetry that increases rapidly (Figure 6 C). I then increased the concentration to 4x, and observed a similar elevated performance of the two cue model, despite the fact that the concentration of the single cue was sufficient to generate a moderate and reproducible asymmetric response. These simulations confirm that, in theory, having more guidance cue when the concentration is limited could enhance the response of a growth cone sensitive to both cues. However, the caveat to this reasoning is that there is not necessarily any synergy between the cues, as this outcome could be explained entirely by the observation that increasing the concentration increases the response (Figure 5F), and that by having two cues you're effectively doubling the concentration. Therefore, I tested a control simulation, where the total number of guidance molecules was kept constant between the 1 cue and 2 cue conditions. In this case, the combined gradient of 1x cue_1 + 1x cue_2 was compared with 2x cue_1. In these simulations, the combined gradient greatly outperforms the single cue gradient (Figure 6E). I repeated this for the 4x concentration condition, such that 2x cue_1 + 2x cue_2 was compared with 4x cue_1, and observed the same trend (Figure 6F). These simulation results corroborate results from model 1 that guidance cues

collaborate when multiple signals converge. Intriguingly, there is a synergy which occurs when signaling pathways converge, and this cannot be explained solely by an increase in the number of guidance molecules (Figure 6E-F).

Conclusions

I have explored two models of how axons could interpret the direction of an external concentration gradient. The first model used a previously derived equation of gradient sensing to model the behavior of a large number of axons in response to gradients of varying concentration and steepness. The second modeled the network of protein interactions and assessed how the Local Excitation, Global Inhibition model could explain how well a growth cone can internally represent the direction of the external gradient. Both models were then extended to the case where multiple gradients of identical parameters portray the same directional information to the growth cone.

I've shown that the axon turning model (model 1) predicts that axonal response increases with increasing steepness, to a maximum value, and that the behavior is relatively consistent across a wide range of sufficient concentrations. In the presence of combined gradients, the combined gradient model surpasses the single gradient model when either the concentration or steepness is insufficient for a single cue to guide, which results in a response curve that rises more rapidly at lower values of the parameter of interest. This demonstrates theoretical precedent

for the existence of concentration-limited and steepness-limited synergy occuring when multiple guidance cues are integrated.

I've confirmed that both models support the role of multiple guidance cues in enhancing gradient sensing when the concentration of guidance cues is low. Furthermore, I've demonstrated that if the concentration of the guidance cue is no longer limiting, then the 2 cue model will still outperform the 1 cue model, beyond merely increasing signaling above a certain threshold. Although model 2 did not show direct theoretical proof of steepness-limited synergy as seen in model 1, it does demonstrate that a summation model of signals converging within the growth cone can theoretically result in a synergistic ability of the growth cone to sense the direction of an external gradient.

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