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Calcium and cAMP Levels Interact to Determine Attraction versus Repulsion in Axon Guidance

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SUMMARY

Correct guidance of axons to their targets depends on an intricate network of signaling molecules in the growth cone. Calcium and cAMP are two key regulators of whether axons are attracted or repelled by molecular gradients, but how these molecules interact to determine guidance responses remains unclear. Here, we constructed a mathematical model for the relevant signaling network, which explained a large range of previous biological data and made predictions for when axons will be attracted or repelled. We then confirmed these predictions experimentally, in particular showing that while small increases in cAMP levels promote attraction large increases do not, and that under some circumstances reducing cAMP levels promotes attraction. Together, these results show that a relatively simple mathematical model can quantitatively predict guidance decisions across a wide range of conditions, and that calcium and cAMP levels play a more complex role in these decisions than previously determined.

INTRODUCTION

The ability of growing axons to accurately locate targets during development or regeneration is critical for the formation of correct neural circuits. Growing axons are tipped with growth cones, which detect distributions of molecular guidance cues in their environment. A key mechanism for guidance is chemotaxis, whereby growth cones detect and respond to concentration gradients of these cues (Tessier-Lavigne and Goodman, 1996; Song and Poo, 2001; Chilton, 2006; Mortimer et al., 2008; O'Donnell et al., 2009). In a gradient, growth cone receptors closer to the source of the guidance cue are bound more frequently, leading to asymmetric intracellular signaling events mediated by second messengers. This leads to polarization of the growth cone, and a turn toward (attraction) or away (repulsion) from the source of the guidance cue.

Calcium signals mediate both growth cone turning and outgrowth (Cohan et al., 1987). Binding of the guidance cue to receptors on the growth cone can trigger the influx of calcium into the cytoplasm from calcium stores in the endoplasmic reticulum by activation of ryanodine receptors or inositol-1,4,5-triphophate receptors, or from extracellular sources via voltage-dependent calcium channels (Berridge et al., 2003) and transient receptor potential (TRP) calcium channels (Henle et al., 2011). Blocking calcium entry through membrane-bound or ryanodine channels can abolish the guidance response, or even switch a normally attractive turning response to a guidance cue to repulsion (Hong et al., 2000). Guidance cues that are normally repulsive do not usually result in calcium release from the endoplasmic reticulum and therefore only result in a shallow intracellular calcium gradient (Tojima et al., 2011). Thus, under normal conditions, a steep intracellular calcium gradient in response to a guidance cue gradient is likely to result in attraction, whereas a shallow intracellular calcium gradient is likely to result in repulsion (Hong and Nishiyama, 2010).

Calcium is quickly buffered by calmodulin, binding to form a calcium/calmodulin complex (Faas et al., 2011). Calcium/calmodulin has many effector molecules. Two of particular relevance for growth cone turning are calcium/calmodulin-dependent protein kinase II (CaMKII) and calcineurin (CaN). CaN has a higher affinity for calcium/calmodulin than CaMKII and thus responds more strongly than CaMKII at low calcium levels, whereas CaMKII signaling predominates over CaN at high calcium levels (Wen et al., 2004). CaMKII and CaN are necessary for attraction and repulsion respectively. Inhibiting CaMKII can block attraction, whereas inhibiting CaN can block repulsion and even convert repulsion to attraction if there are high levels of calcium influx (Wen et al., 2004). Therefore, the ratio of CaMKII to CaN appears to be crucial for determining attraction versus repulsion in guidance responses, rather than the absolute activity of each of these molecules. CaMKII and CaN can also regulate activity of one another at different calcium levels through CaN inhibition of the protein inhibitor 1 (I1), an inhibitor of protein phosphatase 1 (PP1), which in turn is an inhibitor of CaMKII (Wen et al., 2004; Figure 1A).

The regulation of growth cone turning becomes even more complex when one considers other important factors such as the baseline levels of calcium and the activity of cAMP and cGMP. Decreasing the baseline calcium level in the growth cone converts attraction to repulsion, implying an interaction between the baseline calcium level and the amount of calcium influx in determining the sign of the response (Zheng, 2000). Furthermore, increasing cAMP on one side of the growth cone

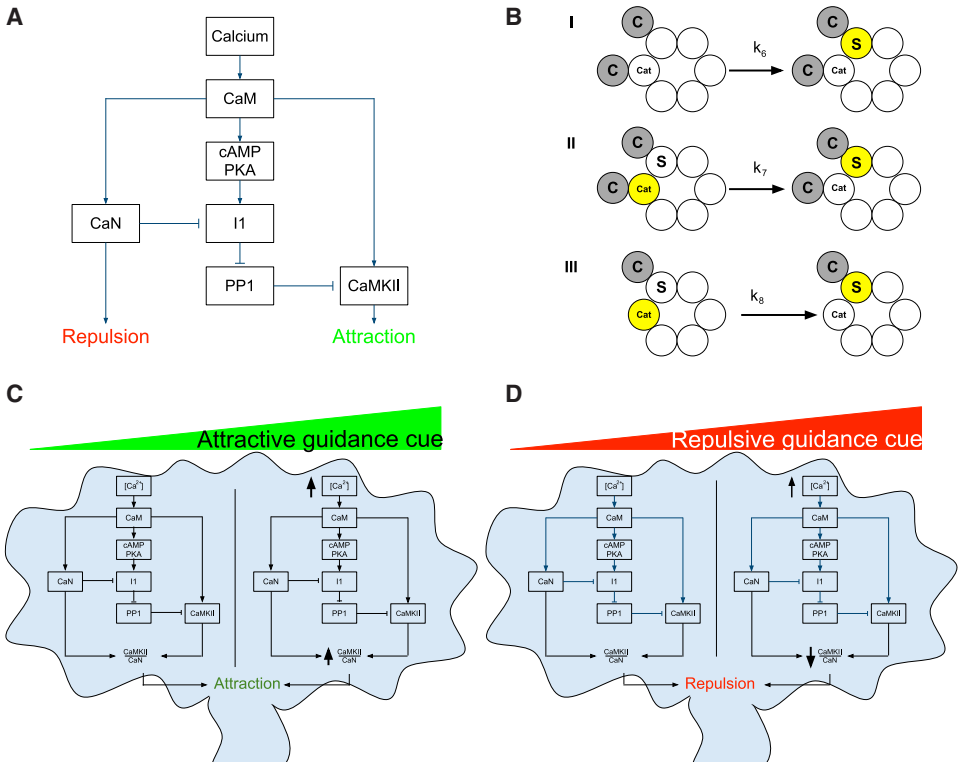

Figure 1. Schematics of the Model

(A) The signal transduction network hypothesized to underlie guidance receptor response switching (Han et al., 2007). CaM, calmodulin; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; I1, inhibitor 1; CaN, calcineurin; PP1, calcineurin phosphotase 1; CaMKII - calcium/calmodulin-dependent protein kinase II. High levels of CaMKII relative to CaN are hypothesized to promote attraction, and the opposite to promote repulsion.

(B) The phosphorylation of the six subunit ring in the CaMKII holoenzyme (white, dephosphorylated; yellow, phosphorylated). One subunit acts as a catalyst (Cat) and the other as a substrate (S). The substrate must be bound to a calcium/calmodulin complex (C, gray circles), but there are three possible states for the catalytic subunit: (I) bound to calcium/calmodulin, II bound to calcium/calmodulin and phosphorylated, III phosphorylated. k6, k7, and k8 are the model parameters giving the respective autophosphorylation rates of the three possibilities. Note that unlabeled subunits are shown here as dephosphorylated; however, the reaction between the catalyst and substrate occurs independently of any other subunits and therefore the state of the unlabeled subunits is unimportant.

(C and D) The division of the growth cone in the model into two compartments, with a reproduction of the signal transduction network in (A) in each compartment. The CaMKII:CaN ratios of each compartment are compared to determine whether the growth cone is attracted or repelled. (C) Attraction at normal baseline calcium (large increase in calcium in the up-gradient compartment leading to a higher CaMKII:CaN ratio in the up-gradient than down-gradient compartment). (D) Repulsion at normal baseline calcium (small increase in calcium in the up-gradient compartment leading to a lower CaMKII:CaN ratio in the up-gradient than down-gradient compartment).

See also Table S1 and Figure S1.

by presenting an extracellular gradient of cAMP promotes attraction (Lohof et al., 1992; Murray et al., 2009), whereas lowering the ratio of cAMP to cGMP activity in the presence of a guidance cue gradient can switch turning from attraction to repulsion (Ming et al., 1997; Song et al., 1997, 1998; Nishiyama et al., 2003). cAMP activates protein kinase A (PKA), which is also known to activate I1 (normally inhibited by CaN), and thus helps to promote attraction by reducing inhibition of CaMKII (Han et al., 2007; Figure 1A). Interpretation of this complex signaling process for guidance must allow for comparison between opposite sides of the growth cone, so that an asymmetric response is possible.

Here, we quantitatively test the hypothesis that turning occurs toward the side of the growth cone with the higher CaMKII:CaN ratio, by constructing a mathematical model of the signaling events discussed above. The model is inspired by previous work modeling the analogous switch between long-term potentiation (LTP) and long-term depression (LTD) based on the relative levels of CaMKII and CaN (Lisman, 1989; Graupner and Brunel, 2007). However, crucially, we consider distinct events occurring on the up-gradient and down-gradient sides of the growth cone, which allows the CaMKII:CaN ratio to be different between the two sides. We first show that this model quantitatively explains the known phenomenology for how calcium and cAMP levels affect the sign of growth cone turning. We then derive predictions from the model for the sign of the response in conditions previously untested experimentally. We test these predictions for both a normally attractive factor (nerve growth factor, NGF) and a normally repellent factor (myelin-associated glycoprotein, MAG) using the standard growth cone turning

assay and show a good match with the model. A particularly surprising result is that, for a normally attractive factor at high levels of baseline calcium, reducing cAMP levels promotes attraction, exactly the opposite response to that previously observed at normal baseline calcium when cAMP levels are reduced. Together, these results generate a unifying quantitative explanation for a large number of previous experimental results, and the model provides a method for quantitatively predicting attraction versus repulsion of growth cone turning in a wide variety of biological situations.

RESULTS

Previous work has shown that a calcium-calmodulin-dependent pathway is responsible for the directional growth of axons during development (Han et al., 2007). This is mediated by CaMKII and CaN, where CaMKII promotes attraction and CaN promotes repulsion (Wen et al., 2004; Henley and Poo, 2004; Gomez and Zheng, 2006). These are both stimulated through the actions of calcium/calmodulin, while the activity of CaMKII is also inhibited by PP1. The activity of PP1 is directly inhibited by I1, which in turn is phosphorylated by cAMP-dependent PKA and dephosphorylated by CaN (Henley and Poo, 2004; Figure 1A). So far, the behavior of this complex network has been studied only qualitatively. We therefore developed a mathematical model of this process to understand quantitatively how calcium and cAMP determine attraction versus repulsion of growth cone responses.

The model allows for analysis of the change in the CaMKII:CaN ratio in the up-gradient and down-gradient sides of the growth cone as the calcium concentration changes (see Figure S1 available online for an example of the activation of the different signaling components of the model as a function of calcium concentration). We assume that a higher CaMKII:CaN ratio in the up-gradient compartment compared with the down-gradient compartment leads to attraction, whereas a lower CaMKII:CaN ratio in the up-gradient compartment compared to the down-gradient compartment leads to repulsion. This means that an increase in calcium in the up-gradient compartment can result in attraction or repulsion, dependent on the resting level of calcium and the magnitude of the increase in calcium. In the model, the numerical value of the CaMKII:CaN ratio is not important, but rather it is the relative values of the CaMKII:CaN ratio between the two compartments that determines attraction or repulsion. The model addresses three separate problems of growth cone guidance: (1) the role of calcium influx into the growth cone during attraction and repulsion, (2) the role of baseline calcium in determining the response to a guidance cue, and (3) the role of cAMP in determining the response to a guidance cue.

Dependence of Attraction versus Repulsion on Calcium Levels

At resting level, a large increase in calcium in one compartment in the model leads to a higher CaMKII:CaN ratio in that compartment and thus attraction (Figure 2A, line 1, and Figure 2C, point M) whereas a small increase in calcium leads to a lower CaMKII:CaN ratio and thus repulsion (Figure 2B, line 1, and Figure 2D, point M). These results are consistent with previous experimental data: a large increase in calcium (using focal laser-induced photolysis to release caged calcium in one side of a growth cone) mediates attraction, whereas a small increase in calcium mediates repulsion (Zheng, 2000; Hong et al., 2000).

However, when neurons were placed in a calcium-free medium, thus reducing intracellular calcium, the same release of caged calcium resulted in repulsion (Zheng, 2000; Wen et al., 2004). When the resting calcium level is reduced in the model, either a small or large local increase in calcium in the up-gradient compartment causes a lower CaMKII:CaN ratio in that compartment compared to the down-gradient compartment, which results in repulsion (Figures 2A and 2B, line 2, and Figures 2C and 2D, point L). Thus, reducing the resting calcium level converts the response to a large increase in calcium from attraction to repulsion, whereas the response to a small increase in calcium remains as repulsion.

Increasing the baseline calcium can also affect the guidance response. MAG is a guidance cue for repulsion, and it causes a small elevation of internal calcium when binding to Nogo-66 receptors (Tojima et al., 2011). If the resting calcium level is increased, then MAG acts as an attractive guidance cue (Henley et al., 2004). This behavior is also reproduced in the model (Figure 2B, line 3, and Figure 2D, point MH). Although attraction could occur in our model at very low levels of calcium, in reality growth cones are unable to turn in either direction in this case, because there is then an insufficient calcium influx to trigger turning (Gomez and Zheng, 2006).

Based on the results of previous experiments, our model therefore confirms that it is not only the magnitude of the calcium increase which is important, but also the baseline calcium. The only way for attraction to occur at biologically plausible calcium concentrations is for one compartment to be over a certain threshold, which occurs due to the bimodal nature of CaMKII (Zhabotinsky, 2000). As in LTP/LTD, the threshold for CaMKII activation acts as a switch between attraction and repulsion (Lisman et al., 2002). The model predicts that increasing the resting levels of calcium in the neuron past that of point H in Figures 2A and 2B leads to repulsion, as now a local increase in calcium in the up-gradient compartment causes a lower CaMKII:CaN ratio in that compartment (Figure 2A, line 3, Figure 2B, line 4, and Figures 2C and 2D, point H).

cAMP-Mediated Directional Switching

cAMP plays a role in determining whether a neuron is attracted or repelled from a gradient, acting as a switch between attraction and repulsion in a steep gradient (Ming et al., 1997; Song et al., 1997, 1998; Nishiyama et al., 2003; Wen et al., 2004; Gomez and Zheng, 2006; Peace and Shewan, 2011; Thompson et al., 2011). Although cAMP concentration is not represented explicitly in our model, one of its downstream targets, PKA, is represented. In addition to calcium, PKA activity is dependent on four constants. Increasing k_{PKA}, the maximum calcium-dependent activity, or decreasing K_{PKA}, the half-activity concentration (calcium/calmodulin-independent base activity k^{o}_{PKA}, and Hill coefficient, unchanged in both cases), results in PKA being more active and thus reflects an increase in the concentration of cAMP. The model shows that increasing the concentration of cAMP, by increasing k_{PKA} or decreasing K_{PKA}, shifts the

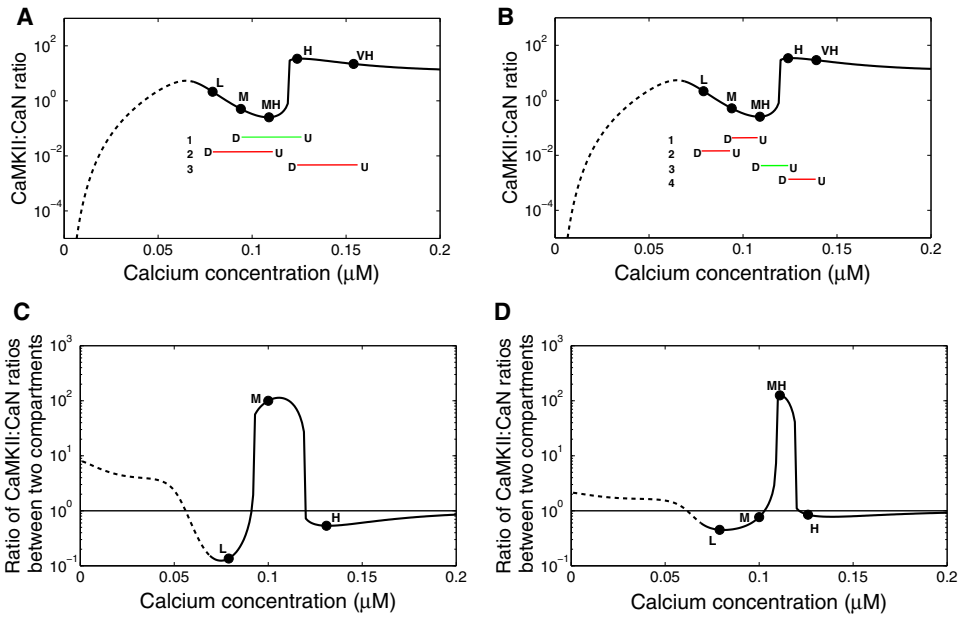

Figure 2. The Effect of Resting Calcium Level on Growth Cone Turning Response

(A and B) CaMKII:CaN ratio plotted against calcium concentration in the model in one compartment with a normal concentration of cAMP (k_{PKA} = 100). The numerical value of the CaMKII:CaN ratio is arbitrary, as the concentration of CaMKII and the activity of CaN do not share the same units; rather, it is the shape of the curve that is important for determining attraction or repulsion. Note that the y axis is on a log scale. The jump in the ratio at $Ca^{2+} \approx 0.13$ μM is caused by bistability of CaMKII activation. The calcium concentration at which this jump occurs can be shifted to higher or lower values by altering the parameters of the model (see Parameter Sensitivity in Results). The red and green lines schematically represent the difference in calcium between the up (U) and down (D) compartments of the growth cone. The length of each line indicates the size of an increase in calcium between the two compartments, the left hand end of the line indicates the resting level calcium, and the color of the line indicates whether the increase in calcium results in attraction (green) or repulsion (red) in the model. Point M ("medium") is the resting calcium concentration (100 nM) we have assumed for this example. The dashed line indicates calcium levels below those which are experimentally relevant, but are included here for completeness. (A) Effect of resting calcium for a normally attractive guidance cue. Line 1: The large increase in calcium from point M to point H ("high") due to an attractive guidance cue results in a higher CaMKII:CaN ratio in the up-gradient compartment compared to the down-gradient compartment, and thus attraction occurs. Line 2: Reducing the intracellular calcium to point L ("low") means that the large increase in calcium from point L to point MH ("medium-high") due to an attractive guidance cue results in a lower CaMKII:CaN ratio in the up-gradient compartment compared to the down-gradient compartment, as the line slopes down, and thus repulsion occurs. Line 3: Increasing the intracellular calcium to point H means the large increase in calcium from point H to point VH ("very high") due to an attractive guidance cue results in a lower CaMKII:CaN ratio in the up-gradient compartment compared to the down-gradient compartment, as again the line slopes down, and thus repulsion occurs. (B) Effect of resting calcium for a normally repulsive guidance cue. Line 1: The small increase in calcium from point M to point MH due to a repulsive guidance cue results in a lower CaMKII:CaN ratio in the up-gradient compartment compared to the down-gradient compartment, as the line slopes down, and thus repulsion occurs. Line 2: Reducing the intracellular calcium to point L means that the small increase in calcium from point L to point M due to a repulsive guidance cue results in a lower CaMKII:CaN ratio in the up-gradient compartment compared to the down-gradient compartment, as the line again slopes down, and thus again repulsion occurs. Line 3: Increasing the intracellular calcium slightly to point MH means the small increase in calcium from point MH to point H due to a repulsive guidance cue now results in a higher CaMKII:CaN in the up-gradient compartment compared to the down-gradient compartment, and thus attraction occurs. Line 4: Increasing the intracellular calcium to point H means that the small increase in calcium from point H to point VH due to a repulsive guidance cue results in a lower CaMKII:CaN ratio in the up-gradient compartment compared to the down-gradient compartment, as the line slopes down, and thus repulsion occurs. It is therefore a combination of the level of resting calcium and the magnitude of calcium increase that determines whether a guidance cue triggers attraction or repulsion.

(C and D) The ratio of CaMKII:CaN ratios between the two compartments, i.e., dividing U/D values for each line in (A) and (B), plotted against calcium concentration, with either a 30% (attractive cue) or 10% (repulsive cue) calcium difference between the compartments (C and D, respectively; see Experimental Procedures). A ratio of one implies that the CaMKII:CaN ratio in each compartment is equal and no guidance response occurs. A ratio greater than one implies that the CaMKII:CaN ratio is higher in the up-gradient compartment, and therefore the growth cone is attracted toward the source of the guidance cue. A ratio less than one implies that the CaMKII:CaN ratio is higher in the down-gradient compartment, and therefore the growth cone is repelled away from the source of the guidance cue. The large peak is caused by the bistability of CaMKII activation. (C) Effect of calcium on a guidance cue for attraction. At normal resting calcium level (point M, "medium"), a 30% increase in calcium in one compartment results in attraction (cf Line 1, A). However, at a lower resting level of calcium (point L, "low"), the result is repulsion (cf Line 2, A), and at a higher resting level of calcium (point H, "high") the result is also repulsion (cf Line 3, A). (D) Effect of calcium on a guidance cue for repulsion. At normal resting calcium level (point M) a 10% increase in calcium in one compartment results in repulsion (cf Line 1, B). At a lower resting level of calcium (point L) the result is still repulsion (cf Line 2, B) and at a high resting level of calcium (point H) the result is also repulsion (cf Line 4, B). However at a moderately high level of calcium (point MH, "medium-high") the response is converted to attraction (cf Line 3, B).

See also Figures S2 and S3.

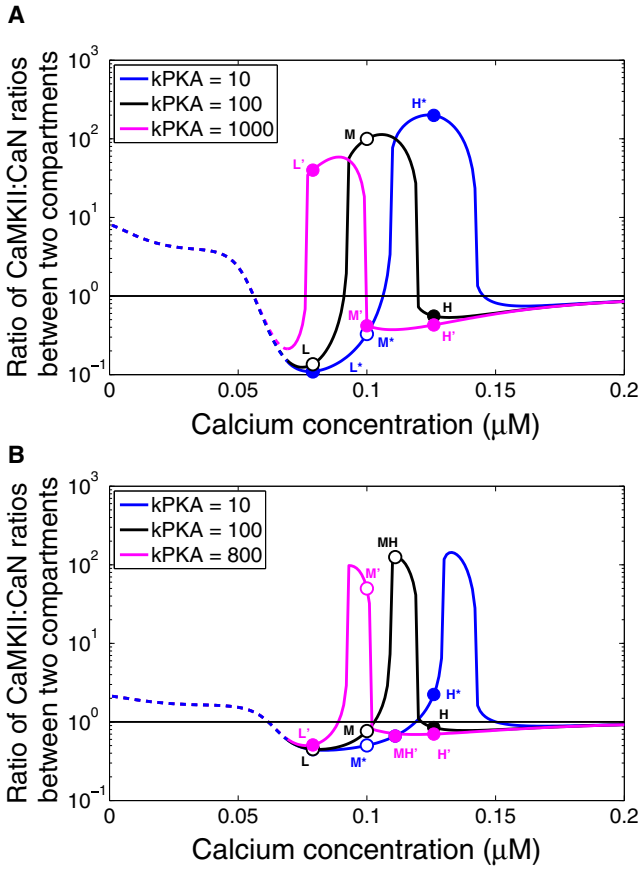

Figure 3. The Effect of cAMP on Growth Cone Turning Response

The ratio of CaMKII:CaN ratios between two compartments plotted against calcium as in Figures 2C and 2D. Hollow points show conditions already tested by others; solid points show predictions of the model.

(A) Effect of cAMP on an attractive guidance cue. For normal PKA levels (black) and thus normal cAMP levels, at a normal calcium level (point M) a 30% increase in calcium in one compartment results in attraction. However, at a lower resting level of calcium (point L, "low") the result is repulsion, and at a higher resting level of calcium the result is also repulsion (point H, "high"). For high PKA levels (pink) and thus high cAMP levels, the peak is shifted to a lower level of calcium so that at resting levels of calcium, attraction is switched to repulsion (point M′). At low levels of calcium, repulsion is switched to attraction (point L′), and at high levels of calcium repulsion still occurs (point H′). Thus, at low calcium levels, increasing the activity of cAMP in the model can cause a switch from attraction to repulsion. For low PKA levels (blue) and thus low cAMP levels, at normal levels of calcium, attraction is switched to repulsion (point M*). At high levels of calcium, repulsion is switched to attraction (point H*), and at low levels of calcium repulsion stays the same (point L*).

(B) Effect of cAMP on a repulsive guidance cue. As seen in Figure 2D, for normal PKA levels (black) and thus normal cAMP levels, at normal resting calcium level (point M) a 10% increase in calcium in one compartment results in repulsion, and at a lower resting level of calcium (point L) the result is still repulsion. However, at a moderately high resting level of calcium the result is now attraction (point MH), but at a high resting level of calcium the result is again repulsion (point H). For high PKA levels (pink) and thus high cAMP levels, the peak is shifted to a lower level of calcium, so that repulsion is switched to attraction at normal levels of calcium (point M′), repulsion still occurs at low levels of calcium (point L′), attraction is switched to repulsion at moderately high levels of calcium (point MH′), and at high levels of calcium repulsion still occurs (point H′). Thus, at moderately high calcium levels, increasing the activity of cAMP in the model can cause a switch from attraction to repulsion.

calcium versus CaMKII:CaN curve to lower levels of calcium (Figure 3, pink curves). Increasing levels of cAMP in the model therefore converts repulsion to attraction at low levels of calcium for a normally attractive guidance cue (Figure 3A, point L′).

Decreasing cAMP activity by applying a cAMP competitor or a specific PKA inhibitor can switch the response to a normally attractive guidance cue from attraction to repulsion (Ming et al., 1997). In the model, decreasing the levels of cAMP by reducing k_{PKA} shifts the CaMKII:CaN versus calcium ratio curve to higher levels of calcium (Figure 3, blue curves). Thus, for a normally attractive guidance cue, reducing levels of cAMP shifts attraction to repulsion at normal levels of calcium (Figure 3A, point M*). However, the model predicts that in a high calcium environment, the decreased cAMP levels will result in attraction (Figure 3A, point H*).

In the case of a repulsive guidance cue, increasing cAMP activity can switch the response to attraction (Song et al., 1998). In the model, a repulsive guidance cue gradient results in a small increase in calcium in the up-gradient compartment, leading to repulsion (Figure 3B, point M). A moderate increase in cAMP shifts the curve to a lower concentration of calcium and converts the repulsive response to attraction (Figure 3B, point M′).

Overall, Figure 3 shows that, in the model, a delicate balance between levels of calcium and cAMP determines whether attraction or repulsion occurs. In particular, the normally attraction-promoting effects of increasing calcium or cAMP are both magnitude dependent and not always additive: increasing both simultaneously can block the attractive effect each would produce individually. Thus, high calcium or cAMP levels do not always promote attraction, and low calcium or cAMP levels do not always promote repulsion. These experimental predictions are tested below, but first we address their robustness to some of the assumptions underlying the model.

Parameter Sensitivity

Many of the parameter values on which the model depends are taken from direct experimental measurements by others (Table S1). These include the parameters controlling calcium-calmodulin dynamics, calmodulin-dependent CaMKII dynamics, the association of I1 with PP1, and the shape of the CaN curve. Other parameters that are more flexible include the rate of CaMKII phosphorylation and thus the position of the curves relative to calcium concentration, the total calmodulin concentration and therefore the value of calcium where the interesting behavior occurs, and the parameters controlling the shape and position of the PKA curve. However, altering these latter parameters does not affect the basic shape of the curves plotted in Figures 2 and 3 or their positions relative to each other on the calcium axis. An example of this is shown in Figure S2, where the

For low PKA levels (blue) and thus low cAMP levels, at normal levels of calcium repulsion still occurs (point M*), at moderately high levels of calcium attraction is switched to repulsion (point MH*, covered by point MH′), at high levels of calcium repulsion is switched to attraction (point H*), and at low levels of calcium repulsion stays the same (L*).

See also Figures S2 and S3.

parameter CaM_o, which is the initial amount of CaM in each compartment, has been reduced from 2.5 μM to 0.25 μM and thus resting calcium has increased from 0.1 μM to 0.4 μM.

PP1 as a Mediator of Repulsion

So far, the model has considered CaMKII to mediate attraction and CaN to mediate repulsion. However PP1, a phosphatase included in our model for its regulatory role, has been suggested to act together with CaN to mediate repulsion (Wen et al., 2004). Including the level of PP1 in the CaMKII:CaN ratio had negligible effects on the predictions of the model at low levels of calcium (Figure S2C, points L and M). However, at higher levels of calcium (Figure S2C, point H) the model predicted attraction where it previously predicted repulsion (Figure 2C, point H), which does not match our experimental results (see below). On the other hand, little is known about the downstream mechanisms or relative roles of CaN and PP1, and thus normalization of their respective activities may be appropriate such that their maximum activities are equal. After normalization, the inclusion of PP1 in the ratio in the model had a minimal effect, and did not change any of the predictions (Figure S2D).

Diffusion of Signaling Components

The model has so far assumed, as a first approximation, that no signaling molecules diffuse between the two sides of the growth cone. To test the robustness of the model to this assumption, we introduced diffusion by sharing a proportion P of the difference of either CaM, PKA, I1, or PP1 between each compartment at each time step, where P = 0.5 corresponds to complete equalization of concentrations in the two compartments (see Experimental Procedures). We did not consider diffusion of calcium, as the sustained spatial difference in calcium between the two compartments is assumed to be driven by the external ligand gradient and thus constant through time, acting as a boundary condition for the model.

For calmodulin, even high levels of diffusion (P = 0.3) had little effect on the outcome of the model (Figure S3A). Diffusion of I1 and PP1 had little effect at resting levels of calcium (Figures S3B and S3C); however, there were larger effects at low levels of calcium. For both I1 and PP1 diffusion, repulsion in the low calcium environment was converted to no turning response at P = 0.1, and this response was converted to attraction at high levels of diffusion (P = 0.3). Little is known about the dynamics of these molecules, but it is likely that their diffusion is slow given that they are large.

The diffusion of PKA, and thus cAMP, had little effect at low levels of diffusion ($P < 0.01$; Figure S3D), but significant alterations to the outcome of the model started to occur at higher levels of diffusion. However, in reality, cAMP diffusion appears quite limited. cAMP achieves high concentrations around its targets while global concentrations remain low (Rich et al., 2000). Although many reasons for this localization may exist, one explanation is the presence of phosphodiesterases which inactivate cAMP and prevent the diffusion of cAMP (Zaccolo et al., 2002). Previous models have found that with unrestricted diffusion cAMP is unable to reach a high enough concentration to substantially activate PKA (Rich et al., 2000). Thus, the lack of diffusion of cAMP could act as a mechanism for amplifying the stimulus. Overall, the model is therefore robust to at least small amounts of diffusion of the signaling components between the two compartments, and strict localization is not a required feature of the model.

Stochastic Movement

So far, the model presented has been deterministic, such that attraction versus repulsion is specified for given conditions with 100% reliability; however, in reality, sensing and movement are corrupted by noise. In particular, the growth cone is not able to measure the concentration gradient of a guidance cue with 100% certainty (Goodhill and Urbach, 1999; Mortimer et al., 2009), and thus one would not expect a deterministic response: although a steep gradient of an attractive cue may be present, a small percentage of growth cones will actually be repelled. To account for this we extended the model to use a bimodal distribution to represent the probabilities of ligand binding (Figure 4A; see Experimental Procedures). This results in a probability distribution for the ratio of bound receptors, and thus the ratio of the calcium concentrations, between the two compartments. When presented with an attractive ligand gradient of 10%, which we assumed corresponds to a calcium gradient of 30%, about 20% of growth cones in the model did not turn in the expected direction (Figure 4B). This fraction is remarkably similar to that observed in a large number of previous experiments using the growth cone turning assay: even when robust attraction or repulsion is observed the cumulative distribution of turning angles tends to cross zero degrees at about 20% (Figure 4C, compare with for example Ming et al., 1997; Song et al., 1998; Gomez et al., 2001; Nishiyama et al., 2003; Robles et al., 2003; Wen et al., 2004; Hong and Nishiyama, 2010). Adjusting the model to specify that a ratio of CaMKII:CaN ratios between 0.9 and 1.1 results in no turning did not significantly affect the percentages of neurons that are predicted to turn in the expected direction (Figure 4D).

Experimental Results

The model makes a number of predictions regarding how changing calcium and cAMP levels will influence attraction versus repulsion in growth cone turning. We tested several of these using the growth cone turning assay (Lohof et al., 1992). As convenient model systems for normally attractive and repulsive turning, we used the response of early postnatal rat superior cervical ganglion (SCG) axons to gradients of NGF (Figure 6A; point M in Figure 3A) and MAG (Figure 7A; point M in Figure 3B), respectively.

We first clarified the intracellular calcium concentration in these growth cones by ratiometric Fura-2 AM imaging (Figure 5). Under our normal culture conditions, this value was ≈75 nM, close to the value of 100 nM assumed in Figures 2 and 3 and previously measured by others (Garyantes and Regehr, 1992). We further verified that the intracellular calcium concentration could be increased by raising the calcium concentration in the bath or by adding potassium to the bath (Figure 5).

We then confirmed that lowering PKA activity using 80 nM KT5720 converted the normal attraction by NGF into repulsion (Figure 6B; point M* in Figure 3A), whereas slightly raising PKA activity using 20 μM Sp-cAMPs maintained attraction (Figure 6C;

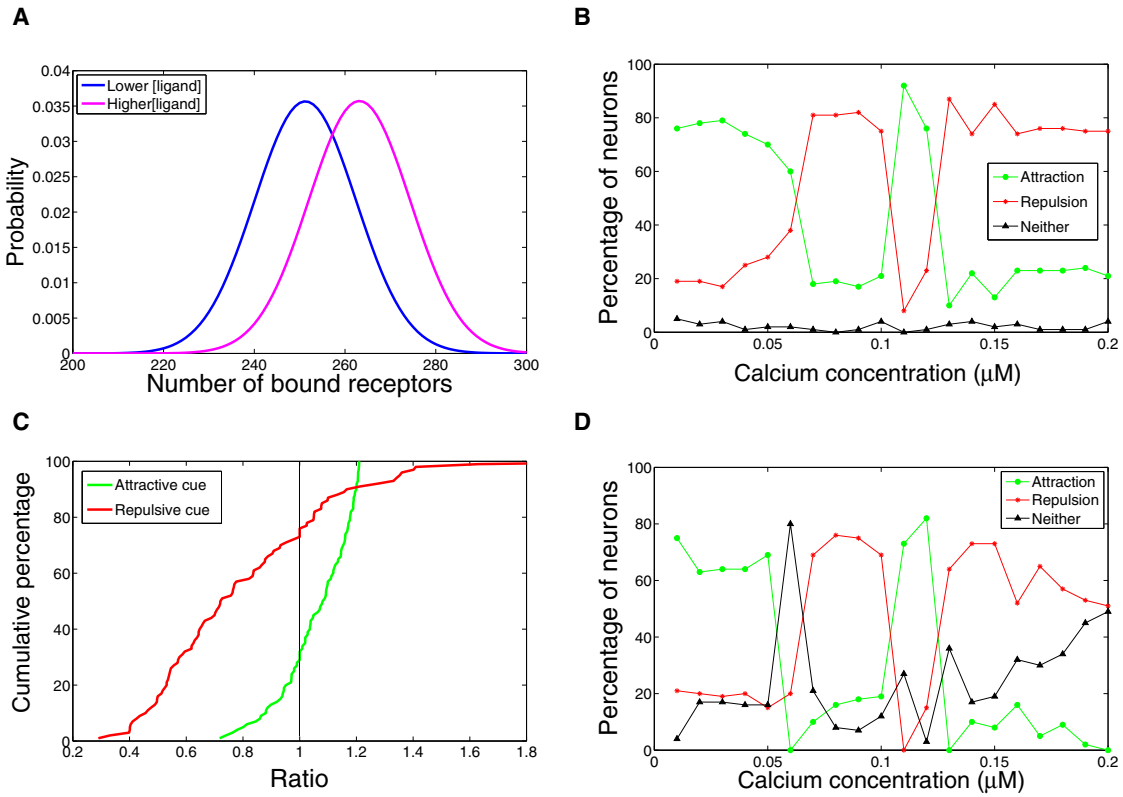

Figure 4. Stochastic Variation of the Model

(A) The two binomial distributions giving the probability for particular numbers of receptors to be bound, assuming a ligand concentration equal to the dissociation constant for the receptor-ligand interaction, and 500 receptors on each side of the growth cone. The probability of a receptor being bound is $k/(k + 1)$ where k is the concentration of ligand relative to the dissociation constant. The compartment which is presented with a 10% greater amount of ligand (pink) is likely to have more receptors bound than the compartment with a lower amount of ligand (blue). Numbers are drawn at random from these distributions in the stochastic simulation.

(B) The percentage of neurons attracted or repelled depending on the concentration of calcium using the stochastic model. The simulation was run 1,000 times for each calcium value.

(C) The cumulative plot of the CaMKII:CaN ratios obtained from the stochastic simulation.

(D) As for (B), except that we now assumed that CaMKII:CaN ratios close to 1 do not cause turning in either direction.

condition not shown in Figure 3A). However, the model predicts that further raising cAMP levels will cause an "overshoot" and converts the attraction into mild repulsion (by shifting point M to point M′ in Figure 3A). Consistent with this, we found that adding 200 μM Sp-cAMPs blocked the normal attraction (Figure 6D). The mean turning angle was slightly negative but was not significantly different from the PBS control gradient, which is consistent with the fact that point M′ in Figure 3A lies only just slightly below the line indicating equal effects in the two compartments.

We next examined the effect of increasing levels of calcium on the normally attractive response to NGF. The model predicts that high calcium at normal cAMP levels should lead to mild repulsion (shifting point M to point H in Figure 3A). Consistent with this, raising calcium from 0.9 mM to 1.3 mM in the bath blocked the normal attraction (Figure 6E). The mean turning angle was not significantly different from the PBS control gradient, but again point H lies only slightly below the line of equal ratios.

In all previous experimental data, across a wide range of guidance systems, reducing cAMP levels converts attraction to repulsion (e.g., Figure 6B). One of the most surprising predictions of the model is therefore that, at high calcium levels, reducing cAMP should produce attraction (point H* in Figure 3A). Consistent with this, using 1.3 mM calcium in the bath in conjunction with 80 nM KT5720 now caused significant attraction (Figure 6F). However, raising calcium levels further (1.7 mM calcium in the bath) with similarly reduced cAMP levels missed the peak for attraction (Figure 6G), again consistent with the model.

MAG is a repulsive factor that produces a shallow calcium gradient in the growth cone (Henley et al., 2004), and we therefore compared this with Figure 3B. For the normally repulsive response of rat SCG axons to MAG (Figure 7A), we first confirmed that slightly raising PKA activity using 40 μM Sp-cAMPs converted repulsion to attraction (Figure 7B; point M′ in Figure 3B). Similarly, raising calcium levels (in this case by using an additional 8 mM potassium in the bath, as in Henley et al. (2004)) also converted repulsion to attraction (Figure 7C; point MH in Figure 3B). However, consistent with the model, raising PKA activity too high using 200 μM Sp-cAMPs caused the peak for attraction to be missed (Figure 7D; point L′ in Figure 3B). Similarly, raising calcium levels too high using 16 mM potassium

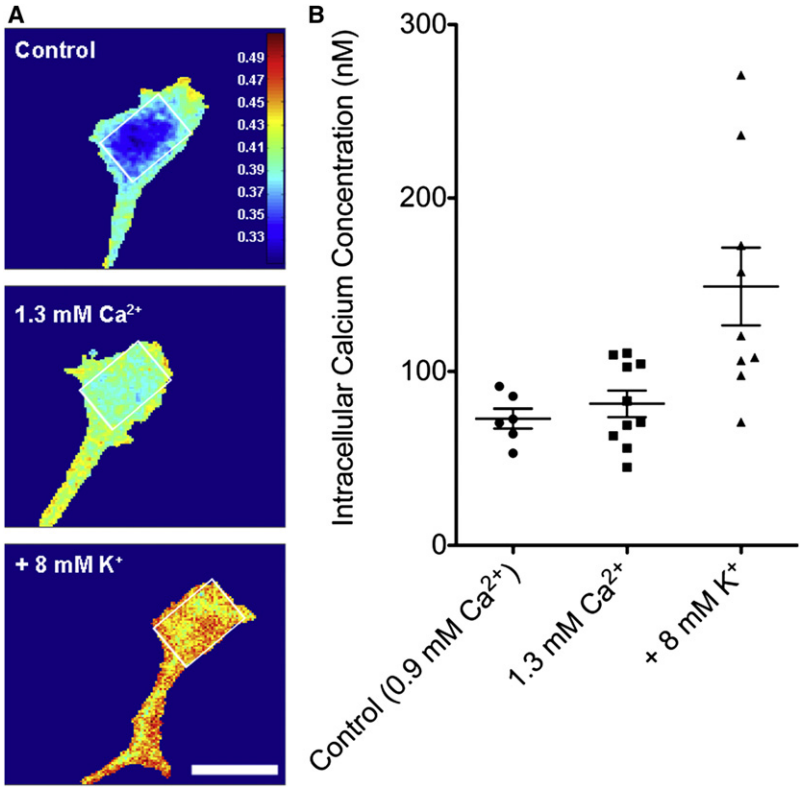

Figure 5. Ratiometric Imaging of Intracellular Calcium Concentration in Early Postnatal Rat SCG Neurons

(A) Pseudocolor images show the ratio of Fura-2 fluorescence intensities at 340 nm excitation divided by 380 nm excitation for example growth cones grown in different media. Blue and red represent the lowest and highest ratios, respectively. Scale bar equals 5 μm.

(B) Increasing the calcium concentration in the bath to 1.3 mM increased intracellular calcium concentration, though this increase did not reach significance in these experiments. Addition of 8 mM potassium caused a significant increase in intracellular calcium concentration ($p < 0.01$ compared to control case, two-tailed Student's t test). Error bars = SEM.

in the bath also caused the peak for attraction to be missed (Figure 7E; point H in Figure 3B). Although moderate increases in calcium levels or PKA activity each individually convert MAG repulsion to attraction, the model predicts that increasing both together will block the attraction (point MH′ in Figure 3B). We confirmed this experimentally using 40 μM Sp-cAMPs combined with 8 mM potassium (Figure 7F).

DISCUSSION

The formation of correct neural circuits requires growth cones to move toward appropriate targets while avoiding inappropriate targets. Previous data have shown qualitatively that whether a growth cone is attracted or repelled by a gradient is crucially affected by three factors: baseline calcium, increase in calcium, and cAMP. Here, we have provided a unifying mathematical model which reproduces and extends these findings, explains quantitatively why they occur, and makes surprising predictions that we have confirmed experimentally. The model applies equally to both bound and diffusible ligand gradients, as it takes as input only differing levels of calcium between the two sides of the growth cone.

A key component of the explanation provided by the model is the bistability of CaMKII (Zhabotinsky, 2000), and it is this that leads to the complex interaction between baseline calcium and the size of the calcium increase in the up-gradient compartment in determining the direction of turning. This bistability is illustrated by the nonmonotonic dependence of the CaMKII:CaN ratio on calcium concentration (Figure 2A and Figure 2B).

Also apparent from these figures is that no attraction can occur unless one side of the growth cone reaches a threshold calcium concentration. This bistability is also the reason for the sharply peaked dependence on calcium concentration of the ratio of CaMKII:CaN ratios between the two compartments (Figures 2C, 2D, and 3).

Figure 3 also makes clear quantitatively why changing cAMP levels causes a switch between attraction and repulsion: the peak is shifted to higher levels of calcium as PKA activity is reduced, and lower levels of calcium as PKA activity is increased. Whereas a small increase in PKA activity shifts the peak only slightly and thus has little effect on attractive responses, a large increase shifts it far enough that, at baseline calcium, the peak has been missed altogether, leading to mild repulsion. This prediction was confirmed experimentally (Figure 6D). This result suggests that increasing cAMP levels to aid regeneration of axons after injury (e.g. Qiu et al., 2002) might be most effective when such increases are small, as large increases could lead to an "overshoot" of the peak for attraction. Related to this the model predicts that, at high levels of resting calcium, reducing cAMP levels can convert repulsion to attraction, which we also confirmed experimentally (Figure 6F). This result is particularly surprising given that previous data have ubiquitously shown that reducing cAMP levels leads to repulsion. Again this arises due to the shift in the peak with PKA activity. Together, these results illustrate the power of mathematical modeling for unraveling the often nonintuitive nature of complex networks of nonlinear interactions.

The Model Predicts Primarily the Sign Rather Than the Magnitude of the Response

The peak for attraction in the ratio of CaMKII:CaN ratios between the two sides of the growth cone has very steep sides (e.g., Figure 2C). Thus, the output of the model is primarily a prediction of the sign rather than the magnitude of the response. One exception to this is where the ratio of ratios drops only slightly below 1, where we suggest the repulsion may be mild and potentially indistinguishable from no net turning. However, given that the

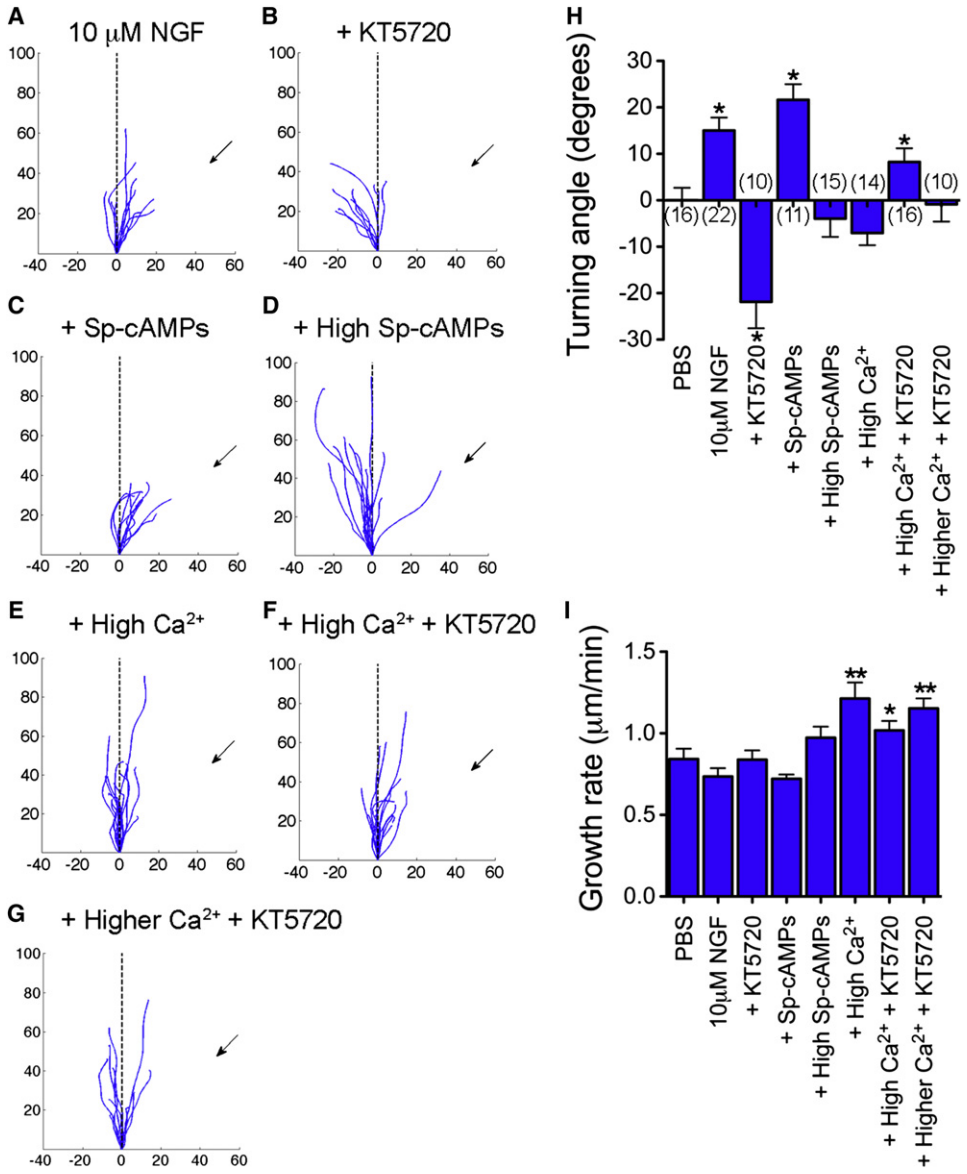

Figure 6. Testing the Predictions of the Model for a Normally Attractive Cue using the Growth Cone Turning Assay

(A) Trajectories of rat SCG axons in response to a gradient of NGF at normal levels of calcium and cAMP (pipette position is shown by the arrow). Ten micromolar NGF in the pipette causes attraction of the growth cones.

(B) Reducing cAMP/PKA activity by addition of 80 nM KT5720 switches the response to repulsion at normal calcium levels.

(C) A small elevation of cAMP/PKA by addition of 20 μM Sp-cAMPs slightly enhances attraction.

(D) A large elevation of cAMP/PKA by addition of 200 μM Sp-cAMPs abolishes the attraction and results in no overall turning.

(E) Elevating baseline calcium (by raising bath calcium from 0.9 mM to 1.3 mM) at normal cAMP/PKA levels abolishes attraction.

(F) Elevating baseline calcium with cAMP levels reduced by addition of 80 nM KT5720 results in attraction. Compared to the normal NGF attraction condition (A), the mean turning angle was smaller, but not significantly so ($p = 0.11$, two-tailed t test).

(G) Elevating calcium further blocks the attraction with cAMP levels reduced. In (A)–(G) x and y axes are distances in microns.

(H and I) Summary of turning angles (H) and growth rates (I) across the different conditions, including the PBS control condition (trajectories not shown). Error bars = SEM, numbers in brackets are growth cones per condition,* indicates $p < 0.05$,** indicates $p < 0.01$, two-tailed Student's t test.

ratio of ratios determines the turning response via several downstream effectors with unknown quantitative dynamics, it is beyond the scope of the model to predict more generally how different ratio values will compare quantitatively in terms of degree of turning. Intriguingly, it appears from the model that the dynamic range of the repulsive condition is substantially smaller than that of the attractive condition: the ratio of ratios attains a highest value of about 100, but a lowest value of about 0.1, a factor of only 10 below unity. This occurs because of the bimodal nature of CaMKII (Figure S1B). When CaMKII has

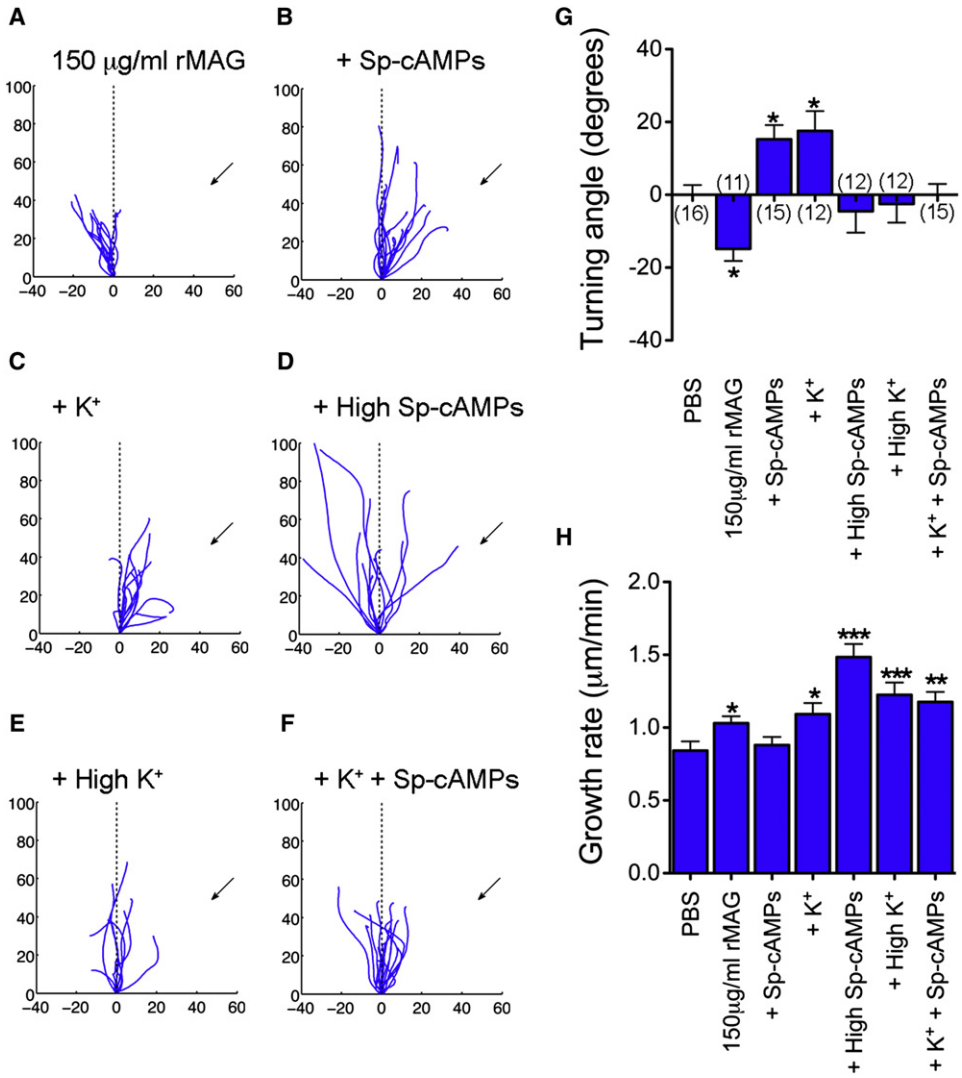

Figure 7. Testing the Predictions of the Model for a Normally Repulsive Cue using the Growth Cone Turning Assay

(A) Trajectories of rat SCG axons in response to a gradient of MAG at normal levels of calcium and cAMP (pipette position is shown by the arrow); 150 μg/ml rMAG in the pipette causes repulsion of the growth cones.

(B) Elevating cAMP/PKA activity by addition of 40 μM Sp-cAMPs switches repulsion to attraction.

(C) Elevating baseline calcium (by addition of 8 mM potassium to the bath) switches repulsion to attraction.

(D) Elevating cAMP/PKA activity to a high level by addition of 200 μM Sp-cAMPs now blocks the attraction.

(E) Elevating baseline calcium to a high level (by addition of 16 mM potassium to the bath) now blocks the attraction.

(F) While elevating calcium and cAMP/PKA separately leads to attraction in each case, elevating both together does not (8 mM potassium, 40 μM Sp-cAMPs). In (A)–(F) x and y axes are distances in microns.

(G and H) Summary of turning angles (G) and growth rates (H) across the different conditions, including for the PBS control condition (trajectories not shown). Error bars = SEM, numbers in brackets are growth cones per condition,* indicates $p < 0.05$,** indicates $p < 0.01$,*** indicates $p < 0.001$, two-tailed Student's t test.

been activated on one side of the growth cone but not the other, there is a very large difference in the ratios between the two compartments. In comparison, CaN does not undergo a dramatic alteration in its activation, so the difference in the ratio between the two compartments is not nearly as great during repulsion. The asymmetry of the dynamic range of attraction versus repulsion in the model thus stems from a fundamental difference in the underlying kinetics of calcium binding by CaN and CaMKII.

Relationship of Growth Cone Switching to LTP/LTD

CaMKII mediates LTP and CaN mediates LTD (Graupner and Brunel, 2010), with a CaMKII/CaN switch also playing an important role in synaptic plasticity (Manninen et al., 2010). We used a mathematical model of the switch between LTP and LTD as the starting point for our model of growth cone switching (Graupner and Brunel, 2007), considering the same bimodal nature of CaMKII but with reactions occurring separately in the two sides of the growth cone. However, α-CaMKII knockout mice show

impaired LTP but normal axon guidance, suggesting that different CaMKII isoforms may be involved in the two processes (Wen et al., 2004). Despite this, it is possible that this quite specific signaling mechanism has been conserved and adapted for different tasks during specific stages of neural function.

cAMP Switching in Shallow Ligand Gradients

It has recently been shown that in very shallow ligand gradients (0.1%–0.3% change in ligand concentration over 10 microns) axons do not respond primarily by turning, but rather by "growth rate modulation," changing their rate of growth depending on whether they are moving up-gradient or down-gradient (Mortimer et al., 2010). Such shallow gradients would cause an insufficient elevation of calcium in the up-gradient compartment of a growth cone to produce a significant difference in the CaMKII:CaN ratios in the two compartments of the growth cone, and thus no turning would be expected in our mathematical model. On the other hand, such a CaMKII:CaN-dependent mechanism could potentially still apply if the two compartments were now parts of the axon shaft with a wider spatial separation than the width of a growth cone. However, reducing cAMP levels does not cause a switch from attraction (mediated by growth rate modulation) to repulsion in shallow gradients (Thompson et al., 2011), suggesting that the signaling network underlying growth rate modulation is not dependent on CaMKII:CaN ratios in the same way as growth cone turning.

cAMP Switching at High Ligand Concentrations

An interesting result to consider in the light of our model is that at high concentrations an attractive cue can cause repulsion (Mai et al., 2009). The application of a high concentration of a guidance cue could potentially open sufficient channels to induce the high calcium condition as seen in Figure 3A, thus causing repulsion. However, in this case we predict that decreasing cAMP would be required to reestablish attraction, whereas Mai et al. (2009) found that increasing cAMP re-established attraction. Alternatively, it is possible that a high concentration of guidance cue saturates the receptors on the growth cone, which makes it difficult for a large calcium gradient to be established across the growth cone. This would result in a small calcium gradient, and thus repulsion. Increasing cAMP would now switch this repulsion to attraction, consistent with the experimental data of Mai et al. (2009), suggesting that this is a more likely explanation.

Alternative Targets of cAMP

The signaling network we have modeled (Figure 1A) is of course simplified. In particular, although in the model the only function of the cAMP-PKA pathway is the activation of I1, other functions for this pathway in growth cone guidance have been proposed. One of these is that the downstream effectors of cAMP-PKA can enhance the activity of L-type calcium channels (Nishiyama et al., 2003). In this case, an increase in cAMP would lead to a greater influx of calcium than normal, which could on its own be enough to trigger attraction. cAMP can also act directly on cyclic-nucleotide-gated ion channels to cause changes in the calcium concentration (Ooashi et al., 2005) and is important in inducing ryanodine-mediated calcium-induced calcium release. Thus cAMP could play a role in mediating the concentration of calcium, leading to amplification or suppression of calcium transients.

However, recent data suggest that the relationship between the concentration of cAMP and calcium varies between the growth cone and the filopodia. A local increase in cAMP causes an increase in calcium in the filopodia, but calcium levels are unaffected in the body of the growth cone (Nicol et al., 2011). In addition, a global internal gradient of cAMP and an internal gradient of cAMP in only the filopodia are both sufficient to trigger attraction, but an internal gradient of cAMP in only the growth cone body is insufficient to trigger any turning (Nicol et al., 2011). This is consistent with our model, in which an increase in cAMP alone is not sufficient to trigger turning, as the lack of calcium means that neither CaN nor CaMKII is activated. Thus, in these terms our model can be interpreted as representing the growth cone body rather than the filopodia, given that we assumed no activation of calcium by cAMP. Although a positive regulation of calcium by cAMP could potentially be added to the model, this would create a positive feedback loop (via CaM) between the two, requiring the addition of further signaling components or other assumptions to control runaway growth. While the specific assumptions in our model about the flow of information between calcium and cAMP may not hold in all circumstances, we have shown that it still predicts growth cone behavior across a broad range of conditions very well.

Another target of cAMP is Epac (exchange protein directly activated by cAMP). Because most PKA inhibitors also inhibit Epac, it has recently been argued that it is difficult to experimentally differentiate between the roles of PKA and Epac in growth cone guidance (Peace and Shewan, 2011). Specific substrates have been used to demonstrate that in the normally attractive response to netrin-1 Epac facilitates attraction, whereas PKA facilitates repulsion (Murray et al., 2009). These authors proposed that Epac requires much higher concentrations of cAMP than PKA; thus, at low concentrations of cAMP, PKA is preferentially activated, leading to repulsion, whereas at high concentrations Epac is preferentially activated, leading to attraction (Murray et al., 2009). Epac facilitates attraction by stimulating CaMKII (Pereira et al., 2007). Because nonspecific inhibitors were used in previous experiments or only cAMP was targeted, there has been little differentiation between the effects of Epac and PKA due to a change in cAMP. However, although our model could potentially be expanded to consider the roles of Epac and PKA separately, their only stimulus is cAMP and it is therefore sufficient to pool their dual roles under just the actions of PKA to explain the phenomena we have considered.

Other Calcium-Dependent Determinants of Axon Guidance

Calcium is an important second messenger in most cells and has many targets (Gomez and Zheng, 2006). Our model only examined CaN:CaMKII-mediated attraction and repulsion, but there are many other calcium-dependent mechanisms involved in growth cone guidance. One of these involves the calcium-dependent protease calpain, which is activated by large calcium transients (Robles et al., 2003). Polarized activation of calpain

results in repulsion as it is a local inhibitor of filopodial motility. Thus calpain has a role in growth cone guidance in response to greater increases in calcium than those that trigger CaMKII/CaN-mediated turning (Gomez and Zheng, 2006). Other potential targets for calcium are the members of the Rho family of small GTPases. Rho GTPases are involved in the regulation of the actin filament network during turning (Gallo and Letourneau, 2004). Activation of Rac1 and Cdc42, the two Rho GTPases suggested to be involved in growth cone advance, is regulated by PKC, which is activated by calcium (Jin et al., 2005). In addition, the inactivation of calpain also promotes activation of Rac1 and Cdc42 (Lokuta et al., 2003), and Rho GTPases can modulate influx of calcium influx by effecting the insertion of membrane calcium channels (Bezzerides et al., 2004). Again, it would be possible in principle to extend our model to include these other signaling molecules. This would be useful if the goal were to understand how manipulations of these molecules affect guidance, but their inclusion is not necessary to understand the phenomena we have addressed.

Models of Growth Cone Signal Transduction

A variety of different theoretical models have previously been proposed to understand different aspects of axon guidance (reviewed in Maskery and Shinbrot, 2005; Simpson et al., 2009; van Ooyen, 2011). A few of these have directly addressed the signal transduction events underlying growth cone chemotaxis. For instance, Sakumura et al. (2005) and Jilkine et al. (2007) considered how the Rho GTPases Cdc42, Rac, and RhoA interact to determine guidance responses. Rho GTPases directly regulate the actin filament network and thus can be considered to act further downstream of the events considered in our model. In contrast, Causin and Facchetti (2009) and Bouzigues et al. (2010) considered the positive feedback loops that may be involved in gradient amplification and cell polarization. Our model considers how this polarization, in terms of a calcium gradient, is then interpreted to determine attraction versus repulsion, and addresses how levels of calcium and cAMP are involved. Integrating elements of these other models could in the future lead to a more comprehensive model of growth cone behavior, although at the expense of adding many additional parameters which are often difficult to directly measure.

Interaction between Cues for Attraction and Repulsion

The interaction of guidance cues may be necessary for correct location of spatial targets. In vitro growth cones do not undergo attraction or repulsion with absolute fidelity in response to single gradients. However, in vivo, connections are made with a higher degree of accuracy (Isbister et al., 2003). Interactions between multiple guidance cues, as well as protein interactions at decision-making locations, could potentially help reduce the error rate (Foa et al., 2001). The remaining error rate could then be accounted for by the stochastic nature and inherent noise of guidance cue binding as considered in the stochastic version of our model, and discussed in more detail in Mortimer et al. (2009).

A particularly intriguing aspect of the model is that it provides a mechanism for integrating information from multiple attractive and repulsive cues. It is known that receptors for guidance cues can interact to determine growth cone responses (Stein and Tessier-Lavigne, 2001). However, alternatively (or in addition) guidance cues could interact via their effect on the calcium signaling pathway we have modeled. For instance, the application of repulsive guidance cues, which individually produce only small calcium influxes, could together produce large influxes, potentially cancelling the repulsion, or even switching it to attraction. This possibility remains to be explored.

EXPERIMENTAL PROCEDURES

Mathematical Methods

The mathematical model of the signaling pathway shown in Figure 1A is adapted from that of Graupner and Brunel (2007), originally proposed for the switch between LTP and LTD. We extended the model to two compartments in order to provide a "distribution" of inputs and outputs over the growth cone. This allows the determination of whether each combination of calcium, cAMP, and spatially nonuniform calcium influx results in attraction or repulsion. For details see Supplemental Experimental Procedures.

Experimental Methods

Cell Culture for Growth Cone Turning Assays

All experimental procedures involving animals were approved by the Animal Ethics Committee of the University of Queensland. SCGs were isolated by microdissection from postnatal day 1–3 Wistar rat pups as per Higgins et al. (1991). The SCGs were then cut into thirds, incubated in 0.25% trypsin (GIBCO, Melbourne, Australia) at 37°C for 15 min and then triturated through flamed-polished Pasteur pipettes for 10 min to dissociate individual cells. The cells were plated in Opti-MEM solution (GIBCO) containing 10 μg/ml natural mouse laminin (Invitrogen, Melbourne, Australia) and 0.5 nM NGF (2.5S mouse NGF; Biosensis, Thebarton, Australia) and incubated overnight at 37°C on 35 mm Petri dishes.

Production of Microscopic Gradients of Guidance Cue

Growth cone turning assays were carried out at 37°C on a heated microscope stage (Fryer Co., Huntley, IL). Growth cones with a straight trailing axon of more than 20 μm were selected for the assay. Steep gradients of 10%– 15% change in concentration across 10 μm were generated using the pulsatile ejection method previously reported by Lohof et al. (1992) (see also Pujic et al., 2008). Forty kilodaltons dextran labeled with fluorescent tetramethylrhodamine (Molecular Probes Inc., Melbourne, Australia) was added to the pipette solution to monitor the chemical gradient produced. KT5720 (Alexis Biochemicals, San Diego, CA) or Sp-cAMPs (BioLog, Bremen, Germany) were added to the prewarmed assay medium when appropriate.

Measurement of Neurite Extension and Growth Cone Turning

Images of the growing axon were taken at 60 s intervals for 1 hr using the 20× objective of a Nikon Eclipse TE200 inverted microscope (Nikon Corporation, Tokyo, Japan) and a Q-Imaging camera and Q-Capture Pro software (Quantitative Imaging Inc., Surrey, Canada) or the 20× objective of a Zeiss AxioObserver inverted microscope and AxioVision 4 software. The trace of each axon, its turning angle, and distance of growth were calculated using Matlab. The center of the growth cone was manually located in each frame and the turning angle was defined as the angle between the original direction of growth and the average position of the growth cone in the final 5 frames in the trace. Only growth cones with more than 15 μm of net growth over the period of the assay were included in the analysis.

Calcium Imaging

Cells were preloaded with Fura-2 AM (2 μM) for 30 min. After removal of excess Fura-2 AM, cells were excited at 340 and 380 nm using a BD Pathway 855 system (BD Bioscience) at 40× and light at 510 nm was collected using a GFP filter. Growth cones were imaged growing in a normal OptiMEM plus 0.5 nM NGF background, or supplemented with 0.4 mM $CaCl_2$ or 8 mM KCl.

Images were analyzed in ImageJ by subtracting background fluorescence from a ROI within the growth cone, then the ratio R of fluorescence intensity at 340 and 380 nm excitation was determined. To calculate absolute calcium

levels, cells preloaded with Fura-2 AM growing in both high calcium medium and calcium free medium were permeablized by adding ionomycin (1 μM) to the media 5 min prior to imaging. The 340:380 fluorescence ratio of cells in high calcium and calcium-free media gave the maximum R_{max} and minimum R_{min} fluorescence ratios, respectively. Calcium levels for each growth cone were then calculated using the formula

$$[Ca^{2+}] = K_d Q \frac{R - R_{\min}}{R_{\max} - R}$$

where K_d = 0.14 μM and Q is the ratio of minimum to maximum fluorescence intensity at 380 nm.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, one table, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.neuron.2012.02.035.

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