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A CaMKII/Calcineurin Switch Controls the Direction of Ca^{2+}-Dependent Growth Cone Guidance

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Summary

Axon pathfinding depends on attractive and repulsive turning of growth cones to extracellular cues. Localized cytosolic Ca^{2+} signals are known to mediate the bidirectional responses, but downstream mechanisms remain elusive. Here, we report that calcium-calmodulin-dependent protein kinase II (CaMKII) and calcineurin (CaN) phosphatase provide a switch-like mechanism to control the direction of Ca^{2+}-dependent growth cone turning. A relatively large local Ca^{2+} elevation preferentially activates CaMKII to induce attraction, while a modest local Ca^{2+} signal predominately acts through CaN and phosphatase-1 (PP1) to produce repulsion. The resting level of intracellular Ca^{2+} concentrations also affects CaMKII/CaN operation: a normal baseline allows distinct turning responses to different local Ca^{2+} signals, while a low baseline favors CaN-PP1 activation for repulsion. Moreover, the cAMP pathway negatively regulates CaN-PP1 signaling to inhibit repulsion. Finally, CaMKII/CaN-PP1 also mediates netrin-1 guidance. Together, these findings establish a complex Ca^{2+} mechanism that targets the balance of CaMKII/CaN-PP1 activation to control distinct growth cone responses.

Introduction

Guided growth of axonal fibers during development constitutes an essential step in the wiring of the complex nervous system. In vivo, a variety of extracellular cues, either permissive/attractive or inhibitory/repulsive, are present in a spatially and temporally regulated pattern to provide directional instructions to elongating axons, guiding them to appropriate targets (Tessier-Lavigne and Goodman, 1996). The motile tip of the axon, the growth cone, serves as the sensory as well as the motile apparatus for translating extracellular signals into directional movement. Ca^{2+} is an important second messenger that has been demonstrated to relay extracellular information to directional motility (Gomez et al., 2001; Hong et al., 2000; Robles et al., 2003; Zheng, 2000; Zheng et al., 1996a). Axon guidance by a number of guidance molecules has been shown to depend on localized Ca^{2+} signals in the growth cone (Hong et al., 2000; Ming et al., 1997; Song et al., 1997; Zheng et al., 1994). Using direct focal photoactivated release of caged Ca^{2+} in the growth cone, we previously found that a localized Ca^{2+} signal in the growth cone is sufficient to induce growth cone attraction as well as repulsion, depending on the resting level of intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) at the growth cone (Zheng, 2000). Similarly, studies on netrin-1 guidance indicated that different Ca^{2+} signals might underlie distinct turning responses induced by netrin-1 gradients (Hong et al., 2000). These results not only demonstrate a crucial role for Ca^{2+} signals in growth cone guidance but also indicate that complex Ca^{2+} mechanisms may operate to control distinct growth cone responses to a wide spectrum of external molecules. How different turning responses are generated by distinct Ca^{2+} signals, however, remains unknown. It is conceivable that different local Ca^{2+} signals, integrated with the baseline level of $[Ca^{2+}]_i$ in the growth cone, activate distinct pathways to mediate attraction and repulsion, respectively. The complexity of Ca^{2+} signaling in axon guidance is further increased by a series of recent studies demonstrating that Ca^{2+}-dependent growth cone responses can be further modulated by the cAMP pathway: elevation of cAMP could lead to switching of repulsion to attraction and vice versa (Song and Poo, 1999). Does the cAMP pathway act upstream to modify the characteristics of Ca^{2+} signals (local and/or global) or target the downstream effectors of Ca^{2+} for the switching? It was recently shown that cAMP/cGMP could affect L-type Ca^{2+} channels to alter intracellular Ca^{2+} signals induced by netrin-1 (Nishiyama et al., 2003), thus placing cAMP/cGMP upstream of Ca^{2+} in mediating bidirectional turning responses. However, whether cAMP also plays a role downstream of Ca^{2+} signaling in guidance remains to be evaluated. Most importantly, the question of what downstream targets mediate distinct Ca^{2+}-dependent turning behaviors is still unanswered. Do attraction and repulsion involve the same or separate downstream signaling cascades? In this study, we utilized a direct local Ca^{2+} elevation approach (Zheng, 2000) to study downstream mechanisms of Ca^{2+}-dependent bidirectional turning responses of nerve growth cones. The use of direct local elevation of intracellular Ca^{2+} concentrations through photoactivated release of caged Ca^{2+} bypasses membrane receptor activation and can largely avoid crosstalk among different signaling pathways, thus allowing us to focus on intracellular Ca^{2+} and its downstream events during distinct turning responses. We now present evidence that Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII) and calcineurin (CaN)-phosphatase-1 (PP1) mediate attraction and repulsion, respectively. Significantly, CaMKII/CaN-PP1 acts as a bimodal switch to control the direction of growth cone turning in response to different Ca^{2+} signals (local and global) by preferentially activating one component over the other. We further show that the cAMP pathway negatively regulates the CaN-PP1 side of the switch to modulate growth cone responses. Finally, we present evidence that the CaMKII/CaN-PP1 mechanism also

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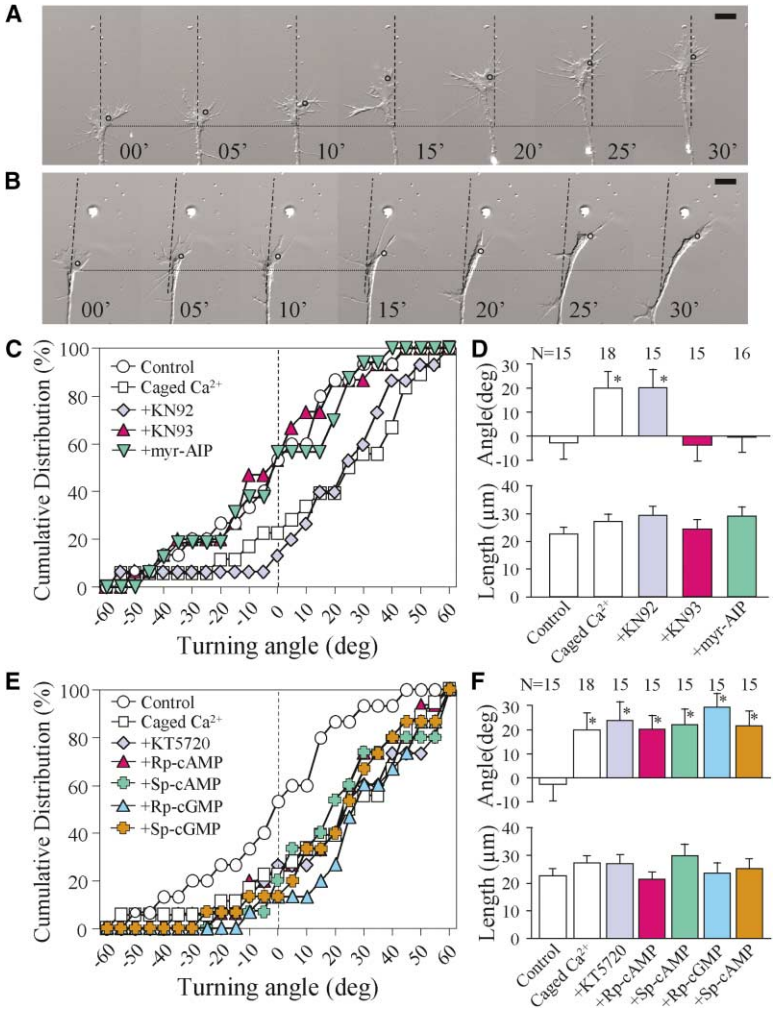


Figure 1. Ca^{2+}-Dependent Growth Cone Attraction Is Mediated by CaMKII

(A and B) Time-lapse sequences showing responses of *Xenopus* growth cones subjected to 30 min of repetitive focal laser irradiation (indicated by the circles). For the growth cone loaded with caged Ca^{2+} compound NP-EGTA, repetitive FLIP caused marked turning toward the laser irradiation side (B), while a control growth cone without loading of NP-EGTA did not show a directional response (A). Dashed lines indicate the original direction for growth cone extension assessed at the start of the assay. Dotted lines represent the corresponding positions of the growth cone at different time points. Digits depict minutes after the onset of the laser irradiation. Scale bars, 10 μm.

(C) Cumulative histograms of turning angles of different groups of growth cones subjected to bath application of different inhibitors. Each point represents the percentage of growth cones with turning angles equal or smaller than that indicated on the x axis. Control group was not loaded with NP-EGTA but was subjected to the same repetitive laser irradiation. All other groups were loaded with NP-EGTA and subjected to 30 min of repetitive FLIP. Different inhibitors were added to the bath 20 min before the onset of the assay.

(D) Average turning angles and neurite lengths of different groups were plotted in bar graphs. Asterisks indicate statistical significance over the control group ($p < 0.01$, Mann-Whitney test).

(E and F) Cumulative histograms of turning angles of growth cones subjected to cyclic nucleotide manipulation during turning assays by direct focal Ca^{2+} elevation (E). The average angles and neurite lengths are presented in the bar graphs in (F).

mediates netrin-1 guidance. These findings thus provide significant insights toward the downstream mechanisms underlying various turning behaviors induced by complex Ca^{2+} signals.

Results

Growth Cone Attraction Depends on CaMKII Activation

To study the downstream signaling cascades involved in Ca^{2+}-dependent growth cone guidance, we utilized focal laser-induced photolysis (FLIP) of caged Ca^{2+} to directly generate spatially and temporally restricted intracellular Ca^{2+} signals for inducing growth cone turning (Zheng, 2000). *Xenopus* neurons grown on laminin-coated coverslips in a serum-free culture medium (SFM) were used for this study because they exhibited relatively large and fast-extending growth cones at room temperature (Guirland et al., 2003). Consistent with the previous report (Zheng, 2000), *Xenopus* growth cones loaded with the caged Ca^{2+} compound NP-EGTA responded to repetitive FLIP with marked turning toward the exposure side in culture medium (hereafter referred to as *attraction*; Figure 1B). Control growth cones without NP-EGTA loading were not affected by the same laser exposure (Figure 1A). Typically, during a 30 min period of repetitive UV exposure on one side of the NP-EGTA-loaded growth cone (focused in a 2 μm spot, one laser pulse every 3 s), the growth cone first exhibited increased protrusion of lamellipodia as well as filopodia on the side of Ca^{2+} uncaging, followed by preferential extension of the growth cone shaft to the same side (Figure 1B). We quantified the turning responses of *Xenopus* growth cones by measuring the turning angle (with respect to the original direction of growth cone extension) and the net extension during the 30 min turning assay. The overall turning responses of a population of *Xenopus* growth cones are illustrated by the cumulative histograms of the turning angles. The majority of growth cones loaded with caged Ca^{2+} and subjected to repetitive FLIP exhibited positive turning angles, shifting the histogram distribution to the positive region (Figure 1C). Control growth cones (i.e., without caged Ca^{2+} loading), on the other hand, did not appear to be affected by the same repetitive laser exposure, as the angle distribution falls evenly around zero degrees. The significant attraction induced by local Ca^{2+} signals was further depicted by the average turning angles (Figure 1D). Statistical comparison of turning angles confirms that repetitive FLIP of caged Ca^{2+} on one side of the growth cone

induced significant attractive turning over the control group ($p < 0.001$, Mann-Whitney test).

We next examined the downstream signals involved in growth cone attraction induced by direct local Ca^{2+} elevation. Since previous studies indicated a role for CaMKII in growth cone turning (Zheng et al., 1994, 1996b), we used KN93, a potent and relatively specific inhibitor of CaMKII, to examine the involvement of CaMKII in Ca^{2+}-induced growth cone attraction. Bath application of 5 μM KN93 completely blocked growth cone attraction induced by repetitive FLIP of caged Ca^{2+}, while the noneffective analog KN92 (5 μM) did not exert any influence (Figures 1C and 1D). To further verify the specificity of CaMKII involvement in Ca^{2+}-induced attraction, we used the membrane-permeable myristoylated-autocamtide-2-related inhibitory peptide (myr-AIP) to inhibit CaMKII without affecting CaMKI and CaMKIV (Ishida and Fujisawa, 1995; Ishida et al., 1995; Uezu et al., 2002). Similar to the KN93 data, bath application of 5 μM myr-AIP completely blocked the growth cone attraction induced by repetitive FLIP of caged Ca^{2+}. The blockade of Ca^{2+}-induced growth cone attraction by CaMKII inhibition is best depicted by the cumulative histograms, as the KN93 and myr-AIP groups exhibited angle distributions that overlapped with that of the control group (Figure 1C). On the other hand, growth cones treated with KN92 still displayed marked attractive turning response (Figure 1C). The average angles also confirm the effective blockade of growth cone attraction by KN93 and myr-AIP (Figure 1D). These results therefore demonstrate that local Ca^{2+} signals act through CaMKII to control growth cone attraction.

Since cyclic nucleotides were shown to modulate Ca^{2+}-dependent growth cone turning in extracellular guidance gradients (Song and Poo, 1999), we examined whether or not growth cone attraction induced by direct local Ca^{2+} elevation can be affected by cyclic nucleotides. We found that manipulation of either cAMP or cGMP pathways by specific agonists and antagonists did not affect the attraction induced by FLIP of caged Ca^{2+} (Figures 1E and 1F). In particular, inhibition of cAMP-dependent protein kinase (PKA) by KT5720 or Rp-cAMP did not block or convert the attraction to repulsion. The angle distributions of growth cones treated with these agonists and antagonists largely overlapped with that of growth cones subjected to FLIP of caged Ca^{2+} only (Figure 1E), resulting in similar positive average turning angles (Figure 1F). Statistical analysis using the Mann-Whitney test on the turning angles showed no difference among those groups ($p > 0.1$) but a significant difference from the control group ($p < 0.01$). Taken together, these results indicate that CaMKII is the downstream target of Ca^{2+} signaling that mediates attractive responses and that cyclic nucleotides do not directly influence the Ca^{2+}-induced growth cone attraction.

Growth Cone Repulsion Is Mediated by Calcineurin Phosphatase

What are the downstream targets that mediate Ca^{2+}-dependent growth cone repulsion? Our previous study has shown that, after reducing the resting level of $[Ca^{2+}]_i$ at the growth cone by removing extracellular Ca^{2+}, the same repetitive FLIP of caged Ca^{2+} induced growth cone repulsion (Zheng, 2000). We therefore used this approach as a model to study the downstream mechanisms underlying Ca^{2+}-induced growth cone repulsion. In this set of experiments, cultured *Xenopus* neurons were loaded with NP-EGTA in the SFM in the same way as above. However, immediately before FLIP and turning assays, we replaced the culture medium with a Ca^{2+}-free Ringer's solution, which effectively lowered the resting level of $[Ca^{2+}]_i$ to about half of the level in culture medium (Zheng, 2000). Since the growth cone extended about twice as fast in Ca^{2+}-free Ringer's saline as those in culture medium or the regular Ringer's solution (Bixby and Spitzer, 1984; Zheng et al., 1994), we accordingly reduced the time of our assay to 15 min so that the similar length of growth cone extension was observed from the turning assay (Zheng, 2000). Consistently, we found that the majority of NP-EGTA-loaded growth cones responded to repetitive FLIP by steering away from the laser irradiation side in Ca^{2+}-free saline (hereafter referred to as *repulsion*; Figure 2A). Similar to that reported previously, the repulsion was accompanied by a local inhibition of filopodia and lamellipodia on the FLIP side as well as an increased protrusion of lamellipodia and filopodia on the opposite side of the growth cone (Zheng, 2000). The repulsive turning responses that were induced by local Ca^{2+} elevation are better depicted by the cumulative histogram of the turning angles (Figure 2E) and the average turning angles (Figure 2F). As a control, growth cones without NP-EGTA loading did not show any directional preference with respect to the repetitive laser exposure (Figure 2E). We first tested whether or not CaMKII is involved in the Ca^{2+}-induced repulsion by bath applying the specific inhibitor KN93 (5 μM). We found that KN93 did not affect the repulsive turning induced by direct local $[Ca^{2+}]_i$ elevation, indicating that Ca^{2+}-dependent growth cone repulsion does not involve CaMKII (Figures 2E and 2F).

Previous studies have suggested a role for CaN, a brain-enriched Ca^{2+}-calmodulin-dependent phosphatase, in growth cone motility (Chang et al., 1995; Lautermilch and Spitzer, 2000); we therefore examined the role of CaN in Ca^{2+}-induced repulsion using specific inhibitors. To our surprise, bath application of cyclosporin A (CsA; 10 nM), a natural inhibitor of calcineurin that forms a complex with endogenous cyclophilin to specifically block CaN activity (Liu et al., 1991), not only abolished repulsion but also converted it to attraction (Figure 2B). Similar conversion of repulsion to attraction was also observed with a different CaN inhibitor, deltamethrin (DM; 10 nM) (Figure 2C). The conversion of repulsion to attraction is clearly illustrated not only by the composite tracings (Figures 2B and 2C) but also by the cumulative histograms of the turning angles (Figure 2E). Moreover, the average turning angles of CsA- and DM-treated groups subjected to FLIP of caged Ca^{2+} are $+25.6° \pm 5.4°$ and $+29.7° \pm 6.5°$, which are the opposite of that of the growth cones exposed to repetitive FLIP of caged Ca^{2+} without any treatment ($-25.6° \pm 6.7°$) in Ca^{2+}-free solution, further demonstrating the marked conversion of Ca^{2+}-induced repulsion to attraction. Since we have shown that CaMKII mediates Ca^{2+}-dependent growth cone attraction, we thus asked whether or not this growth cone attraction (i.e., converted by CaN inhibition) also depended on CaMKII. We found that bath

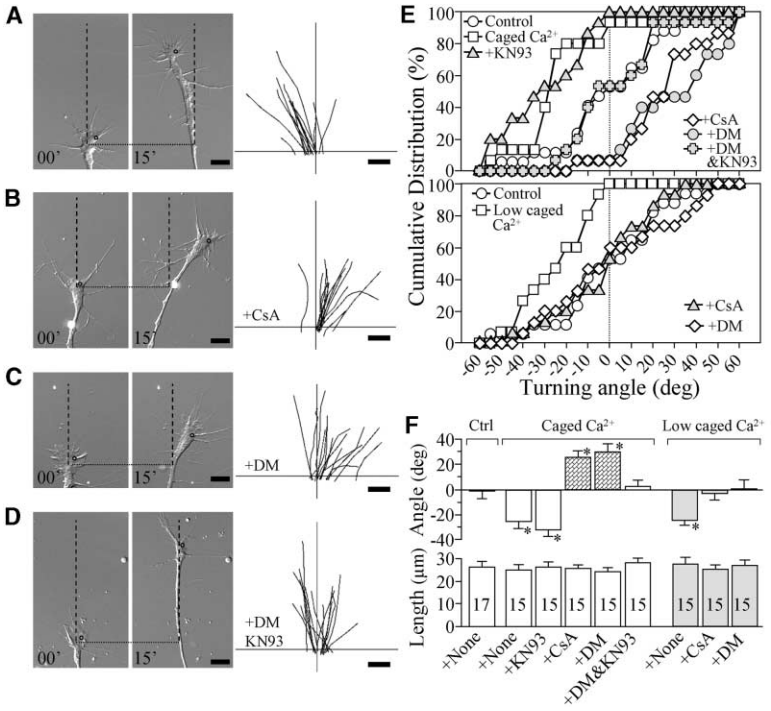


Figure 2. Growth Cone Repulsion in Ca^{2+}-Free Saline Induced by Direct Focal Ca^{2+} Elevation Depends on Calcineurin Phosphatase

(A) Repetitive FLIP of caged Ca^{2+} in calcium-free saline results in turning away from the FLIP side. The pair of DIC images shows a representative *Xenopus* growth cone turning away from the FLIP side. The composite traces of all growth cones examined are shown on the right, of which the original direction of growth is vertical and the laser is always on the right side.

(B–D) Growth cone responses in Ca^{2+}-free solution subjected to repetitive FLIP of caged Ca^{2+} with the presence of different inhibitors in bath. To inhibit calcineurin, 10 nM cyclosporin A (CsA) or 10 nM deltamethrin (DM) was added to the bath 20 min before the assay. To inhibit both CaMKII and CaN, both DM and KN93 were added to bath 20 min before the assay. Digits indicate the time (min) after the onset of assay. Scale bars, 10 μm.

(E) Cumulative histograms of turning angles summarize the growth cone responses for each condition.

(F) Average turning angles and neurite lengths for each group examined. Bars filled with the diagonal brick pattern indicate switching from the opposite turning response. For groups under "Caged Ca^{2+}," neurons were loaded using 6 μM NP-EGTA-AM. For groups under "Low caged Ca^{2+}," the cells were loaded with 2 μM NP-EGTA-AM (for small local Ca^{2+} elevation, see text).

application of both 10 nM DM (for inhibiting CaN) and 5 μM KN93 (for inhibiting CaMKII) totally eliminated the turning response (Figures 2D and 2E), resulting in an average turning angle of 1.9° ± 5.5° (Figure 2F; $p > 0.1$, compared to the control group). These results indicate that direct local Ca^{2+} elevation by repetitive FLIP of caged Ca^{2+} activates both calcineurin and CaMKII. However, at the low resting level of $[Ca^{2+}]_i$ (through removal of extracellular Ca^{2+}), CaN activation by local Ca^{2+} signals predominates over CaMKII activation to induce repulsive turning; only when the CaN pathway is blocked can Ca^{2+} activation of CaMKII become effective in inducing attraction.

Since CaN is known to require much smaller $[Ca^{2+}]_i$ elevation than CaMKII for activation (Groth et al., 2003; Hudmon and Schulman, 2002; Rusnak and Mertz, 2000), we hypothesized that the local Ca^{2+} elevation in the above case primarily activated CaN but was high enough to have also activated some CaMKII. As such, a smaller local Ca^{2+} elevation might be able to solely activate CaN to induce growth cone repulsion, which should not be switched to attraction after CaN inhibition. To test this hypothesis, we produced a small local Ca^{2+} elevation (hereafter referred as the *small local Ca^{2+} elevation*) by reducing the concentration of the caged compounds in the cell while maintaining the same laser exposure (see the Experimental Procedures). We found that *Xenopus* growth cones responded similarly to the small local Ca^{2+} elevation with repulsion in Ca^{2+}-free solution (Figures 2E and 2F). However, inhibition of CaN by bath application of 10 nM DM completely abolished the repulsive turning without switching it to attraction (Figures 2E and 2F). Similar blockade of repulsion without conversion was also observed with the other CaN inhibitor, CsA. These results confirm that CaN mediates Ca^{2+}-induced repulsion of growth cones and that local Ca^{2+} signals could activate both CaN and CaMKII or CaN alone depending on their amplitudes. In the former case, however, CaN predominates over CaMKII to generate repulsion, which can be converted to attraction by CaN inhibition.

Phosphatase-1 Is Part of the Repulsive Signaling Pathway

The phosphatase calcineurin is known to regulate the activity of other serine-threonine phosphatases, such as phosphatase-2A (PP2A) and PP1 (Klee et al., 1998; Oliver and Shenolikar, 1998). We therefore investigated whether these phosphatases are the downstream effectors of CaN in inducing growth cone repulsion. We first used tautomycin, a specific phosphatase inhibitor that has higher affinity for PP1 over PP2A (MacKintosh and MacKintosh, 1994). When used at 4 nM to inhibit PP1 but not PP2A, tautomycin converted growth cone repulsion to attraction in the Ca^{2+}-free solution (Figure 3A), which is consistent with CaN inhibition. We also used a different phosphatase inhibitor, okadaic acid (OA), to further examine the role of PP2A and PP1 in growth cone repulsion. Different from tautomycin, OA has a higher affinity for PP2A over PP1 (MacKintosh and MacKintosh, 1994). At 3 nM, a concentration that effectively inhibits PP2A but not PP1, OA did not affect the growth cone repulsion induced by direct local Ca^{2+} elevation (Figure 3B), indicating that PP2A is not involved in Ca^{2+}-dependent repulsion. However, after bath application of 30 nM OA, which inhibits both PP2A and PP1, Ca^{2+}-induced growth cone repulsion was effectively switched to attraction (Figure 3B). Similarly, we

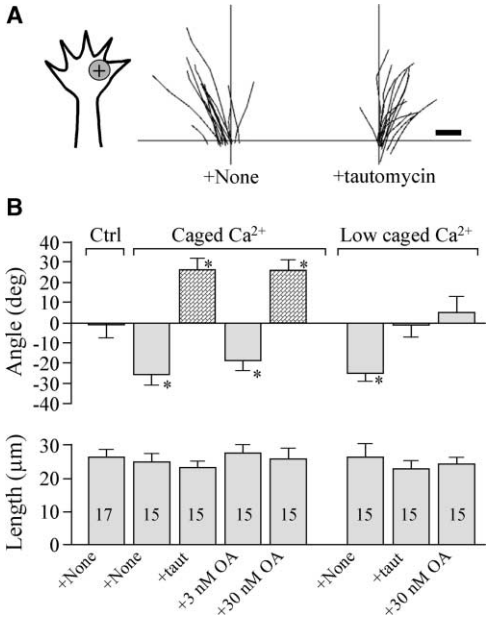


Figure 3. The Involvement of Phosphatase-1 in Repulsion

(A) Growth cone repulsion induced by repetitive FLIP of caged Ca^{2+} in Ca^{2+}-free solution was switched to attraction by phosphatase-1 (PP1) inhibition using tautomycin. The laser is focused on the right, the traces represent the trajectories of all growth cones examined, and the original direction of growth cone extension is vertical.

(B) Average turning angles and net lengths of growth cone extension for all the growth cones examined under different treatments. PP1 inhibition was achieved by bath application of 4 nM tautomycin (taut) or 30 nM okadaic acid (OA). PP2A inhibition was achieved by 3 nM OA. Bars in the graph that are filled with the diagonal brick pattern indicate switching from the opposite responses.

have also examined growth cone repulsion induced by the small local Ca^{2+} elevation and found that PP1 inhibition by either tautomycin at 4 nM or OA at 30 nM effectively blocked the repulsion without switching it to attraction (Figure 3B). Taken together, these results indicate that CaN-PP1 mediates growth cone repulsion induced by small local Ca^{2+} signals.

Switching Growth Cone Repulsion to Attraction by cAMP

In the first part of our study, we have shown that cyclic nucleotides did not affect growth cone attraction induced by direct focal Ca^{2+} elevation. Here, we examined whether or not cyclic nucleotides affect Ca^{2+}-induced growth cone repulsion in Ca^{2+}-free solution. At first, we examined growth cone repulsion induced by the "large" local Ca^{2+} elevation (standard loading of NP-EGTA). We found that bath application of 20 μM Sp-cAMP converted the Ca^{2+}-induced repulsion to attraction, while Rp-cAMP did not exert significant effects on the repulsion (Figure 4). The conversion of repulsion to attraction by Sp-cAMP is clearly depicted by the composite tracings (Figure 4A), cumulative histograms (Figure 4B), and average angles (Figure 4C). Significantly, the average turning angle of Sp-cAMP-treated group in response to repetitive FLIP of caged Ca^{2+} is $+20.8° \pm 6.0°$, which is opposite of the repulsion in the group only exposed to repetitive Ca^{2+} elevation ($-25.6° \pm 6.7°$). On the other hand, manipulation of the cGMP pathway by Sp-cGMP or Rp-cGMP did not affect the growth cone repulsion (Figures 4B and 4C). We next examined whether the attraction that resulted from cAMP elevation depended on CaMKII by bath applying both Sp-cAMP and KN93. We found that concurrent application of Sp-cAMP and KN93 abolished the turning response; neither repulsion nor attraction was observed (Figure 4). Finally, we examined cAMP regulation of growth cone repulsion induced by the small local Ca^{2+} elevation. Consistently, bath application of Sp-cAMP abolished the repulsive turning without switching it to attraction (Figures 4B and 4C, indicated by "Low caged Ca^{2+}"). These observations are very similar to those of CaN or PP1 inhibition and further confirm that local Ca^{2+} elevation of different amplitudes could activate both CaN and CaMKII or CaN alone to produce repulsion, but only the former can be converted to attraction. Furthermore, the parallel results of cAMP elevation and CaN or PP1 inhibition suggest that the cAMP pathway negatively regulates the CaN-PP1 pathway for switching growth cone repulsion to attraction.

CaMKII/CaN-PP1 in Growth Cone Guidance at Normal Resting Level of $[Ca^{2+}]_i$

Growth cone repulsion in the above studies was induced by focal Ca^{2+} elevation in neurons with reduced resting levels of $[Ca^{2+}]_i$ through the removal of extracellular Ca^{2+}. Using this model, we have identified the CaN-PP1 pathway in mediating growth cone repulsion. We next tested whether or not growth cone repulsion can be induced by focal Ca^{2+} elevation in growth cones with normal resting levels of $[Ca^{2+}]_i$ observed in culture medium. Previous studies have found that small local Ca^{2+} elevations are associated with growth cone repulsion induced by extracellular gradients (Hong et al., 2000). We therefore used FLIP to produce local Ca^{2+} elevation with reduced amplitude (see above) and examined turning responses in regular culture medium. We found that the majority of growth cones exposed to the small local Ca^{2+} elevation turned away from the FLIP side, exhibiting marked repulsion as depicted by the cumulative histogram (Figure 5A, marked as "Low caged Ca^{2+}"). Similar to repulsion induced in Ca^{2+}-free medium, we found that the repulsion involved preferential activation of CaN over CaMKII, as it was converted to attraction by the specific CaN inhibitor DM (Figures 5A and 5C). Furthermore, when both CaN and CaMKII were inhibited by simultaneous application of DM and KN93, no turning was observed. Finally, the growth cone repulsion induced by small local Ca^{2+} elevation was also converted to attraction by bath application of cAMP analogs, thus indicating that the cAMP pathway inhibits CaN-PP1 signaling underlying repulsion. Taken together, these results show that the CaMKII/CaN-PP1 mechanism also operates in growth cones with a normal baseline of $[Ca^{2+}]_i$: a small local Ca^{2+} signal at the normal baseline $[Ca^{2+}]_i$ induced repulsion by preferentially activating CaN over CaMKII, whereas a relatively large Ca^{2+} elevation activates CaMKII to induce attraction (shown in the first part of the study).

One interesting question regarding growth cone turning at the normal Ca^{2+} resting level (in culture medium)

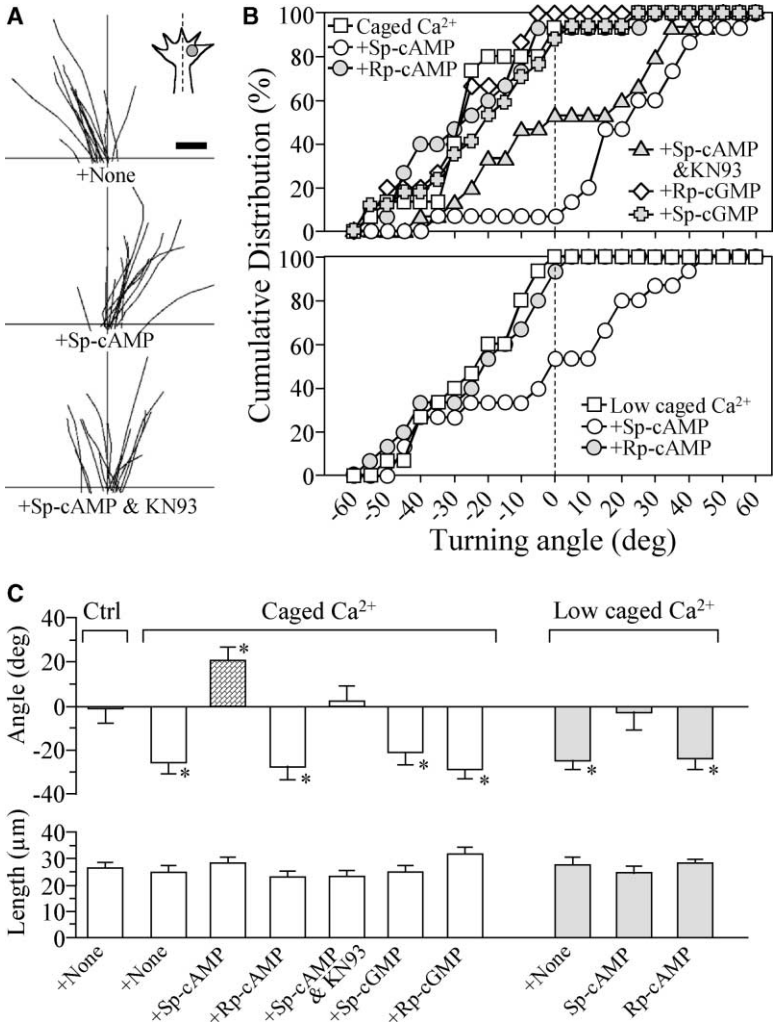


Figure 4. Regulation of Growth Cone Repulsion by Cyclic Nucleotides

(A) Responses of growth cones to direct focal Ca^{2+} elevation in Ca^{2+}-free solution without (top) and with Sp-cAMP (middle) or Sp-cAMP + KN93 (bottom) in bath. Scale bar, 10 μm.

(B) The cumulative distributions show the different responses of growth cones to direct focal Ca^{2+} in Ca^{2+}-free solution. The top panel represents the histograms from groups of growth cones loaded with the typical concentration of NP-EGTA, and the bottom panel represents the growth cones loaded with a reduced concentration of NP-EGTA. Different analogs and antagonists were added to the bath 20 min before the onset of the turning assay.

(C) Average angles and net lengths of extension for each group examined. The bar filled with the diagonal brick pattern represents the conversion from the repulsion.

is why attraction was not converted to repulsion after CaMKII inhibition (see Figure 1). In principle, both CaN and CaMKII would have been activated by the relatively large local Ca^{2+} signals during attraction; inhibition of CaMKII would have then uncovered CaN-PP1 activation to result in repulsion. Previous evidence indicates that large Ca^{2+} elevation could act upon Ca^{2+}-dependent adenylate cyclase to activate the cAMP pathway (Wayman et al., 1994; Yovell et al., 1992), which could in turn negatively regulate the CaN-PP1 pathway as we described above. We therefore directly tested this possibility by concurrently inhibiting CaMKII and PKA (using both KN93 and Rp-cAMP) and examined the growth cone response to the "large" focal Ca^{2+} elevation (standard NP-EGTA loading). As shown above (Figure 1), inhibition of CaMKII alone blocked the turning response without switching it to repulsion, and inhibition of PKA alone did not affect attractive responses. However, simultaneous inhibition of both CaMKII and PKA resulted in switching of Ca^{2+}-induced attraction to marked growth cone repulsion, as depicted by the cumulative histograms of turning angles (Figure 5B) as well as the averages (Figure 5C). Significantly, the conversion to repulsion could be further blocked by CaN inhibition through simultaneous application of KN93, Rp-cAMP, and DM. These results thus illustrate that the large local Ca^{2+} signals elicit a negative control on the CaN-PP1 repulsive pathway through PKA at normal resting $[Ca^{2+}]_i$. Such negative feedback could be an important part of the guidance mechanisms for generating appropriate responses.

Netrin-1 Guidance Involves the Same Mechanisms

The conclusion that CaMKII and CaN-PP1 mediate Ca^{2+}-dependent growth cone attraction and repulsion, respectively, is based on our experiments employing direct local Ca^{2+} manipulation. Does this CaMKII/CaN-PP1 mechanism operate in growth cone guidance by in vivo guidance cues? We therefore examined Ca^{2+}-dependent growth cone turning induced by netrin-1 gradients using an in vitro turning assay (Guirland et al., 2004; Ming et al., 1997). When *Xenopus* neurons were cultured on laminin, netrin-1 induced marked growth cone repulsion (Guirland et al., 2004; Hopker et al., 1999). We found that inhibition of CaN or PP1 resulted in immediate conversion of repulsion to attraction (Figure 6). In the presence of 2 nM DM or 3 nM tautomycin in bath, netrin-1 gradients induced turning responses with the average turning angles of $+9.6° \pm 4.9°$ and $10.3° \pm 5.3°$,

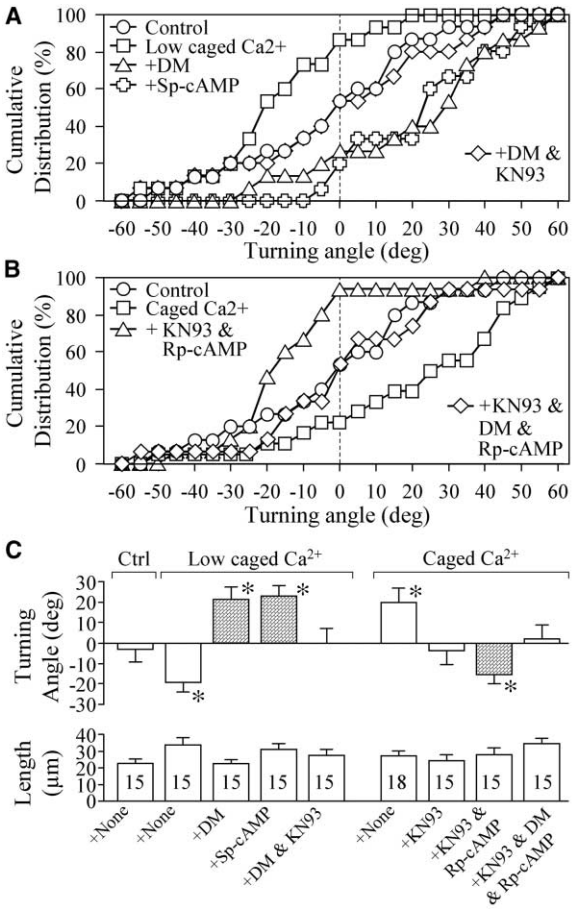


Figure 5. Growth Cone Responses to Direct Focal Ca^{2+} Elevation at the Normal Resting $[Ca^{2+}]_i$ Level in Culture Medium

(A) Cumulative histograms of turning angles from groups of growth cones loaded with a low concentration of caged Ca^{2+} compound NP-EGTA (for small local Ca^{2+} elevation).

(B) Cumulative histograms of turning angles from groups of growth cones loaded with the normal concentration of caged Ca^{2+} compound NP-EGTA.

(C) Average angles and lengths of growth cone extension for each group examined.

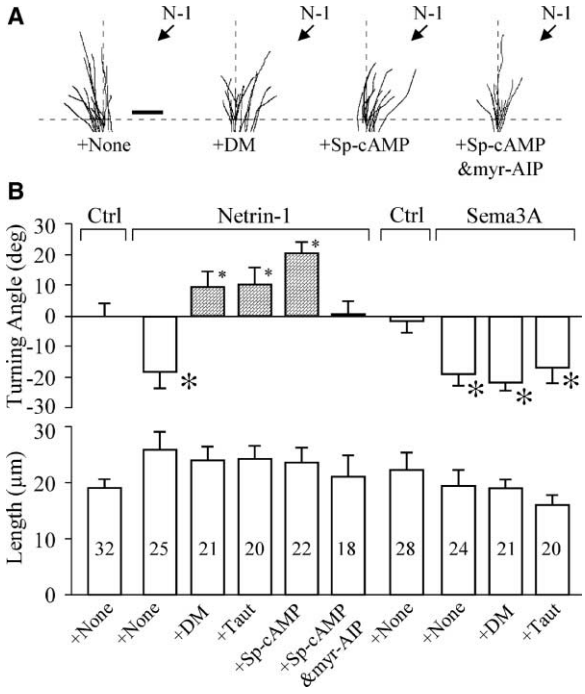


Figure 6. Growth Cone Turning Responses Induced by Extracellular Gradients of Netrin-1 and Sema3A

(A) Composite traces of the trajectories of growth cone extension during the 30 min turning assay in a netrin-1 gradient (N-1).

(B) Average angles and lengths of neurite extension of each group are summarized in the bar graph. Bars filled with the diagonal brick pattern indicate switching from the opposite responses. Numerals represent the total number of cells examined for each group.

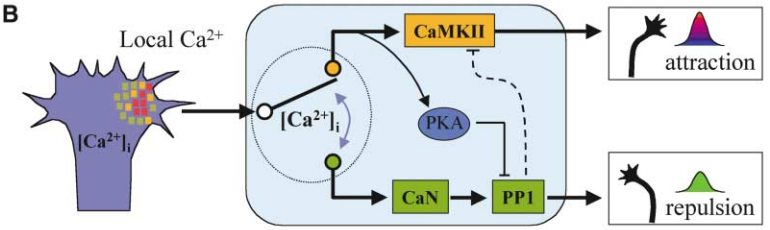
respectively, which contrast largely to the repulsion ($-18.0° \pm 5.4°$) induced by netrin-1 without any treatment ($p < 0.01$, Mann-Whitney test; Figure 6B). Statistical comparison to the control group also shows that netrin-1 induced significant attraction in the presence of DM or tautomycin ($p < 0.01$). Furthermore, bath application of Sp-cAMP converted netrin-1-induced repulsion to attraction, which is consistent with the previous report (Ming et al., 1997). The conversion of turning responses by Sp-cAMP appeared to be more effective than those by inhibition of CaN or PP1, as it resulted in a larger average angle of $+20.3° \pm 3.8°$. Moreover, we found that the attraction from cAMP conversion in netrin-1 gradients depended on CaMKII, as it was blocked by addition of the inhibitory peptide myr-AIP (Figure 6). Finally, CaN and PP1 appear to be specifically involved in Ca^{2+}-dependent repulsive guidance, as growth cone repulsion induced by Sema3A gradients was not affected by inhibition of either CaN or PP1 (Figure 6B). It has been reported previously that Sema3A repulsion does not depend on Ca^{2+} and cannot be switched by cAMP (Song et al., 1998). Given the recent finding that netrin-1 can activate CaN in vivo (Graef et al., 2003), our results thus indicate that CaMKII/CaN-PP1 plays an important role in mediating Ca^{2+}-dependent netrin-1 guidance.

Discussion

In this study, we have identified CaMKII and CaN-PP1 as the downstream effectors of localized Ca^{2+} signals in mediating attractive and repulsive turning responses of growth cones, respectively. Based on our experimental data involving direct focal elevation of $[Ca^{2+}]_i$ (summarized in Figure 7A), we conclude that CaMKII/CaN-PP1 acts like a bimodal switch that responds to different local Ca^{2+} signals to control the direction of growth cone extension (Figure 7B). Importantly, the resting level of $[Ca^{2+}]_i$ at the growth cone directly influences the switch operation, as it biases the local Ca^{2+} signal for differential activation of CaMKII and CaN. At the normal resting level of $[Ca^{2+}]_i$ ($\sim$130 nM for neurons in culture medium [Zheng, 2000]; experimental sets *I* and *III* in Figure 7A), the amplitude of the local Ca^{2+} signals dictates the difference in CaMKII/CaN activation: small local Ca^{2+} signals activate CaN over CaMKII for repulsion (*Set III* in Figure 7A), while a large Ca^{2+} elevation preferentially

A

Set	Resting	Local	Turning	Proteins inhibited	Result of inhibition
I	norm	high	+	CaMKII	⊗
				CaMKII & PKA	−
				CaMKII & PKA & CaN	⊗
II	low	high	−	CaN or PP1	+
III	norm	low	−	(CaN or PP1) & CaMKII	⊗
IV	low	low	−	CaN or PP1	⊗



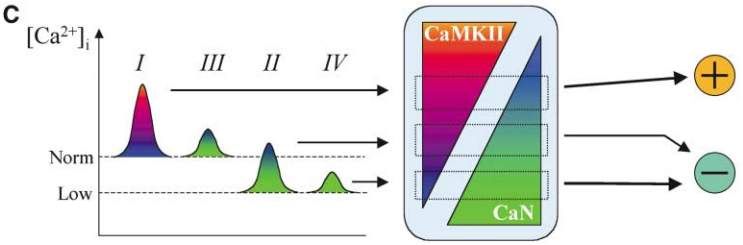


Figure 7. Schematic Diagrams Illustrating the CaMKII/CaN-PP1 Switch Model

(A) Summary of our experimental data from combinations of different local Ca^{2+} elevation and resting Ca^{2+} level (four sets: I–IV). "+," "−," and "×" represent attraction, repulsion, and no turning response, respectively.

(B) Local Ca^{2+} elevation activates CaMKII and CaN-PP1 for attraction and repulsion, respectively. The negative feedback of these two pathways is also proposed, of which PKA activation inhibits CaN-PP1 and PP1 potentially inhibits CaMKII.

(C) Different local Ca^{2+} signals and baseline levels of $[Ca^{2+}]_i$ (sets I–IV as indicated in [A]) differentially activate CaMKII and CaN-PP1 to control the direction of growth cone turning. The activities of CaMKII and CaN are illustrated as gradients in a reversed order due to their differences in Ca^{2+} affinity for activation.

elicits CaMKII signaling for attraction (*Set I* in Figure 7A). However, when the resting level of $[Ca^{2+}]_i$ is significantly reduced (~60 nM for neurons in Ca^{2+}-free solution [Zheng, 2000]; experimental sets *II* and *IV* in Figure 7A), both small and large local Ca^{2+} signals would primarily activate CaN for inducing repulsive turning responses. This switch model is consistent with the different Ca^{2+} affinities of CaMKII and CaN for activation (Groth et al., 2003; Hudmon and Schulman, 2002; Rusnak and Mertz, 2000) and suggests that the balance between CaMKII and CaN-PP1 activities determines the output of the switch (Figure 7C). Significantly, negative feedback appears to be implemented in the switch for its proper functioning (Figure 7B). Our experimental data demonstrate a negative feedback from the attractive pathway to the repulsive pathway through PKA (Figure 7B), which was activated during attraction by large local Ca^{2+} elevation at normal resting level. In parallel, a negative feedback from PP1 to CaMKII may also exist (Figure 7B), as direct dephosphorylation of CaMKII by PP1 has been well documented (Shields et al., 1985). These negative feedback loops may play an integral part in the switch operation, as they can assure a single output of the switch and thus generate specific growth cone responses. Finally, the integration of local Ca^{2+} signals with global (resting) Ca^{2+} levels for switch operation potentially provides the growth cone with the flexibility of generating distinct responses to the same local signals under different environmental settings. For example, other globally available factors (e.g., matrix molecules and growth factors) could alter the resting $[Ca^{2+}]_i$ to modulate the growth cone responses to the limited number of local cues.

While our conclusion that CaMKII/CaN-PP1 mediates Ca^{2+}-dependent growth cone turning is primarily based on experiments involving direct local Ca^{2+} elevation by FLIP of caged Ca^{2+}, we have also accumulated evidence that the CaMKII/CaN-PP1 mechanism operates in Ca^{2+}-dependent guidance by in vivo cues. Specifically, we tested the involvement of CaMKII/CaN-PP1 in guidance by netrin-1 and Sema3A. As previously demonstrated, guidance by netrin-1 but not Sema3A depends on Ca^{2+} and can be switched by cAMP manipulation (Ming et al., 1997; Song et al., 1998). Consistently, we found that only netrin-1-induced growth cone repulsion on the laminin substrate was converted to attraction by inhibition of either CaN or PP1, whereas inhibition of CaN or PP1 had no effects on Sema3A-induced repulsion. These results further support the notion that CaMKII/CaN-PP1 is selectively involved in Ca^{2+}-dependent guidance. It should be noted that young *Xenopus* spinal growth cones (6 hr cultures) responded to netrin-1 with repulsion on bare coverglass (Ming et al., 2001) or on different substrates, including laminin (Buck and Zheng, 2002; Guirland et al., 2004). It was proposed that the repulsive effects of netrin-1 on *Xenopus* growth cones with or without laminin are partially due to a lower level of endogenous cAMP (Hopker et al., 1999; Ming et al., 2001). In relatively old *Xenopus* spinal cultures (16–24 hr), attraction was induced by netrin-1 in a Ca^{2+}-dependent manner (Ming et al., 1997, 2001). Furthermore, the attraction could be converted to repulsion by PKA inhibi-

tion, suggesting that these *Xenopus* spinal neurons may change the endogenous cAMP level over time in culture. Consistently, netrin-1-induced repulsion was converted to attraction by cAMP elevation in our culture, which was subsequently blocked by CaMKII inhibition (Figure 6). We therefore believe that the netrin-1-induced repulsion in this study is similar to the netrin-1-induced attraction in older cultures: both likely involve Ca^{2+} signaling through the CaMKII/CaN-PP1 switch and can be converted by the cAMP pathway. While CaN and its downstream NFAT transcription factor have been shown to mediate netrin-1-dependent neurite outgrowth (Graef et al., 2003), it is unknown whether or not these long-term effects on neurite outgrowth directly depended on netrin-1-induced Ca^{2+} signals. On the other hand, our CaN-PP1-mediated repulsion is a short-term effect that is directly elicited by local Ca^{2+} signals. It is entirely possible that different ways/magnitudes of CaN activation and/or specific spatiotemporal patterns may contribute to distinct netrin-1 effects on outgrowth and guidance. Furthermore, there is ample evidence that growth cone guidance and neurite outgrowth are not necessarily related (Chang et al., 2004; Guirland et al., 2003; Ming et al., 1999). Consistent with this notion, we did not observe significant inhibition of growth during repulsion in netrin-1 gradients (Figure 6B). We therefore propose that the CaMKII/CaN-PP1 switch also mediates growth cone steering in netrin-1 guidance. However, given the complexity of intracellular signaling elicited by extracellular molecules, the existence of multiple routes of Ca^{2+} entry into the cytosol, and crosstalk among different pathways, it is reasonable to speculate that other signaling pathways may contribute to and/or regulate the Ca^{2+}-dependent guidance of growth cones. For example, CaMKI has been shown to play an important role on axonal extension and growth cone motility (Wayman et al., 2004). CaN was shown to be cleaved by calpain (Wu et al., 2004), a protease that has been implicated in Ca^{2+} transients-induced growth cone repulsion (Robles et al., 2003). Therefore, how various signaling pathways contribute to and/or interact with CaMKII/CaN-PP1 observed here awaits further investigation.

One of the interesting findings in this study is the negative regulation of the CaN-PP1 repulsive pathway by the cAMP pathway, which represents, arguably, the first direct evidence that the cAMP pathway acts downstream of the Ca^{2+} signals in guidance. Our conclusion is based on experimental data on growth cone responses to direct local Ca^{2+} elevation, which is not affected by cAMP manipulation (imaging data not shown). An established body of evidence has indicated a role for PKA in regulating the activity of phosphatases, especially that of PP1. For example, PP1 is inhibited by inhibitor-1 (I-1), a protein that is the target of PKA phosphorylation (to inhibit PP1) and CaN dephosphorylation (to remove PP1 inhibition) (Oliver and Shenolikar, 1998). Given the known PKA inhibition of PP1 and, most importantly, the parallel effects on turning responses by PKA activation, CaN inhibition, and PP1 inhibition shown here, we conclude that CaN and PKA oppositely regulate PP1 activity to regulate growth cone repulsion (Figure 7B), potentially through the common targets, such as I-1 (Huang and Glinsmann, 1976) or Darpp-32 (Walaas et al., 1983). It should be noted that our findings do not rule out the possibility that the cAMP pathway may also act upstream of the Ca^{2+} signals, in particular, during growth cone attraction induced by extracellular cues (Hong et al., 2000; Nishiyama et al., 2003). In our experiments involving netrin-1-induced growth cone turning, we found that inhibition of CaN or PP1 was able to switch the repulsion to attraction, but not as effectively as did Sp-cAMP application. It is conceivable that elevation of intracellular cAMP concentrations exerted dual roles in converting repulsion to attraction: it not only activated PKA to inhibit CaN-PP1 signaling to block repulsion (this study), but also amplified netrin-1-induced Ca^{2+} signals for more effective attraction (Hong et al., 2000; Nishiyama et al., 2003). Similarly, conversion of attraction to repulsion by PKA inhibition likely involves both reduction of local Ca^{2+} signals and the removal of PP1 inhibition. In our preliminary studies on BDNF-induced attraction, we found that switching of BDNF-induced attraction to repulsion by Rp-cAMP was eliminated after PP1 inhibition by tautomycin, confirming the role of CaN-PP1 pathway in Ca^{2+}-dependent repulsion (data not shown). These findings further support our CaMKII/CaN-PP1 model in Ca^{2+}-dependent guidance and predict that PKA inhibition of the CaN-PP1 pathway plays an essential step in the conversion of guidance responses.

It is of great interest to note that a similar CaMKII/CaN-PP1 switch appears to exist and play an important role in synaptic plasticity involving long-term potentiation (LTP) and long-term depression (LTD) (Isaac, 2001; Lisman and Zhabotinsky, 2001; Malleret et al., 2001; Morishita et al., 2001). In particular, CaMKII and CaN-PP1 were shown to mediate LTP and LTD, respectively. This apparent parallel between attraction/repulsion and LTP/LTD is also striking, as they both represent, in a broad sense, positive/negative regulation of a particular neuronal behavior. On the other hand, there are clear differences between the CaMKII/CaN-PP1 switch in LTP/LTD and the one observed in growth cone turning here. For example, the CaMKII/CaN-PP1 switch observed here appears to operate in a much smaller Ca^{2+} range (nanomolar range) than that in synaptic plasticity (micromolar range). Furthermore, there is evidence that CaMKII/CaN-PP1 targets neurotransmitter receptors in LTP/LTD (Isaac, 2001; Lisman and Zhabotinsky, 2001), whereas our turning responses by direct local Ca^{2+} elevation apparently did not involve surface guidance receptors. Finally, α-CaMKII knockout mice were impaired in LTP and spatial learning but exhibited no gross defects in pathfinding (Silva et al., 1992a, 1992b). Since multiple isoforms of CaMKII are expressed in the brain (Hudmon and Schulman, 2002), it is possible that different CaMKII isoforms may be involved in the CaMKII/CaN-PP1 switch regulating growth cone turning. The fact that α-CaMKII is not expressed in *Xenopus* (Tombes et al., 2003) further suggests that other isoforms participate in the growth cone switching. Despite these differences, our experimental data make a good case for a CaMKII/CaN-PP1 switch in growth cone turning induced by localized Ca^{2+} elevation. Such a CaMKII/CaN-PP1 switch may represent an important signaling mechanism that is conserved as well as adapted for specific tasks at different stages during development and maintenance of the nervous system.

At present, the downstream target(s) of CaMKII/CaN-PP1 remains unknown. While CaN has a narrow range of targets, PP1 is known to act on a wide spectrum of substrates that may be involved in growth cone turning. For example, growth cone steering depends on localized cytoskeletal dynamics, and CaMKII/Calcineurin-PP1 may directly act on the cytoskeleton for local modification of the dynamics. Previous studies have shown that CaMKII and CaN-PP1 can phosphorylate and dephosphorylate tubulin proteins, respectively, to affect the ability of tubulin to assemble into microtubules (Goto et al., 1985; Yamamoto et al., 1985). Furthermore, the microtubule-associated proteins MAP2 and *tau* can be phosphorylated and dephosphorylated by CaMKII/CaN to affect their affinity of binding to microtubules, resulting in modification of microtubule stability (Goto et al., 1985; Mandelkow et al., 1995; Yamamoto et al., 1985). As we showed previously, such local modification of microtubule dynamics and stability could potentially initiate growth cone steering (Buck and Zheng, 2002). Moreover, the growth cone-associated protein GAP43 has been shown to be the target of CaMKII/Calcineurin (Lautermilch and Spitzer, 2000). The CaMKII and PP1 serine/threonine kinase and phosphatase pair could potentially converge their effects on the same targets to regulate the balance of phosphorylation/dephosphorylation of target proteins, which may be critical for growth cone motility and directionality. Clearly, further identification of the downstream targets would greatly enhance our understanding of the molecular and cellular mechanisms underlying guidance of developing axons that leads to proper construction of the intricate nervous system.

Experimental Procedures

Cell Culture

Embryonic *Xenopus* spinal neurons were cultured on coverslips that were precoated with poly-d-lysine and laminin in SFM containing 1% BSA as described previously (Guirland et al., 2003). The SFM consisted of 50% (v/v) Leibovitz L-15 medium (Invitrogen, Gaithersburg, MD), 50% (v/v) Ringer's solution (115 mM KCl, 2 mM $CaCl_2$, 2.5 mM KCl, 10 mM HEPES [pH 7.4]), and 1% (w/v) BSA (Sigma). *Xenopus* cultures were maintained at 20–22°C, and the neurons normally started to sprout neurite processes within 2–3 hr after plating.

Growth Cone Turning Induced by Focal Laser-Induced Photolysis of Caged Ca^{2+}

At about 3 hr after plating, the neurons were loaded with either 6 μM or 2 μM acetoxymethyl (AM) ester derivative of NP-EGTA (Molecular Probes) for 30 min, followed by three washes and a 2 hr recovery in the SFM (Zheng, 2000). The two different loading concentrations of NP-EGTA-AM were used for generating relatively large (experimental sets *I* and *II* in Figure 7A) or small (sets *III* and *IV* in Figure 7A) local Ca^{2+} elevation. Growth cone turning induced by repetitive FLIP of caged Ca^{2+} was done on a Nikon TE300 inverted microscope equipped with a 40×/1.3 oil immersion objective and a nitrogen-pulsed UV laser as described before (Zheng, 2000). For turning in the culture medium, we exposed the growth cone to repetitive FLIP (one pulse every 3 s) for 30 min. For turning in Ca^{2+}-free Ringer's solution, the growth cone was examined for 15 min.

Growth Cone Turning Induced by Extracellular Guidance Gradients

Growth cone turning induced by netrin-1 or Sema3A gradients was performed according to the method described previously (Guirland et al., 2003; Ming et al., 1997), except assays were done in a modified Ringer's solution (Ming et al., 1999). Microscopic gradients of chemicals were produced by the pipette application method described previously (Lohof et al., 1992; Zheng et al., 1996b). A standard pressure pulse of 3 psi was applied to a glass pipette (1 μm opening) at a frequency of 2 Hz with pulse duration of 20 ms. The direction of growth cone extension at the beginning of the experiment was defined by the distal 20 μm segment of the neurite. The pipette tip was positioned 45° from the initial direction of extension and 100 μm away from the growth cone. Purified netrin-1 proteins were a gift from Dr. Marc Tessier-Lavigne (Genentech) and used at 5 μg/ml in the pipette. Sema3A was collected from the supernatant of confluent COS cells expressing human Sema3A (Kolodkin et al., 1993) and dialyzed into Ringer's saline for pipette application. The vehicle control solution was obtained from the supernatant of mock-transfected COS cells that expressed the same vector lacking the hSema3A insert. Different agonists and antagonists were added to the bath medium 20 min before the onset of turning assays. The agonists and antagonists were purchased from Calbiochem (San Diego, CA), except myristoylated-AIP, which was purchased from Biomol Research Laboratories (Plymouth Meeting, PA).

Quantification of Growth Cone Turning

The digital images of the growth cone at the onset and end of the turning assay (either FLIP-induced or pipette application) were acquired and overlaid with pixel-to-pixel accuracy, and the trajectory of new neurite extension was traced using Adobe Photoshop (Adobe Systems). The turning angle was defined by the angle between the original direction of neurite extension and a line connecting the positions of the growth cone at the onset and the end of the experiment. Neurite extension was quantified by measuring the entire trajectory of net neurite growth of the assay period. Only growth cones that extended 5 μm or more were scored and analyzed for turning responses. We used the nonparametric Mann-Whitney test to analyze turning angles, since they do not follow a normal distribution.

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