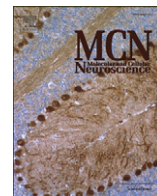




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Cyclic nucleotide-dependent switching of mammalian axon guidance depends on gradient steepness

Andrew W. Thompson^a, Zac Pujic^a, Linda J. Richards^{a,b}, Geoffrey J. Goodhill^{a,c,*}^a Queensland Brain Institute, The University of Queensland, St Lucia 4072, Queensland, Australia^b School of Biomedical Sciences, The University of Queensland, St Lucia 4072, Queensland, Australia^c School of Mathematics and Physics, The University of Queensland, St Lucia 4072, Queensland, Australia

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ABSTRACT

Correct wiring of the nervous system during development requires axons to respond appropriately to gradients of attractive and repulsive guidance cues. However, the steepness and concentration of these gradients vary *in vivo*, for instance, with distance from the target. Understanding how these changing conditions affect the navigation strategies used by developing axons is important for understanding how they are guided over long distances. Previous work has shown that cyclic nucleotide levels determine whether axons are attracted or repelled by steep gradients of the same guidance cue, but it is unknown whether this is also true for shallow gradients. We therefore investigated the guidance responses of rat superior cervical ganglion (SCG) axons in both steep and shallow gradients of nerve growth factor (NGF). In steep gradients we found that cyclic nucleotide-dependent switching occurred, consistent with previous reports. Surprisingly however, we found that in shallow NGF gradients, cyclic nucleotide-dependent switching did not occur. These results suggest that there may be substantial differences in the way axons respond to gradient-based guidance cues depending on where they are within the gradient.

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Introduction

In the developing or regenerating nervous system, neurons extend axons that must navigate through complex environments to find their final targets (Tessier-Lavigne and Goodman, 1996; Mueller, 1999; Plachez and Richards, 2005). Some of the most important cues used to steer axons appropriately are molecular gradients (Yu and Bargmann, 2001; Dickson, 2002; Chilton, 2006; Flanagan, 2006). Such gradients induce differences in receptor binding across the growth cone, which are then converted into an appropriate behavioral response (Mortimer et al., 2008; O'Donnell et al., 2009). Although quite specific gradient conditions are required for effective guidance of axons (Mortimer et al., 2009), variations in both steepness and concentration are likely to exist *in vivo* depending on the distance of the axon from the target (Goodhill, 1997; Kennedy et al., 2006).

Differences in gradient steepness can influence pathfinding decisions *in vivo* (Isbister et al., 2003), and more recent work suggests that there may be fundamental differences in the response to gradients for steep *versus* shallow gradients. In particular, it was recently proposed that growth cone turning dominates the guidance response for steep gradients, while growth rate modulation without biased turning dominates for shallow gradients (Mortimer et al.,

2010). Here, axons grown from dorsal root ganglion explants in the presence of a shallow NGF gradient were found on average not to exhibit biased turning. Rather, axons growing up the gradient were found to be longer than those growing down the gradient, even when those growing down the gradient were at a higher NGF concentration. These experiments showed that the crucial factor controlling neurite length is the direction of growth relative to the gradient direction, not the absolute concentration of NGF, confirming that growth rate modulation is a chemotropic guidance effect rather than a purely trophic effect. The mechanisms leading to growth rate modulation however remain unknown, and may involve different signaling pathways than those responsible for the guidance of axons in steep gradients.

Many effector molecules have been implicated in axon guidance to molecular gradients (Song and Poo 1999, 2001; Huber et al., 2003; Guan and Rao, 2003; Gomez and Zheng, 2006; Zheng and Poo, 2007; O'Donnell et al., 2009; Hong and Nishiyama, 2010). In particular, cyclic nucleotide levels can influence the sign of the chemotactic response (Ming et al., 1997; Song et al., 1997, 1998; Wen et al., 2004; Mai et al., 2009; Murray et al., 2009; Tojima et al., 2009). It has been shown that high levels of cAMP relative to cGMP lead to growth cone attraction, while the opposite leads to repulsion (Nishiyama et al., 2003). High relative cAMP was thought to produce attraction through its downstream effector protein kinase A (PKA), although recent evidence suggests that Epac (exchange protein activated by cAMP) may be the primary transducer of this response (Murray et al., 2009).

* Corresponding author at: Queensland Brain Institute, The University of Queensland, St Lucia 4072, Australia.

E-mail address: g.goodhill@uq.edu.au (G.J. Goodhill).

In either case, this cyclic nucleotide-dependent switching effect has become a classic result in the field of axon guidance, with potential consequences for regenerating axons after injury (Neumann et al., 2002; Qiu et al., 2002; Lu et al., 2004).

However, studies of cyclic nucleotide-induced switching have generally utilized only the very steep gradients of the “pipette” or “growth cone turning” assay. When Moore and Kennedy (2006) investigated the role of PKA in an explant co-culture-type assay, they were notably unable to induce switching from chemoattraction to chemorepulsion. Instead, they found a PKA-dependent effect on the sensitivity of the attractive response. This raised the question as to exactly what role cyclic nucleotides and their effectors play in mediating guidance decisions, and what role gradient and environmental conditions might play in this process. Here we compare cyclic nucleotide-dependent switching of the same type of growth cone in steep gradients using the growth cone turning assay with both 2- and 3-dimensional substrates, and in shallow gradients with a 3-dimensional collagen matrix. We show that guidance switching occurs in steep but not shallow gradients and thus that the intracellular mechanisms leading to guidance also depend fundamentally on the properties of the gradient itself. These results demonstrate that important aspects of the behavior of axons *in vivo* may depend sensitively on the specific gradient conditions present, and thus may vary depending on the position of the axon within the gradient and hence proximity to its target.

Results

We used three different assays to determine how the guidance response of mammalian axons is affected by modulation of cyclic nucleotide levels. We first used the standard growth cone turning assay for axons growing in a steep gradient on a 2-D substrate, and compared these results with a collagen gel printing assay for axons growing in a shallow gradient in 3-D. Finally, we investigated switching in a modified growth cone turning assay for axons growing in a steep gradient in a 3-D matrix. For our experiments we used the superior cervical ganglion (SCG) of P0–3 rat. This is a sympathetic population of neurons, 97% of which have been reported to express mRNA for the high-affinity nerve growth factor (NGF) receptor, TrkA (Wetmore and Olson, 1995), and are robustly responsive to NGF (Ohta et al., 1990; Yu et al., 2010).

Modulation of PKA and PKG activity switches SCG responses to steep NGF gradients

We began our investigation into the role of gradient steepness on axon guidance by first establishing whether SCG neurons are capable of cyclic nucleotide-induced switching *in vitro*. To do this we utilized the well-established growth cone turning assay on dissociated SCG cells. This assay produces gradients with steepness of roughly a 10–15% change in concentration across 10 μm , and is commonly used to assess growth cone turning over about one hour of growth (Lohof et al., 1992; Pujic et al., 2008). When we used a concentration of 10 μM NGF in the pipette, growth cones from dissociated SCG neurons showed significant attraction up the gradient (mean turning angle = $14.6 \pm 4.7^\circ$) compared to the control condition (mean turning angle = $0.02 \pm 2.7^\circ$; $p = 0.02$, Kolmogorov–Smirnov (KS) test, Fig. 1A, B).

The growth cone turning assay has revealed a cyclic nucleotide-dependent switch between attractive and repulsive responses to the same guidance cues for *Xenopus* spinal neurons (Ming et al., 1997; Song et al., 1997, 1998). Specifically, PKA inhibitors and PKG activators have both been shown to switch a normally attractive response to repulsion using this assay (Nishiyama et al., 2003). Consistent with these results, we found that bath application of the PKA inhibitor KT5720 to dissociated rat SCG neurons at 80 nM for 30 min prior to the assay with 10 μM NGF switched growth cone responses from

attraction to repulsion (mean turning angle = $-21.9 \pm 5.7^\circ$, Fig. 1A, B). Bath application of the second PKA inhibitor, Rp-8-CPT-cAMPs (5 μM), or a PKG activator, 8-Br-cGMP (20 μM), prior to exposure to an NGF gradient, also switched growth cone responses from attraction to repulsion (mean turning angles = $-13.45 \pm 4.1^\circ$ and $-14.24 \pm 3.8^\circ$ respectively, Fig. 1A, B). Growth rates in all of the experimental conditions above were not significantly changed from the control condition ($p > 0.05$, KS test, Fig. 1C). Thus, mammalian SCG growth cone turning is strongly influenced by internal protein kinase signaling when responding to steep gradients of NGF.

Modulation of PKA activity does not switch SCG responses in shallow NGF gradients

The above results confirm that cyclic nucleotide-modulated switching of guidance responses occurs for mammalian axons in the steep gradients of the pipette assay. However, questions have been raised about the ability of axons to utilize this switching mechanism when guided by shallower gradients in a 3D environment (Moore and Kennedy, 2006). To address this, SCG explants were embedded in a collagen gel matrix and grown for 48 h in a shallow NGF gradient, with an approximately 0.3% change in concentration over 10 μm , as per the printing method described in Rosoff et al. (2004, 2005) and Mortimer et al. (2009) (Fig. 2A). Outgrowth was measured as a ratio of neurite pixels to explant pixels, and the guidance ratio (GR) was measured by the number of neurite pixels on the high side of the gradient relative to the center of the explant, minus the number on the low side, divided by the total number of neurite pixels (see Experimental methods).

We found that a shallow NGF gradient produced significant attraction of neurites grown from SCG explants ($\text{GR} = 0.14 \pm 0.01$, Fig. 2B, C). However, when the PKA inhibitors were added to the collagen prior to gelling, in similar concentrations to those used in the growth cone turning assays above, we saw only a reduction in attractive guidance (positive guidance ratio values) rather than a switch to repulsion (negative guidance ratios) (Fig. 2C). While we found no change in outgrowth with the PKA inhibitors, we did observe a small decrease in outgrowth with 20 μM 8-Br-cGMP (Fig. 2D), but no change in the guidance ratio.

To examine whether the effects of these drugs were concentration-dependent, we increased the concentrations used ten-fold. We observed a significant decrease in the attractive guidance with both 1 μM KT5720 and 200 μM 8-Br-cGMP compared to the no drug gradient condition, however 50 μM Rp-8-CPT-cAMPs did not result in a significantly different guidance ratio (Fig. 2B, C). Remarkably, though some significant effects on the magnitude of guidance in shallow gradients were evident, none of the compounds resulted in a switch from attraction to repulsion. At the higher drug concentrations we also found significant inhibition of growth for both KT5720 and 8-Br-cGMP, but not Rp-8-CPT-cAMPs (Fig. 2B, D). Similar to previous observations of DRG neurite growth in the same assay (Mortimer et al., 2009), the guided SCG explants were found to show no observable neurite turning in the direction of the gradient (Fig. 2E).

Neurites in shallow gradients are guided by growth rate modulation

Given the above results, we tested whether these SCG neurites were being guided by growth rate modulation. To do this, we performed zigzag collagen printing assays as described by Mortimer et al. (2010). Briefly, explants were cultured in a collagen matrix in the presence of a 0.3% NGF gradient as before, however the explants were positioned so that half would extend neurites from roughly a 0.2 nM concentration toward a roughly 0.3 nM concentration, and half would extend neurites from roughly a 0.4 nM concentration to the roughly 0.3 nM concentration (shown schematically in Fig. 3A). We found that SCG neurites growing up the NGF gradient extended more than those

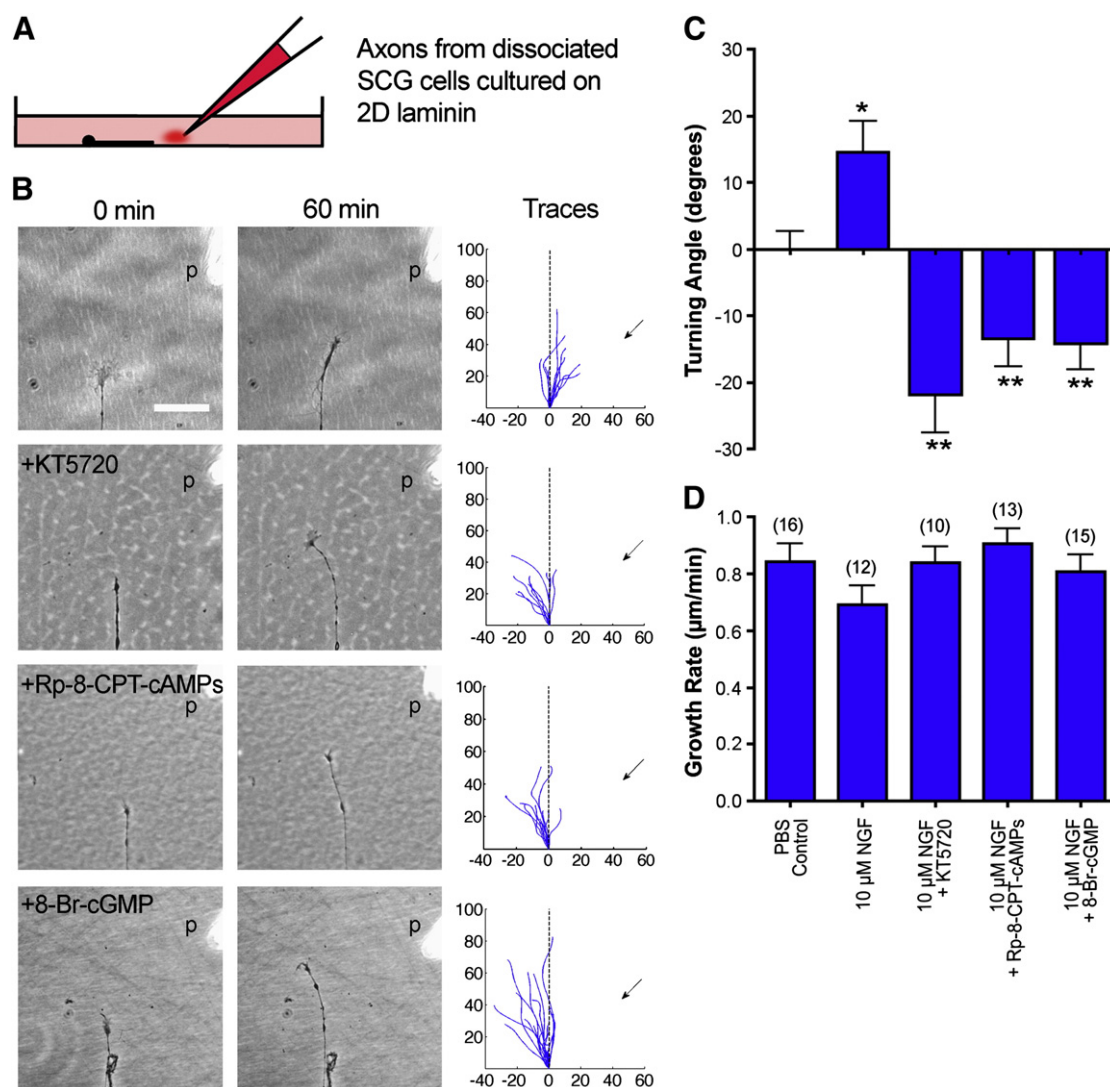


Fig. 1. Modulation of PKA and PKG activity switches SCG chemotactic responses to steep NGF gradients. A, Schematic of the standard growth cone turning assay in which dissociated cells are grown on a 2D substrate and a steep gradient of a chemotropic factor (red) is produced from a glass micropipette. B, Example images and traces of growth cone trajectories in an NGF gradient with PKA and PKG modulation (KT5720 was bath applied at 80 nM; Rp-8-CPT-cAMPS was bath applied at 5 μ M; 8-Br-cGMP was bath applied at 20 μ M). Left = prior to gradient application; middle = one hour following gradient exposure; right = smoothed traces of each growth cone path. Scale bar = 40 μ m; p, pipette tip, arrow indicates direction of gradient from the pipette, x- and y-axes on traces are distance (μ m). C–D, Summary of the mean turning angles (C) and growth rates (D) of SCG axons in the growth cone turning assay under different gradient and bath conditions. Error bars = SEM, numbers in brackets = number of assays per condition. * $p < 0.05$, ** $p < 0.01$, Kolmogorov–Smirnov test compared to control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

growing down the gradient, despite originating from a region of lower NGF concentration (Fig. 3B, C). Taken together, these data suggest that the lack of cyclic nucleotide-induced switching in shallow gradients may be a result of SCG axons being guided by growth rate modulation rather than turning under these conditions.

Dissociation of explants to individual cells does not prevent guidance switching

The growth cone turning assay and collagen printing assay systems used above differ in more than simply gradient steepness. We therefore investigated whether these other differences could account for the change in switching behavior between the two systems. First, while the collagen assay measures the response of neurites originating from explants, our pipette assays described so far have only used dissociated cells. Dissociation has been reported to change levels of Trk expression (Genc et al., 2005) and responsiveness to PKA and PKG (Zheng et al., 2007). We therefore examined whether switching was also achievable in axons from intact SCG explants

presented under normal pipette assay conditions. We found that axons originating from explants were switched from attraction in a steep 10 μ M NGF gradient to repulsion by application of KT5720 (Fig. 4). Thus we show there is no difference in ability to undergo switching between explants and dissociated cells.

Chronic PKA inhibition does not prevent guidance switching

The second difference between the standard growth cone turning assay and our collagen printing assay is the duration of drug application. We investigated whether the response changed between the PKA inhibitor KT5720 being applied acutely, as in the pipette assays, or chronically, as in the collagen assay. Following the incubation of explants overnight in the presence of 80 nM KT5720, growth cones were exposed to steep NGF gradients as for the pipette assays above. We found no difference between the mean turning angles of growth cones exposed to acute or chronic KT5720 ($p = 0.56$, KS test, Fig. 4), eliminating this as a source of the difference in

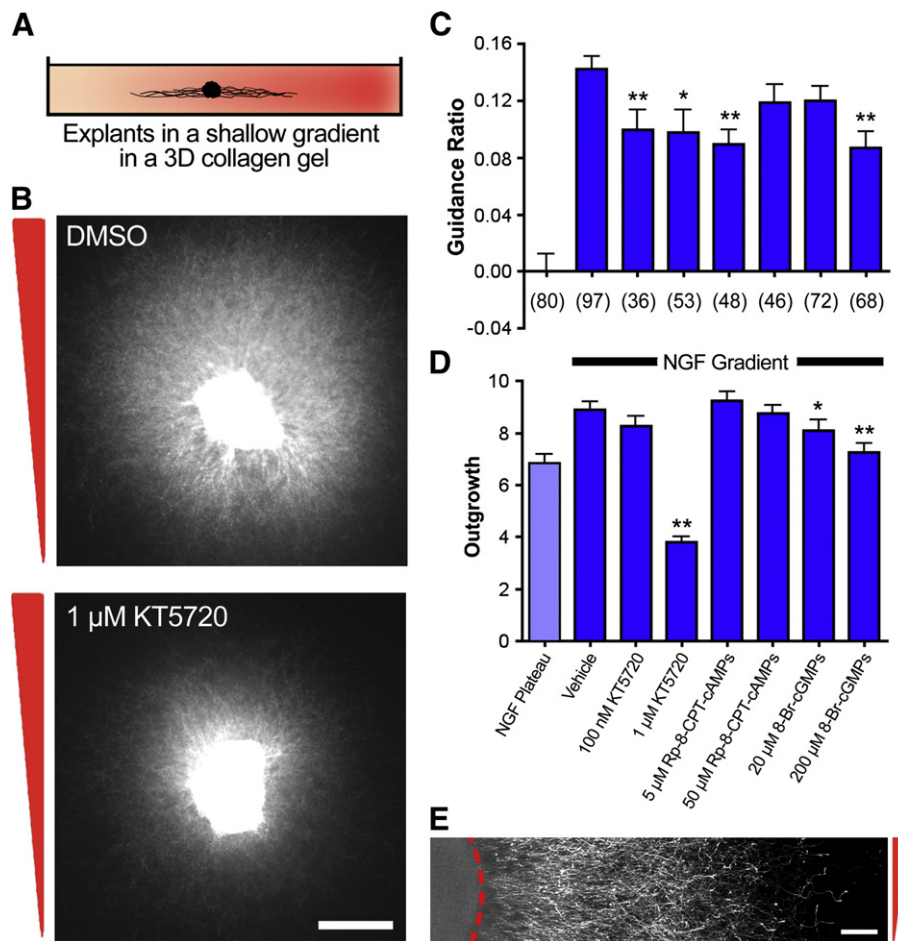


Fig. 2. Modulation of PKA and PKG activity does not switch SCG guidance responses to shallow NGF gradients. **A**, Schematic of a 35 mm dish in which SCG explants are cultured for 48 h in collagen gel in the presence of a shallow NGF gradient (red). **B**, Representative SCG explants in a 0.3% gradient of NGF (direction indicated on the left in red) in the presence of vehicle (DMSO) or 1 μ M KT5720. Explants were stained for β -3 tubulin and imaged from above. Scale bar = 500 μ m. **C**, KT5720 tended to reduce attractive guidance in this assay, but did not switch attraction to repulsion. Guidance ratio was measured by the number of neurite pixels on the high side of the gradient relative to the center of the explant, minus the number on the low side, divided by the total number of neurite pixels. Attraction is indicated by positive guidance ratios whereas repulsion is indicated by negative guidance ratios ($n \geq 24$ explants per condition). **D**, High, but not low concentrations of KT5720 significantly reduced neurite outgrowth from SCG explants. Neurite outgrowth was measured by the ratio of the number of neurite pixels to the number of explant pixels. * $p < 0.05$, ** $p < 0.01$, Kolmogorov–Smirnov test compared to vehicle. **E**, ApoTome (Zeiss) image of a 50 μ m section of neurite outgrowth from the side of an SCG explant in the presence of a 0.3% NGF gradient. No visible neurite turning in the direction of the gradient is apparent (gradient direction indicated on the right in red), consistent with the behavior of DRG explants in shallow gradients (Mortimer et al., 2010). Scale bar = 100 μ m; dotted line indicates side of explant. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

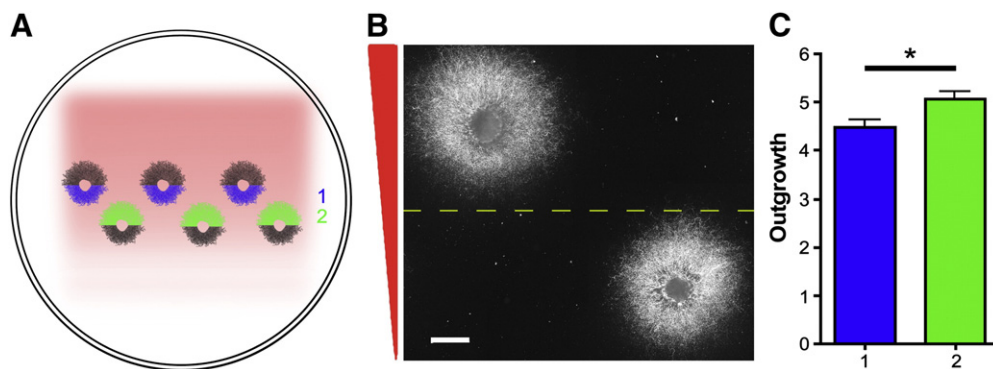


Fig. 3. SCG neurites in shallow gradients are guided by growth rate modulation. **A**, Top-view schematic of the zigzag collagen printing assay. SCG explants were cultured in a 3-D collagen gel in the presence of a 0.3% NGF gradient (red). They were positioned so that half would extend axons down the gradient from a concentration of roughly 0.4 nM NGF toward a concentration of roughly 0.3 nM NGF (blue), and half would extend axons up a gradient from a concentration of roughly 0.2 nM NGF toward a concentration of roughly 0.3 nM NGF (green). **B**, Representative SCG explants cultured in a 3-D collagen gel in the presence of a 0.3% NGF gradient stained for β -3 tubulin. Gradient direction indicated on the left in red. Dashed yellow line = region of approximately 0.3 nM NGF. Scale bar = 500 μ m. **C**, Measured outgrowth for the two groups of neurites labeled in (A). Group 2 has significantly higher outgrowth than group 1 despite being at a slightly lower NGF concentration. This eliminates a trophic effect explanation for the guidance of these explants and suggests that the neurites may be responding to the gradient by growth rate modulation. $n = 97$ – 98 per group, averaged over three separate experiments. Error bars = SEM. * $p < 0.05$, Kolmogorov–Smirnov test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

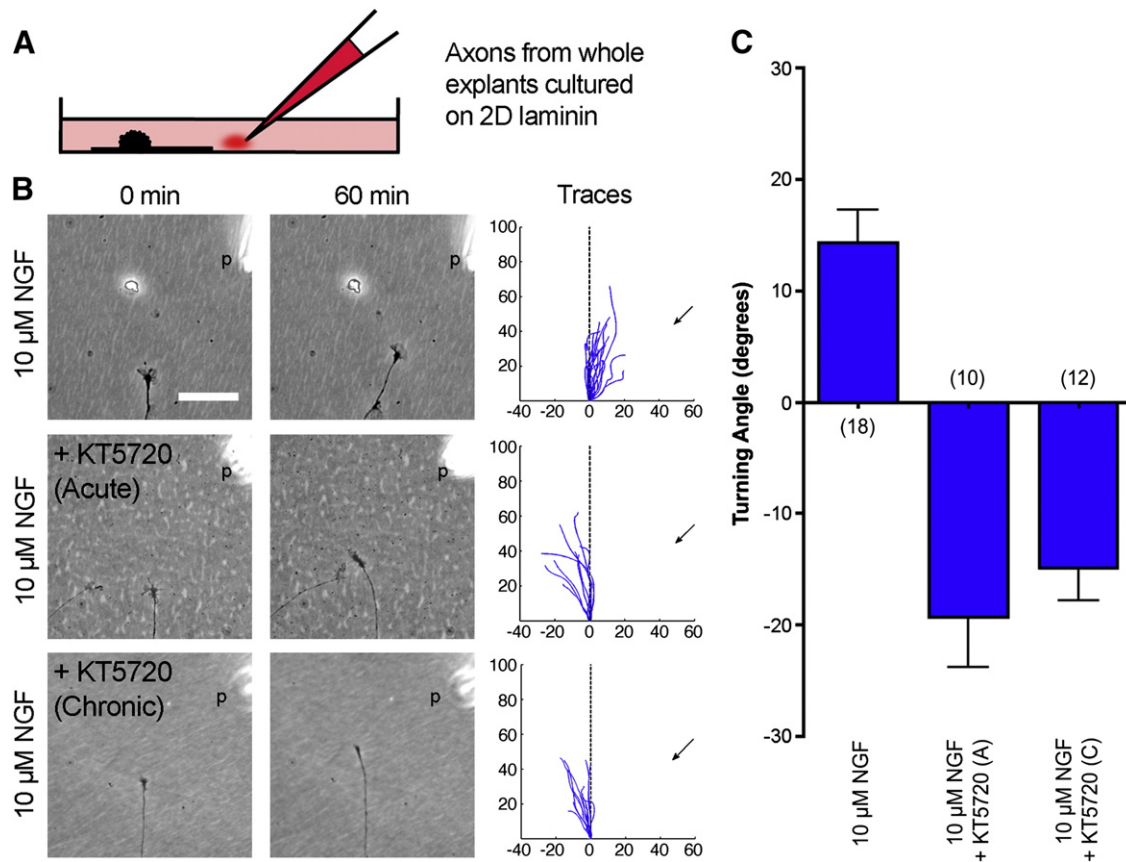


Fig. 4. Acute and chronic PKA inhibition switches the turning response of growth cones from SCG explants in steep gradients. A, The standard growth cone turning assay was modified to culture explants of SCG tissue in liquid media. Individual axons originating from the explant were assayed as per standard growth cone turning assays for dissociated cells. B, Example images and traces of growth cone trajectories from SCG explants in an NGF gradient with and without PKA modulation. KT5720 was bath applied at 80 nM for either 30 min (acute) or overnight (chronic). Scale bar = 40 μm; p: pipette tip, arrow indicates direction of gradient from the pipette, x- and y-axes on traces are distance (μm). C, Summary of the mean turning angles of neurites from SCG explants in the growth cone turning assay in the presence of acute (A) or chronic (C) application of KT5720. Error bars are SEM and numbers in brackets are number of assays per condition.

switching between the growth cone turning assay and the shallow gradient collagen assay.

A 3-dimensional matrix does not prevent the ability to undergo guidance switching

The growth cone turning assay examines the response of axons growing on a 2D substrate, while the collagen assay examines the response in a 3D matrix. We therefore employed a hybrid of the two assays by using a pipette to create a steep gradient in a 3D agarose/collagen environment, which allowed better visualization of live neurite growth than a purely collagen environment (see [Experimental methods](#)) (Fig. 5A). Under these conditions, timelapse imaging demonstrated that KT5720 induced a switch from attraction to repulsion, comparable to that in the 2D substrate condition (Fig. 5B, C). Since this hybrid assay is largely similar to the 3D collagen assay except for gradient steepness, this leaves gradient steepness as the determining factor controlling whether or not attraction will be converted to repulsion by varying cyclic nucleotide levels.

Discussion

Axon guidance by gradients is important both for correct development of the nervous system, and potentially for aiding repair after nervous system disease or injury (Yaron and Zheng, 2007). Examination of growth cone turning mechanisms under acute, steep gradient conditions *in vitro* has revealed that cyclic nucleotide levels within the cell control a switch in guidance responses between attraction and

repulsion (Ming et al., 1997; Song et al., 1997, 1998). It is also known that a developmentally regulated decline in basal cAMP levels between embryonic and postnatal CNS tissue is accompanied by a fall in regenerative capacity (Cai et al., 2001). Thus, several experiments have manipulated cyclic nucleotide signaling in regenerating axons to change the way they respond to inhibitory guidance cues to overcome injury (Neumann et al., 2002; Qiu et al., 2002; Lu et al., 2004). However, environmental and gradient conditions can vary considerably depending on the type, location and extent of neuronal damage. Thus, we investigated the interaction of gradient parameters with cyclic nucleotide levels in determining axonal response to gradients.

First, we found that the turning response of SCG growth cones in a steep NGF gradient was switched from attraction to repulsion by application of either the PKA inhibitors KT5720 and Rp-8-CPT-cAMPs, or the PKG activator 8-Br-cGMP. This result is consistent with the previous understanding of the guidance switching mechanism, which relied upon *Xenopus* spinal neurons as a model (Nishiyama et al., 2003). However, more recently Murray et al. (2009) used high specificity cAMP analogs in rat DRG axons to show that the attractive guidance response previously attributed to the actions of PKA may actually be due to the guanine nucleotide exchange factor Epac (exchange protein activated by cAMP). The authors also suggested that the switch to repulsion previously generated by KT5720 application might be due to non-specific inhibition of proteins other than PKA. While the precise downstream effectors of guidance switching remain unclear, both paradigms still propose that high relative cAMP activity leads to attraction and the opposite leads to repulsion, which is in agreement with our current data.

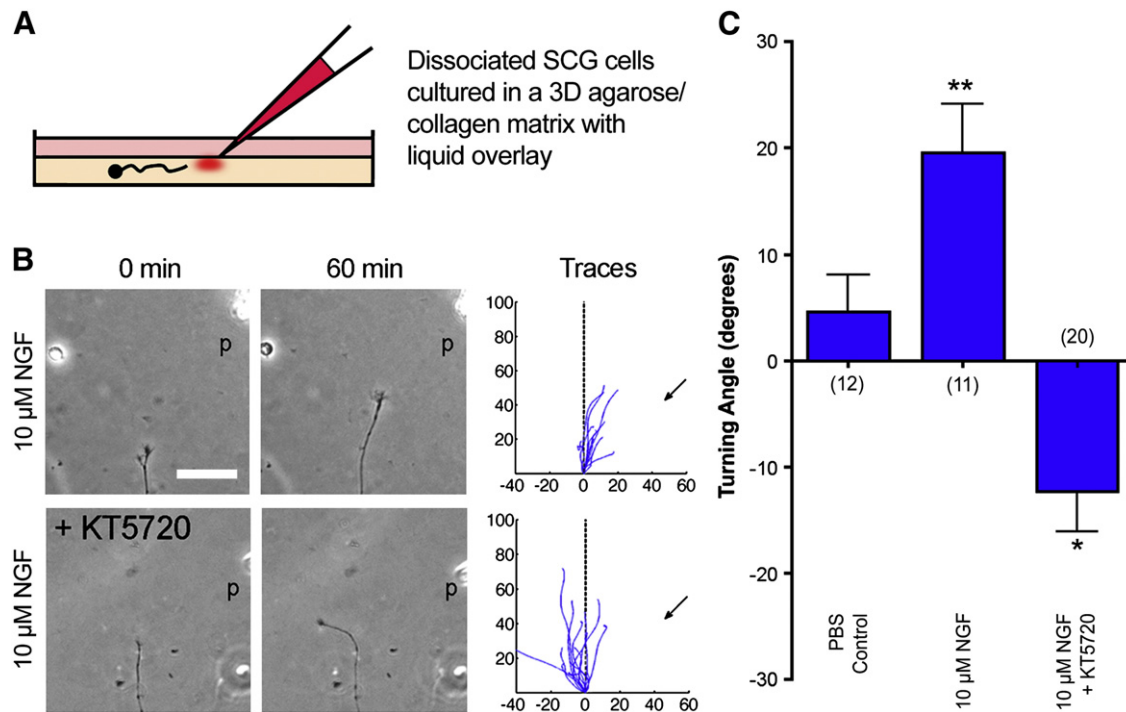


Fig. 5. A 3-dimensional environment does not prevent the ability of SCG axons to switch guidance response in steep gradients. **A**, The standard growth cone turning assay was modified to culture dissociated SCG cells in a thin layer of agarose/collagen gel overlaid with liquid media. The pipette was positioned with the tip on the surface of the gel and only axons to a depth of 50 μm from the surface were assayed. This arrangement was optimal for reproducibly generating gradients similar to the standard 2D assay as visualized by fluorescently labeled dextran. **B**, Example images and traces of growth cone trajectories from SCG axons growing through a 3-D agarose/collagen matrix in an NGF gradient with and without PKA modulation (KT5720 was bath applied to the liquid overlay at 80 nM). Scale bar = 40 μm; p: pipette tip, arrow indicates direction of gradient from the pipette, x- and y-axes on traces are distance (μm). **C**, Summary of the mean turning angles of neurites from SCG explants in the 3-D growth cone turning assay in the absence or presence of KT5720. Error bars are SEM and numbers in brackets are number of assays per condition. * $p < 0.05$, ** $p < 0.01$, Kolmogorov–Smirnov test compared to control.

Previous attempts to investigate cyclic nucleotide-dependent switching in a biologically more realistic, 3-dimensional *in vitro* assay revealed that the situation was more complicated than first thought. Moore and Kennedy (2006) discovered that embryonic commissural spinal axons exposed to a gradient of netrin-1 were not switched from attraction to repulsion by application of KT5720 in their cultures. Our present results may help to explain the differences between this observation and data from previous growth cone turning assays (Ming et al., 1997; Song et al., 1997, 1998). For the first time, we have directly compared the switching response of the same axons under both steep gradients in 2D and 3D environments, and shallow gradients in a 3D environment.

Consistent with the observations of Moore and Kennedy (2006), we found that no switching of the guidance response of mammalian axons occurred under shallow gradient conditions. Similarly, we also found that cyclic nucleotide-dependent signaling altered the sensitivity of the guidance response in our shallow gradient assays. While guidance can be achieved in shallow gradients without biased growth cone turning (Mortimer et al., 2010), guidance switching has previously only been shown in growth cones turning in the presence of steep gradients. Although we cannot rule out other factors in the embryonic spinal cord cultures of Moore and Kennedy (2006) affecting the guidance response, we suggest that the lack of observed guidance switching was a result of these axons predominantly responding by growth rate modulation to shallow gradients.

To control for the different timescales of our two steep gradient assays versus our shallow gradient assay is fundamentally impractical. The response of axons to a 0.3% gradient for only one hour would be immeasurably small given the inherently large variability in axon growth rates. Conversely for a long-term assay with 10% gradient steepness, SCG axons would enter biologically unrealistic concentrations within just a few hours. However, any time-dependent difference in response with altered cyclic nucleotide levels would be revealed in the trajectories of axons in our long-term assay, and we saw no

indication of this (Fig. 2E). We have controlled for differences in the chronic application of drug, dissociation of the tissue, and a 2- versus 3-dimensional environment and found that none of these issues affects the ability of SCG growth cones to undergo guidance switching by KT5720 application (Figs. 4, 5). We therefore conclude that the different responses must be attributed to the differences in gradient steepness between the growth cone turning assay and the explant printing assay.

We recently argued that axons guided by shallow gradients respond by altering their rate of growth, while those guided by steep gradients respond by biased growth cone turning (Mortimer et al., 2010). While cAMP is a potent second messenger that has been shown to be involved in the acute turning response of axons to steep gradients, we have shown here that it does not play the same role in the guidance of axons by growth rate modulation in shallow gradients. Growth rate modulation is therefore not subject to switching by cyclic nucleotide modulation. While previous work has noted there may be different strategies used by growth cones under different gradient conditions (Isbister et al., 2003; Mortimer et al., 2010), this is the first time a molecular mechanism unique to guidance in steep gradients but not shallow gradients has been demonstrated. This suggests that axonal responses to gradients and related modulatory cues could vary significantly as they travel through regions of varying gradient steepness and concentration. Understanding more about such potential differences is crucial for deciphering the many complex guidance events that occur during development and regeneration.

Experimental methods

Cell culture for steep gradient assays

SCGs were isolated by microdissection from P1 to P3 Wistar rat pups as per Higgins et al. (1991). For the studies involving dissociated

cells, 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 (2.5 S mouse NGF; GroPep, Adelaide, Australia) and incubated overnight at 37 °C on 35 mm Petri dishes. For the assays of growth cones of SCG explants, the whole SCGs were transferred to Petri dishes containing Opti-MEM with 10 µg/ml laminin and 0.5 nM NGF. The SCGs were then cut into about 6 equal size pieces, approximately 500 µm in diameter, and incubated overnight at 37 °C.

Production of steep gradients on a 2-dimensional substrate

30 min prior to the onset of the assays, the growth medium was exchanged for a prewarmed assay medium (50% Opti-MEM and 50% RPMI supplemented with 0.5 mM L-glutamine (BioWhittaker Inc., Walkersville, MD), KT5720 (Alexis Biochemicals, San Diego, CA), Rp-8-CPT-cAMPs and 8-Br-cGMP (BioLog, Bremen, Germany) were added to the prewarmed assay medium when appropriate. The growth cone turning assays were carried out at 37 °C on a Fryer 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 reported previously by Lohof et al. (1992) (see also Pujic et al., 2008). 70 kDa dextran labeled with fluorescent tetramethylrhodamine (Molecular Probes Inc., Melbourne, Australia) was added to the pipette solution to monitor the chemical gradient produced.

Production of steep gradients in a 3D substrate

SCG cells were dissociated as above and resuspended in a mixture of Opti-MEM with agarose with (1.5% w/v), collagen (90 µg/ml), laminin (20 µg/ml), and NGF (0.5 nM). 100 µl of this was plated onto glass-bottom culture dishes and allowed to set into a 3-dimensional gel at 4 °C. A liquid overlay containing Opti-MEM and 0.5 nM NGF was then added and the cells were incubated overnight at 37 °C. Axons growing straight for greater than 50 µm roughly parallel to the bottom of the plate, between 15 and 50 µm below the surface of the gel, were chosen for 3D growth cone turning assays. The pipette was then positioned with the tip resting on the surface of the gel for the duration of the assay. Where applicable, KT5720 was bath-applied to the liquid overlay 30 min prior to gradient application. Movies were analyzed in 2D as described above.

Measurement of neurite extension and growth cone turning in steep gradient assays

Images of the growing axon were taken using the 20× objective of a Nikon Eclipse TE200 inverted microscope (Nikon Corporation, Tokyo, Japan) at 60 s intervals for 1 h using a Q-Imaging camera and Q-Capture Pro software (Quantitative Imaging Inc., Surrey, Canada). The trace of each axon, its turning angle and distance of neurite outgrowth were calculated using Matlab. The pipette location, initial direction of growth and center of the growth cone were manually identified in the first frame of the movie, after which the center of the growth cone was manually located in each subsequent frame. The turning angle was defined as the angle between the original direction of growth and the average position of the growth cone in the last 5 frames in the trace. The cumulative distributions of turning angles for each condition were statistically compared using a nonparametric test of significance, the two-sample Kolmogorov–Smirnov (KS) test. All statistical analyses were two-tailed. Only growth cones with more

than 15 µm of net growth over the period of the assay were included in the analysis.

Production of shallow gradients in a 3D substrate

SCG explants were cut into thirds and six of these were embedded in a 35 mm Petri dish in a gel containing Opti-MEM and 0.2% collagen I (w/v) (BD Bioscience, Sydney, Australia). A gradient of NGF of steepness 0.3% change in concentration per 10 µm was generated with 0.3 nM NGF at the center of the explants using the printing assay of Mortimer et al. (2009). Briefly, a GeSim nanoplotter was used to print 12 lines of NGF 1 mm apart in decreasing concentrations perpendicular to the line of explants. A smooth gradient is established by diffusion within 1 h and is relatively stable for up to 48 h (Rosoff et al., 2004).

Analysis of neurite growth from explants in shallow gradients

After a 48 hour growth at 37 °C, tissue was fixed and neurites were stained for neuronal β3-tubulin. Explants were photographed with an AxioCam HRm (Zeiss) camera on a Zeiss Imager Z1 fluorescence microscope. The response to the NGF gradient was assessed using the “Guidance Ratio”, given by the number of neurite pixels on the high side of the gradient relative to the center of the explant, minus the number on the low side, divided by the total number of neurite pixels. Outgrowth was measured by the ratio of the total number of neurite pixels to the number of explant pixels (for further details see Rosoff et al., 2004; Mortimer et al., 2009). A slight negative offset consistent over the plateau experiments was subtracted from the measured guidance ratio in the gradient conditions. Where appropriate, the drugs were added to the collagen solution prior to gelling.

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