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

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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.

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).

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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 ± 4.7°) compared to the control condition (mean turning angle = 0.02 ± 2.7°; $p = 0.02$, 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 ± 5.7°, 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 ± 4.1° and −14.24 ± 3.8° 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 (GR = 0.14 ± 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