The Interdependent Roles of Ca^{2+} and cAMP in Axon Guidance

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ABSTRACT: **Axon guidance is a fundamental process in the developing and regenerating nervous system that is necessary for accurate neuronal wiring and proper brain function. Two of the most important second messengers in axon guidance are Ca^{2+} and cAMP. Recently experimental and theoretical studies have uncovered a Ca^{2+}- and cAMP-dependent mechanism for switching between attraction and repulsion. Here, we review this process and related Ca^{2+} and cAMP interactions, the mechanisms by which necessary intracellular calcium elevations are created, and the pathways, which effect attractive and repulsive responses to the switch.** © 2013 Wiley Periodicals, Inc. Develop Neurobiol 75: 402–410, 2015

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INTRODUCTION

The development of appropriate neural wiring during development is critical for normal brain function (Kalil et al., 2011), and incorrect wiring has been linked to a number of severe neurological conditions (Stoeckli, 2012). In addition to the context of normal development, understanding the mechanisms underlying axon guidance is also critical for understanding how to optimize axonal regeneration and recovery following injury to the nervous system (Horner and Gage, 2000).

While a large number of second messenger signaling molecules are known to be important for axon guidance (see for instance Akiyama and Kamiguchi in this issue), two of the most crucial are cyclic adenosine monophosphate (cAMP) and Ca^{2+}. These have a wide range of physiological functions (Berridge et al., 2003; Hofer, 2012) and interactions (Borodinsky and Spitzer, 2006), but in particular cAMP and Ca^{2+} have interdependent and significant effects on growth cone turning and axon guidance (Gorbunova and Spitzer, 2002; Wen et al., 2004; Nicol et al., 2011). Here, we review what has been discovered in the last few years about the mechanisms involved.

THE FOUNDATIONS OF Ca^{2+} AND cAMP MEDIATED GROWTH CONE TURNING

cAMP activity was first implicated in axon guidance via experiments where an extracellular gradient of dB-cAMP, a membrane permeable cAMP analogue, induced attraction while the same gradient of native cAMP had no effect on growth cone turning (Lohof et al., 1992). Attraction was also induced by extracellular gradients of isobutylmethylxanthine, used to inhibit degradation of intracellular cAMP by phosphodiesterases, and forskolin, used to increase cAMP production through adenylate cyclase activation (Lohof et al., 1992). Together, these results indicated that cAMP is likely to be a second messenger in axon guidance, acting via a spatial gradient inside the growth cone.

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Surprisingly however, bath (i.e., spatially uniform) application of cAMP analogues was also found to have an effect on growth cone turning induced by acetylcholine (ACh), brain-derived neurotrophic factor (BDNF), netrin-1, and myelin associated glycoprotein (MAG; Ming et al., 1997; Song et al., 1997, 1998; Han et al., 2007). In particular, Rp-cAMPS, a membrane permeable competitive and antagonistic cAMP analogue, or KT5720, which inhibits cAMP-dependent protein kinase (PKA), converted attraction by BDNF, ACh and netrin-1 to repulsion (Ming et al., 1997; Song et al., 1997; Han et al., 2007). In contrast, Sp-cAMPS, a membrane permeable agonistic analogue of cAMP, slightly enhanced attraction (Song et al., 1997). Therefore activation of PKA by cAMP is crucial for growth cone attraction. Bath application of Sp-cAMPS converted repulsion by MAG to attraction (Song et al., 1998; Han et al., 2007), while application of Rp-cAMPS had no effect on repulsion (Henley et al., 2004). It is thus clear that cAMP levels/PKA activity is crucial for determining the polarity of growth cone turning.

An important role for Ca^{2+} began emerging when it was found that growth cone turning induced by neurotransmitters was abolished in calcium free medium, and attraction via ACh induced an increase in cytosolic calcium (Zheng et al., 1994). Subsequent studies confirmed that both attraction and repulsion by a variety of ligands are abolished by low extracellular calcium (Zheng et al., 1996; Ming et al., 1997; Song et al., 1997; Hong et al., 2000). Intracellular calcium was further implicated when inhibition of a known effector of calcium pathways, Ca^{2+}-calmodulin dependent protein kinase II (CaMKII), similarly abolished the ACh induced attraction (Zheng et al., 1994). Gomez et al. (1995) found that removal of extracellular Ca^{2+} also blocked intracellular Ca^{2+} transients, which influence growth cone migration. General Ca^{2+} channel blockers disrupted intracellular transients while blocking voltage-gated Ca^{2+} channels had no effect on transients (Gomez et al., 1995). The frequency of Ca^{2+} transients was found to control growth cone extension and pathfinding *in vivo*, where high frequencies of transients slowed growth, stalled growth cones or even caused retraction (Gomez and Spitzer, 1999). Thus, removal of extracellular Ca^{2+} may abolish guidance by blocking intracellular Ca^{2+} transients.

Using netrin-1-induced attraction, Hong et al. (2000) further developed understanding of the influence of Ca^{2+} on growth cone turning. Pre-incubation of neurons with benzyhydroquinone or thapsigargin to deplete intracellular calcium stores resulted in loss of turning, as did blockade of plasma membrane Ca^{2+} channels with Cd^{2+} (Hong et al., 2000). Hence both intracellular calcium stores and influx of calcium from extracellular sources are required for turning. However, specific blockade of L-type Ca^{2+}-channels with nimodipine, or bath application of ryanodine, an inhibitor of Ca^{2+}-induced Ca^{2+} release (CICR), switched netrin-1 induced attraction to repulsion. In the presence of a (low concentration) ryanodine gradient both attraction and repulsion were observed depending on the gradient steepness (Hong et al., 2000). Through step changes in the substrate and release of caged Ca^{2+}, Gomez et al. (2001) demonstrated that evenly distributed filopodial Ca^{2+} transients slow axonal outgrowth while asymmetric filopodial Ca^{2+} transients induce turning away from the site of increased filopodial transients. Combined, these results argue that intracellular Ca^{2+} patterns control turning (Hong et al., 2000; Gomez et al., 2001).

To further analyze, local Ca^{2+}, Zheng (2000) used focal laser-induced photolysis (FLIP) to produce precisely localized increases in Ca^{2+} concentration by releasing caged Ca^{2+} with a laser. Using NP-EGTA, a caged Ca^{2+} compound, Zheng showed that in a medium containing 1 m*M* Ca^{2+}, focal Ca^{2+} release resulted in significant attractive turning (turning towards the side of Ca^{2+} release), while in a Ca^{2+}-free medium the same focal Ca^{2+} release resulted in repulsion (Zheng, 2000). The turning responses were such that growth cones could be led, by laser, in a zigzag pattern of movement (Zheng, 2000).

Using FLIP of caged Ca^{2+}, much of the Ca^{2+}-mediated turning mechanism was discovered (Wen et al., 2004). With the same basic experimental design as Zheng (2000), Wen et al. (2004) applied specific inhibitors to a number of known downstream effectors of calcium to determine their effect. When using a (relatively) large focal elevation of Ca^{2+} (an attractive stimulus), CaMKII inhibition with specific inhibitors KN93 and myristoylated-autocamtide-2-related inhibitory peptide abolished attraction (Wen et al., 2004). Dual inhibition of PKA and CaMKII switched attraction to repulsion (Wen et al., 2004), suggesting that PKA and CaMKII both have important roles in Ca^{2+}-mediated attraction.

FLIP of the same concentration of caged Ca^{2+} in Ca^{2+}-free medium and FLIP of a low concentration of caged Ca^{2+} in standard 1 m*M* Ca^{2+} medium both resulted in growth cone repulsion (Wen et al., 2004). Inhibition of calcineurin (CaN), a well known CaM-dependent phosphatase, or activation of PKA by Sp-cAMPS switched repulsion to attraction in the Ca^{2+}-free medium and abolished repulsion induced by a small focal intracellular Ca^{2+} increase (Wen et al.,

2004). Similar results were found by inhibiting phosphatase-1 (PP1) (Wen et al., 2004), which is regulated by CaN (Ceulemans and Bollen, 2004) and inhibits CaMKII (Blitzer et al., 1998; Colbran, 2004), but not by inhibiting phosphatase-2A (PP2A), also regulated by CaN (Wen et al., 2004). CaN and PP1 inhibition were also found to switch netrin-1 induced repulsion to attraction, an effect, which was abolished by simultaneously inhibiting CaMKII (Wen et al., 2004). Thus, CaN and PP1, but not PP2A, are important for Ca^{2+}-dependent repulsion.

CaN, PKA, and PP1 are linked by inhibitor-1 (I1) (Han et al., 2007), which is an inhibitor of PP1 when phosphorylated by PKA, but is inactive when dephosphorylated by CaN (Ceulemans and Bollen, 2004). Inhibition of I1 thus abolished both attraction induced by an intracellular cAMP gradient and repulsion induced by MAG (Han et al., 2007). It is thus clear that cAMP activates I1 (through PKA), which, when active, inhibits PP1, to switch Ca^{2+}-mediated repulsion to attraction. Conversely, inhibition of PKA (through KT5720 or Rp-cAMPS) or activation of CaN causes inhibition of I1, and allows PP1 to inhibit CaMKII, switching Ca^{2+}-mediated attraction to repulsion. This pathway is summarized in Figure 1.

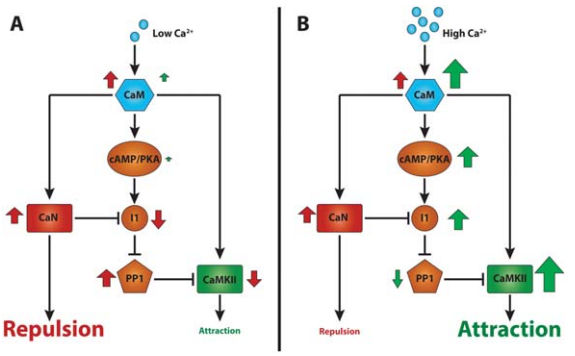

Figure 1 The molecular switch proposed to locally determine attraction versus repulsion. A: When a low Ca^{2+} elevation occurs, CaN is much more responsive to Ca^{2+}/CaM than CaMKII, and little PKA involvement is observed. Hence CaN inhibits I1 and PP1 inhibits CaMKII, making a net repulsive result. B: When a high Ca^{2+} elevation occurs (over the bistability threshold of CaMKII), CaMKII is activated by Ca^{2+}/CaM much more than CaN. Further, cAMP and PKA are more activated, hence I1 inhibits PP1 and the net response in attractive. However, CaN activity in this scenario is no less than CaN activity resulting from a low Ca^{2+} elevation; the critical difference is that CaMKII is activated much more, promoting attraction. Arrows show activity, with red arrows indicating a change, which favors repulsion and green arrows indicating a change which favors attraction. Abbreviations: CaM: calmodulin; CaMKII: Ca2+-calmodulin dependent protein kinase II; CaN: calcineurin; I1: inhibitor 1; PKA: protein kinase A; PP1: protein phosphatase 1.

A UNIFYING MODEL

Many of the above findings were recently brought together by a unifying mathematical model (Forbes et al., 2012). As discussed by Wen et al. (2004), the signaling network these authors proposed to underlie the switch between attractive and repulsive responses to chemotactic gradients (Fig. 1) is similar to that suggested to underlie the switch between long-term potentiation (LTP) and long-term depression (LTD) in synaptic plasticity (Lisman and Zhabotinsky, 2001; Malleret et al., 2001; Morishita et al., 2001). Graupner and Brunel (2007) proposed a mathematical model of synaptic plasticity mediated by this signaling network, providing a starting point for Forbes et al. (2012) to develop a similar model in the context of understanding switching of guidance responses. The mathematical model consists of a set of ordinary differential equations quantitatively describing the interactions between the different components of the signaling network, including the binding of calcium to calmodulin, and the autophosphorylation and dephosphorylation of CaMKII. Expressing these interactions quantitatively allows much more precise statements than can be provided by purely qualitative reasoning for how levels of one component affect levels of other components. The key input to the network is Ca^{2+} concentration, and the key output is the resulting ratio of CaMKII to CaN levels. Although for synaptic plasticity the precise temporal dynamics of this network are relevant, the timescale over which growth cones make guidance decisions is much longer, such that it is only the final output of the network that is important in this case.

A critical difference between the LTP/LTD and guidance response cases is that the former does not involve a spatial component: one is only interested in the resulting CaMKII/CaN ratio. However, determining a guidance direction involves generating differences in signaling component levels across space. The simplest way to introduce a spatial difference, adopted by Forbes et al. (2012), is to discretize space into just two positions, which can be referred to as the up-gradient and down-gradient sides of the growth cone [Fig. 2(A)]. The mathematical model can then be run separately at these two positions, based on different input levels of Ca^{2+} produced by the external gradient. The difference in these levels is assumed to be generated by differential activation of calcium channels in the membrane, and/or

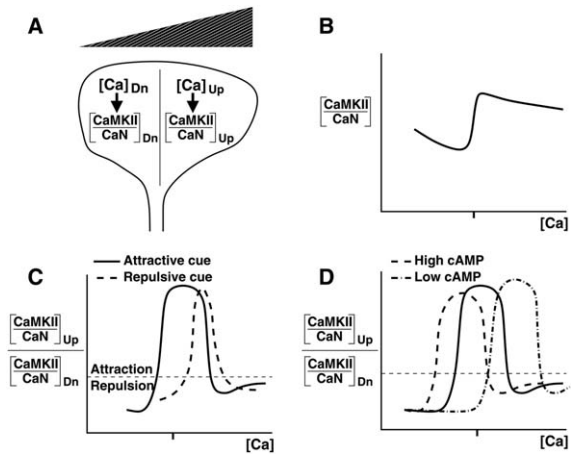

Figure 2 A mathematical model explains how calcium and cAMP interact to determine guidance decisions (Forbes et al., 2012). A: An external ligand gradient causes different levels of calcium on the up and down (Dn) sides of a growth cone. Via the signaling network shown in Figure 1 this leads to a different ratio of CaMKII to CaN levels on the two sides. B: CaMKII/CaN ratio as a function of background calcium concentration [shown schematically; for actual model output see (Forbes et al., 2012)]. For low or high background levels of calcium the CaMKII/CaN ratio on the up-gradient side of the growth cone is lower than on the down-gradient side, causing repulsion. However at normal values (indicated by the tick mark on the calcium axis) attraction occurs, where the change in calcium concentration between the two compartments spans the jump in the curve. C: The ratio of CaMKII/CaN ratios between the two sides of the growth cone. The solid curve is for a normally attractive factor, that is, assuming a steep internal calcium gradient, while the dashed curve is for a normally repulsive factor, that is, assuming a shallow internal calcium gradient. D: Changing cAMP levels shifts the curve for an attractive cue along the calcium axis, so that background calcium levels that previously caused attraction can now cause repulsion, and vice versa, depending on cAMP level. Similar shifts occur in the repulsive case.

differential release of calcium from intracellular stores. However, ultimately the source of the calcium elevation is not used directly by the model, only the spatial location and magnitude of the elevation. For the model, Forbes et al. (2012) assumed that a normally attractive gradient produces a 30% increase in Ca^{2+} concentration in the up-gradient side compared to the down-gradient side, while a normally repulsive factor produces a 10% increase. The key hypothesis is that the growth cone then turns towards the side with the larger CaMKII/CaN ratio. It is thus the ratio of CaMKII/CaN ratios between the two sides of the growth cone that is the critical factor driving the guidance decision.

Figure 2(B) shows schematically the change in the CaMKII/CaN ratio produced by the model as a function of input calcium concentration. Due to the higher affinity of CaN than CaMKII for calcium, small increases in calcium lead to activation of CaN without significant increases of CaMKII, and a reduction in the CaMKII/CaN ratio (Forbes et al., 2012). However at a certain Ca^{2+} concentration, due to bistability of CaMKII activation (Zhabotinsky, 2000), CaMKII rapidly increases to its maximum level, and the CaMKII/CaN ratio jumps suddenly. Beyond this point the CaMKII level is saturated but the CaN level continues to increase steadily with calcium concentration, and the CaMKII/CaN ratio then decreases again. In this picture the growth cone can be thought of as spanning a small distance along the calcium axis, corresponding to the calcium concentrations in its up- and down-gradient compartments. If this span straddles the calcium concentration at which the curve rapidly increases then the growth cone is attracted. At all other positions along the calcium axis (when a gradient is present) the growth cone is repelled since elsewhere the curve is always decreasing, corresponding to a lower CaMKII/CaN ratio in the up-gradient compared to down-gradient compartment. The position on the calcium axis at which the rapid increase occurs can be adjusted by altering the parameters of the model (Forbes et al., 2012), to appropriately match the varying resting calcium concentrations inside different types of neurons (Henley and Poo, 2004). Forbes et al. (2012) also considered the random noise due to the stochastic nature of receptor binding, and showed that this could lead to some random variability in the outcome, including no turning.

An alternative way of viewing the same information is shown in Figure 2(C), which plots the ratio of CaMKII/CaN ratios between the up- and down-gradient compartments as a function of Ca^{2+} concentration (Forbes et al., 2012). Effectively this is the ratios of all positions on the curve in Figure 2(B), which are separated by a 30% difference in calcium concentration. This picture explains why there is only a limited range of calcium levels for which attraction occurs. The effect on the output of the model of changing overall levels of cAMP is shown in Figure 2(D). Reducing cAMP shifts the curve in the direction of increasing calcium, while increasing cAMP shifts the curve in the opposite direction. This reveals why reducing cAMP switches attraction to repulsion, and why attraction is lost for large increases in cAMP. Furthermore it explains the surprising result that at high calcium levels, which normally block attraction, reducing cAMP can restore attraction.

Thus, this model provides a way of integrating many disparate findings regarding the effect of calcium and cAMP levels on whether axons are attracted or repelled by gradients, and shows that calcium and cAMP levels interact in a nonlinear way to determine the guidance response.

The model proposed by Forbes et al. (2012) made a number of novel predictions, which they confirmed experimentally. These include that while small increases in cAMP or Ca^{2+} maintain attraction large increases do not, and that attraction at high Ca^{2+} levels can be recovered by lowering cAMP activity. The latter is a particularly surprising result, since inhibition of PKA or cAMP had previously been ubiquitously observed to reduce attraction.

PRODUCING Ca^{2+} ELEVATIONS

A critical step in Ca^{2+}-mediated growth cone turning is intracellular Ca^{2+} elevation (Wen et al., 2004; Han et al., 2007; Forbes et al., 2012). It is thus important to understand the mechanisms that induce Ca^{2+} elevation (summarized in Fig. 3). One of these mechanisms involves voltage operated Ca^{2+} channels (VOCCs; Hong et al., 2000; Gomez and Zheng, 2006), in particular L-type Ca^{2+} channels (LCCs; Hong et al., 2000; Nishiyama et al., 2003). LCCs are activated by cAMP, and inhibited by cGMP, so Ca^{2+} influx through these channels is determined by the cAMP/cGMP ratio (Nishiyama et al., 2003). However, VOCCs are not responsible for the majority of Ca^{2+} influx in axon guidance; other mechanisms include release of intracellular Ca^{2+} stores, and store operated Ca^{2+} entry (SOCE) from extracellular sources (Gomez et al., 1995, Li et al., 2005, Gomez and Zheng, 2006, Gasperini et al., 2009).

One of the key ways in which cytoplasmic Ca^{2+} increases are induced is through release of intracellular Ca^{2+} stores in a phospholipase C (PLC) dependent manner. Attractive chemical gradients of netrin-1, BDNF and NGF have been shown to activate PLCγ (Li et al., 2005; Xie et al., 2006; Mizoguchi et al., 2009). PLCγ is responsible for hydrolyzing phospholipids into inositol-1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG) (Li et al., 2005; Gomez and Zheng, 2006). $InsP_3$ binds selectively into $InsP_3$ receptors ($InsP_3R$) in the endoplasmic reticulum (ER) to cause the release of ER Ca^{2+} stores. In addition to the $InsP_3$-induced Ca^{2+} release (IICR) of Ca^{2+} stores, Ca^{2+}-induced Ca^{2+} release (CICR) of internal stores is activated by an initial release of Ca^{2+} binding to ryanodine receptors (RyRs) in a positive feedback mechanism (Ooashi et al., 2005; Gomez and Zheng, 2006). However, RyR CICR is dependent on cAMP and PKA activation of RyRs, as application of Rp-cAMPS or KT5720 attenuated the CICR (Ooashi et al., 2005). A crucial molecule in Ca^{2+} store release is Homer1, a scaffold protein that is known to interact with both RyRs and $InsP_3$Rs (Gasperini et al., 2009). Indeed, Homer1 knockdown removed cytoplasmic Ca^{2+} elevation in response to bath application of BDNF, and switched both BDNF and netrin-1 induced attraction to repulsion (Gasperini et al., 2009). Therefore, Homer1-mediated store release of Ca^{2+} is crucial for growth cone turning.

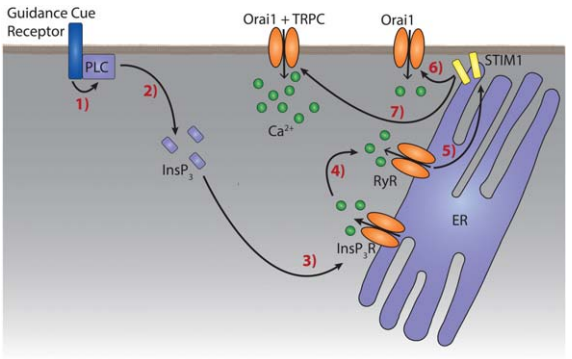

Figure 3 Key mechanisms that produce Ca^{2+} elevations. (1) A guidance cue receptor activates PLC through a receptor specific mechanism. (2) PLC hydrolyzes phospholipids into DAG (not shown) and $InsP_3$. (3) $InsP_3$ activates ER $InsP_3R$ channels to induce IICR of Ca^{2+}. (4) Ca^{2+} from IICR activates RyR and cause CICR. IICR and CICR are potentially mediated by Homer (not shown). (5) Depletion of ER Ca^{2+} stores cause STIM1 to localize to the junction of the plasma membrane and ER. (6) STIM1 mediates the activation of SOC channels, which use Orai1, in the plasma membrane. (7) STIM1 mediates the activation of CRAC channels, which use both Orai1 and TRPC1 in the plasma membrane. Abbreviations: CICR: Ca2+ induced Ca2+ release; CRAC: Ca2+ release activated Ca2+; ER: endoplasmic reticulum; IICR: InsP3 induced Ca2+ release; InsP3: inositol-1,4,5-trisphosphate; InsP3R: InsP3 receptor; PLC: phospholipase C; RyR: ryanodine receptor; SOC: store operated Ca2+; STIM: stromal interaction molecule; TRPC: transient receptor protein canonical.

However, internal Ca^{2+} stores are not the only mechanism for intracellular Ca^{2+} elevation. In non-neuronal cells depletion of ER calcium stores initiates two different SOCE mechanisms, Ca^{2+}-release-activated Ca^{2+} (CRAC) channels and store operated Ca^{2+} (SOC) channels (Salido et al., 2009; Cheng et al., 2011). These mechanisms are also active in the growth cones of neurons and are necessary for correct Ca^{2+}-mediated turning (Gasperini et al., 2009; Mitchell et al., 2012). CRAC channels

are highly specific to Ca^{2+}, while SOC channels are less specific Ca^{2+} permeable cation channels, which are responsible for greater Ca^{2+} influx (Salido et al., 2009; Cheng et al., 2011).

Both CRAC and SOC channels rely heavily on stromal interaction molecule 1 (STIM1), an ER resident protein that can sense Ca^{2+} depletion in the ER (Zhang et al., 2005; Mitchell et al., 2012). Once ER Ca^{2+} stores are depleted STIM1 localizes to ER/plasma membrane junctions (Cheng et al., 2011), where it interacts with transient receptor potential canonical (TRPC) channels and Orai1, two other proteins crucial for SOCE (Mitchell et al., 2012). Inhibition of STIM1 thus reduces Ca^{2+} elevations from BDNF gradients, switching attraction to repulsion (Mitchell et al., 2012). However, STIM1 has also been demonstrated to increase adenylyl cyclase activity and thus increase cAMP levels and PKA activity in non-neuronal cells (Lefkimmiatis et al., 2009). An increase in PKA activity is known to be favorable for attraction (Wen et al., 2004; Han et al., 2007) in most cases (Forbes et al., 2012), suggesting that cAMP production could be another possible mechanism for STIM1 to influence growth cone turning. Regardless, it is clear that STIM1 pathways are crucial in Ca^{2+}-mediated axon guidance.

Orai1, a subunit of plasma membrane channels, has been implicated in both CRAC and SOC channels (Salido et al., 2009; Cheng et al., 2011; Mitchell et al., 2012) and is thus an important component of intracellular Ca^{2+} elevation. In contrast, TRPC channels are thought to be crucial for raising intracellular Ca^{2+} levels through SOC, but not CRAC (Li et al., 2005; Wang and Poo, 2005; Cheng et al., 2011; Mitchell et al., 2012). In *Xenopus* neurons xTRPC1, an analogue of human TRPC1 is known to play a crucial role in turning and guidance. While knockdown of xTRPC1 has been shown to switch attraction to repulsion and abolish repulsion (Shim et al., 2005), it has also been shown to abolish attraction (Wang and Poo, 2005). The different responses may be due to different extracellular Ca^{2+} concentrations or different extents of knockdown. TRPC channels have also been demonstrated as crucial for growth cone turning, where both the cytoplasmic Ca^{2+} elevation and attraction induced by BDNF were abolished by TRPC inhibition (Li et al., 2005). Hence, both Orai1 and TRPC1 are crucial for Ca^{2+}-mediated growth cone turning.

RESPONSE TO THE SWITCH

The signaling mechanisms that lead from the Ca^{2+}- and cAMP- dependent molecular switch to growth cone turning are not yet fully understood. Many recent studies have implicated membrane trafficking in both repulsive and attractive turning (Tojima et al., 2007, 2010; Akiyama and Kamiguchi, 2010; Cotrufo et al., 2011). Utilizing photolysis (similar to FLIP) of caged Ca^{2+}, Tojima et al. (2007) found that attraction to Ca^{2+} signals involved microtubule-dependent, vesicle-associated membrane-protein (VAMP) 2 mediated, exocytosis, which was localized to the side of the growth cone with a larger Ca^{2+} elevation. Inhibition of phosphatidylinositol 3-kinase (PI3K), which catalyzes phosphorylation of phosphatidylinositol 4,5-biphosphate (PIP_2) to form phosphatidylinositol 3,4,5-triphosphate (PIP_3) disrupts Ca^{2+} induced attractive growth cone turning (Akiyama and Kamiguchi, 2010), although it has also been suggested that PI3K (and thus PIP_3) are involved upstream of Ca^{2+} signals in intracellular Ca^{2+} elevation mechanisms (Henle et al., 2011). However, as many other proteins, such as PKA, have dual upstream and downstream functions in this pathway (Bouchard et al., 2004; Bashaw and Klein, 2010) it is possible that PI3K and PIP_3 are active both in generating Ca^{2+} elevations and responding to them. Attractive turning in response to netrin-1 has also been shown to use an exocytotic mechanism involving VAMPs (Cotrufo et al., 2011). This suggests that exocytosis towards the up gradient side of growth cones is involved in response to attractive signals.

In contrast to attractive signals, repulsive Ca^{2+} elevation patterns cause Clathrin-mediated endocytosis on the side of the growth cone with a larger Ca^{2+} elevation (Tojima et al., 2010). It has been suggested that this process could be CaN-dependent (Tojima et al., 2010) and while this has not been demonstrated in axon guidance, CaN has previously been implicated in Clathrin-mediated endocytosis (Lai et al., 1999). Hence, attraction and repulsion may be effected through different mechanisms, rather than the same mechanism with the polarity of the growth cone reversed.

The Rho family of small GTPases, which are regulators of the actin cytoskeleton, are involved in axon guidance in responses to cues from all four major guidance cue families (Bashaw and Klein, 2010). In particular, attractive gradients of netrin-1 have been shown to activate Rac1 and CDC42 (Bashaw and Klein, 2010), while intracellular Ca^{2+} gradients generated by ryanodine also activated Rac1 and CDC42 while inhibiting RhoA (Jin et al., 2005). Uniform overexpression of CDC42 inhibited turning responses (Jin et al., 2005) suggesting that cytoplasmic CDC42 distributions are important for turning. GTPase activity required CaMKII activity (Jin et al., 2005),

suggesting that the Rho GTPases are indeed downstream effectors of the Ca^{2+}/cAMP molecular switch described above.

SOME UNANSWERED QUESTIONS

While the mechanisms underlying growth cone turning in steep gradients have been rigorously studied, mechanisms for axon guidance in shallow gradients are less clear. Mortimer et al. (2010) argued that in shallow gradients (<1% change in concentration across 10 microns) axons are guided by growth rate modulation rather than turning. Consistent with important differences in chemotactic response mechanisms between steep and shallow gradients, Thompson et al. (2011) found that growth cones in shallow gradients did not exhibit the same cAMP-dependent switching of responses to an NGF gradient that was apparent in steep gradients. Similarly, to shallow gradients, it has also been observed that some guidance molecules (e.g., Sema3A) use different, CaMKII/CaN-independent pathways to induce turning (Song et al., 1998; Nishiyama et al., 2003, Tojima et al., 2011). Thus, the Ca^{2+}/cAMP-dependent switch is not involved in all types of axon guidance.

Another largely unanswered question is the role in axon guidance played by the exchange protein activated by cAMP (Epac). While the main effector of cAMP has been thought to be PKA, a recent study has shown that Epac may in fact be responsible for many of the cAMP-mediated effects on axon guidance (Murray and Shewan, 2008; Peace and Shewan, 2011). Gradients of a membrane permeable cAMP analogue that selectively activates Epac and not PKA were sufficient to induce attractive growth cone turning (Murray and Shewan, 2008), similar to previously observed turning in response to intracellular cAMP gradients (Lohof et al., 1992). This suggests that Epac has a significant role in axon guidance and may be responsible for some responses previously attributed to PKA.

Some roles attributed to CaN have also been linked to calpain (Lautermilch and Spitzer, 2000; Robles et al., 2003). Calpain has been demonstrated to slow neurite outgrowth in response to Ca^{2+} signals or local activation. While it has not so far been determined if it is part of the CaN repulsion pathway, calpain could in fact regulate CaN; it is known that CaN acts as a substrate of calpain in some cases (Robles et al., 2003).

CONCLUDING REMARKS

Since the first observations of cAMP and Ca^{2+} involvement in growth cone turning and axon guidance in the 1990s many important discoveries have been made. The mechanism through which growth cones determine whether to exhibit attractive or repulsive responses to guidance cues has been fairly comprehensively described, and an accurate mathematical model, which has made unexpected predictions, has been developed and experimentally verified. However, a number of links are still missing in this pathway, particularly downstream of the cAMP and Ca^{2+} switch. Future work will seek to extend our knowledge in these areas, answer the above questions, and develop an understanding how this axon guidance pathway is integrated with other axon guidance pathways in the dynamic *in vivo* environment.

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