Nerve growth cone guidance mediated by G protein–coupled receptors

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Growing axons navigate by responding to chemical guidance cues. Here we report that growth cones of rat cerebellar axons in culture turned away from a gradient of SDF-1, a chemokine that attracts migrating leukocytes and cerebellar granule cells via a G protein–coupled receptor (GPCR). Similarly, *Xenopus* spinal growth cones turned away from a gradient of baclofen, an agonist of the $GABA_B$ receptor. This response was mediated by G_i and subsequent activation of phospholipase C (PLC), which triggered two pathways: protein kinase C (PKC) led to repulsion, and inositol 1,4,5-triphosphate (IP_3) receptor activation led to attractive turning. Under normal culture conditions, PKC-dependent repulsion dominated, but the repulsion could be converted to attraction by inhibiting PKC or by elevating cytosolic cGMP. Thus, GPCRs can mediate both repulsive and attractive axon guidance *in vitro*, and chemokines may serve as guidance cues for axon pathfinding.

The growth cones of growing axons are guided by attractive and repulsive extracellular chemical signals[1,2]. Several families of guidance factors and their membrane receptors have been identified[3–5], intracellular transduction mechanisms are beginning to be determined[2,6], and there is increasing evidence that axon guidance and neuronal migration share similar signaling mechanisms[2,7]. For example, the Slit and Netrin families of axon guidance proteins also guide neuronal migration during development[7,8]. Although it seems likely that neuronal migration and immune-cell chemotaxis share similar signaling mechanisms[9,10], most chemotactic factors in the immune system signal through seven-transmembrane GPCRs[11,12]—a receptor type that has not, as yet, been implicated in axon guidance. We therefore investigated the possibility that GPCRs mediate axon guidance by extracellular diffusible factors, using an *in vitro* growth cone turning assay.

The chemokine stromal-derived factor 1 (SDF-1), which induces leukocyte chemotaxis[13–15], triggers chemoattraction of cultured cerebellar granule cells[16,17]. The action of SDF-1 is mediated by CXCR4, a specific GPCR[18] that is widely expressed in both immune and nervous systems[9,10,19]. In the developing cerebellum, *in situ* hybridization shows that SDF-1 is expressed in the pia, whereas CXCR4 is expressed in the external granule cell layer (EGL)[16,17]. The localized expression of SDF-1 suggests that it may function in a gradient fashion to affect axon guidance and neuronal migration. Targeted disruption of the gene encoding either SDF-1 or CXCR4 in mice leads to defects in blood cell formation (hematopoiesis) and migration of proliferating cerebellar granule cells[9,10], suggesting that SDF-1 and CXCR4 are involved in neural development. We first asked whether SDF-1 can serve as an axon guidance cue for cultured cerebellar granule cells. To generalize our findings to other GPCRs, we also examined the effects of activating $GABA_B$ receptors in the growth cones of cultured *Xenopus* spinal neurons. Further studies of intracellular signaling mechanisms underlying the growth cone guidance by these two GPCRs led to the identification of PKC as the key mediator of growth cone chemorepulsion. Our results also showed that GPCR-mediated signaling can trigger either chemorepulsion or chemoattraction of the growth cone, depending on the levels of PKC activation and cytosolic cGMP.

Results

Chemorepulsion induced by SDF-1 or baclofen

Microscopic gradients of SDF-1 were applied to isolated cerebellar granule cells by pulsed application of SDF-1 in a standard growth cone turning assay (Methods). We observed marked repulsive turning of growth cones within 30 min, as shown by the neurite trajectory and the angle of growth cone turning (**Fig. 1a**). When PBS or a lower concentration of SDF-1 (1 μg/ml instead of 20 μg/ml) was used in the pipette, growth cones showed no significant turning (**Fig. 1g** and data not shown). The SDF-1 effect was specific because another chemokine, RANTES (20 μg/ml), had no effect on the growth cone direction when applied in a gradient (**Fig. 1g**). Incubation with a specific antibody against CXCR4 (but not normal IgG) or with pertussis toxin (PTX), a specific inhibitor of the G protein G_i, completely abolished the turning response (**Fig. 1b, c** and **g**). Thus, the turning induced by SDF-1 was due to activation of CXCR4, whose presence on these granule cells was confirmed by immunostaining (data not shown). The SDF-1 effect was cell-type specific, as the

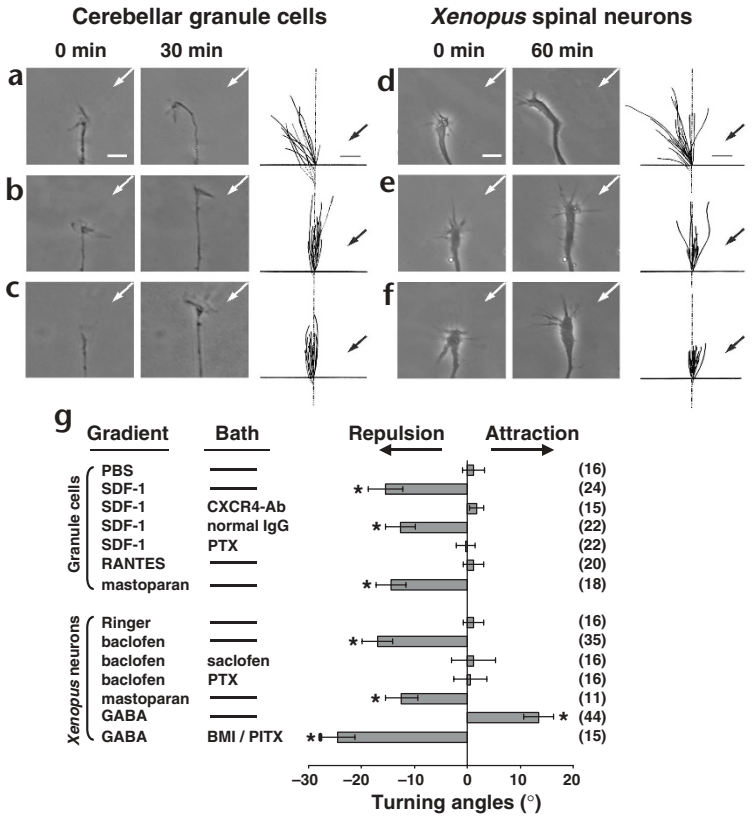

Fig. 1. Chemorepulsion of growth cones induced by a gradient of SDF-1 or baclofen. (**a**) Images of the growth cone of a cerebellar granule cell at the onset (left) and 30 min after onset (right) of exposure to a SDF-1 gradient (arrow, 20 μg/ml in the pipette). Scale bar, 10 μm. (**b**, **c**) The medium also contained antibody against CXCR4 (**b**, 2 μg/ml) or PTX (**c**,1 μg/ml). (**d**) Same as (**a**) except that a cultured *Xenopus* spinal neuron was used and the turning response to a baclofen gradient (20 mM in the pipette) was assayed. (**e**–**f**) The medium also contained saclofen (**e**, 50 μM) or PTX (**f**, 1 μg/ml). Superimposed traces (right, in **a**–**f**) depict samples of the neurite trajectory at the end of the turning assay. The origin is the center of the growth cone at the onset of assay and the original direction of growth was vertical. Dashed traces depict shifted neurite shank during the assay. (**g**) Summary of average turning angles (± s.e.m, number of growth cones in parentheses) for data shown in (**a**–**f**), and for assays with the following: PBS and Ringer solution (Ringer) in the pipette as controls for granule cells and spinal neurons, respectively, using a gradient of RANTES (20 μg/ml in the pipette), a gradient of mastoparan (1 mM in the pipette), a gradient of GABA (10 mM in the pipette) in the absence or presence of bicuculline methiodide (BMI, 10 μM) and picrotoxin (PITX, 50 μM). *P < 0.001 compared to respective control (PBS or Ringer alone).

same SDF-1 gradient had no effect on the growth cone of cultured *Xenopus* spinal neurons. Finally, to determine whether a gradient of G protein activation is sufficient to cause repulsive turning, we applied an extracellular gradient of mastoparan, a membrane-permeable peptide that causes growth cone collapse through activation of PTX-sensitive G proteins[20]. The mastoparan gradient indeed repelled the growth cones of these cerebellar granule cells (**Fig. 1g**).

To examine the generality of GPCR-dependent axon guidance, we tested the effect of activating the $GABA_B$ receptor, another specific GPCR, in *Xenopus* spinal neurons. When we applied a gradient of baclofen, a $GABA_B$ receptor agonist, to *Xenopus* growth cones, there was marked repulsion within 1 hour (**Fig. 1d** and **g**). Moreover, bath-addition of saclofen (a specific $GABA_B$ receptor antagonist) or PTX completely abolished the repulsive effect of baclofen (**Fig. 1e–g**). Notably, in the presence of a gradient of GABA, these *Xenopus* growth cones showed marked chemoattraction (**Fig. 1g**). The attraction was mediated by ionotropic $GABA_A$ receptors, as addition of the specific $GABA_A$ receptor antagonists bicuculline and picrotoxin (**Fig. 1g**) resulted in repulsive turning. Furthermore, the baclofen gradient markedly turned away the growth cones of cultured cerebellar granule cells (data not shown). As seen in cerebellar granule cells, an extracellular gradient of mastoparan induced chemorepulsion of *Xenopus* spinal neuron growth cones (**Fig. 1g**). Together with the SDF-1 results, these findings showed that activation of two specific neuronal GPCRs by two different extracellular factors can trigger repulsive turning of the growth cone.

cGMP converts repulsion to attraction

Knockout studies[9,10] and *in vitro* chemotaxis assays[16,17] suggest that SDF-1 attracts rather than repels migrating granule cells. Previous studies have also shown, however, that the level

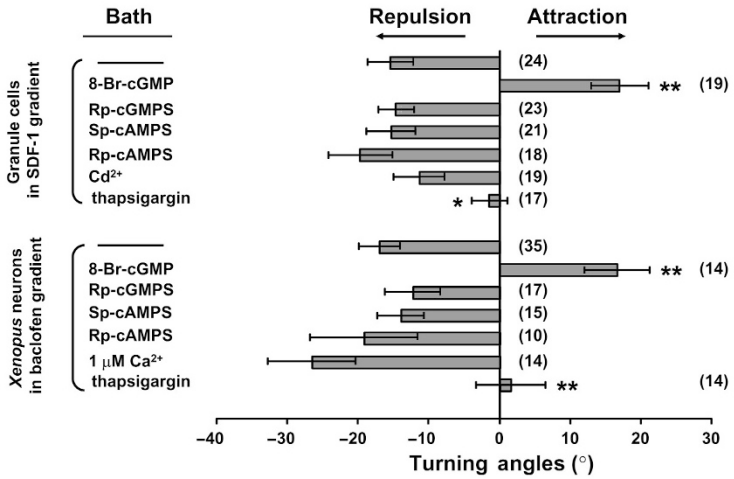

Fig. 2. The modulatory role of cyclic nucleotides. Average turning angles for various experimental conditions. The bath contained normal medium with or without 8-Br-cGMP (100 μM), Rp-cGMPS (10 μM), Sp-cAMPS (20 μM), Rp-cAMPS (20 μM), thapsigargin (1 μM) or Cd^{2+} (50 μM), or the bath contained low Ca^{2+} (1 μM) medium. Numbers in parentheses refer to the total number of growth cones examined. Results that differed significantly from control (exposed to the SDF-1 or baclofen gradient alone) are marked by asterisks (*P < 0.05, **P < 0.001).

of cytosolic cyclic nucleotides is critical in determining growth cone behavior toward many guidance cues[21,22]. Attractive turning can be converted to repulsion and vice versa, depending on the level of either cAMP or cGMP, which often depends on other coincident incoming signals to the neuron[23]. Furthermore, T lymphocytes can either migrate toward or away from SDF-1, depending on SDF-1 concentration[24]. Here we found that in the presence of 8-Br-cGMP, a membrane-permeable cGMP analog, growth cone repulsion induced by the SDF-1 or baclofen gradient was converted to attraction. However, addition of Rp-cGMPS, a non-hydrolyzable analog competitor of cGMP, had no effect (**Fig. 2**). This cGMP-dependent turning behavior resembles that found for group II guidance cues[25], which include semaphorin 3A, neurotrophin 3 and Slit. Rp-cAMPS and Sp-cAMPS (a cAMP analog that activates PKA), which reversed the turning response to group I guidance cues (netrin-1, brain-derived neurotrophic factor (BDNF) and myelin-associated glycoprotein)[25], were all ineffective in modulating the effects of SDF-1 or baclofen (**Fig. 2**).

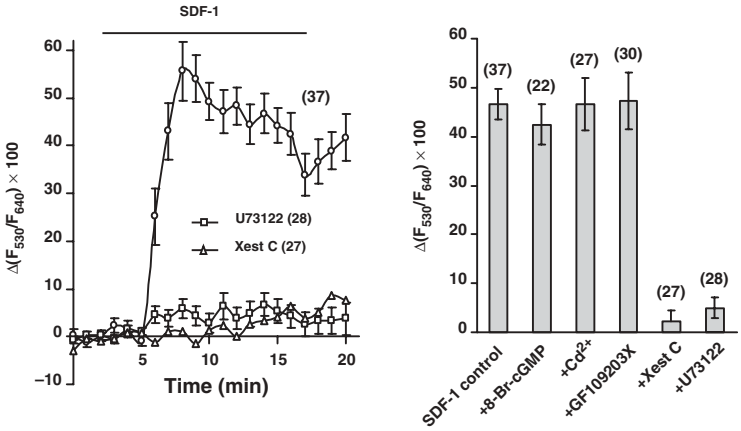

Fig. 3. Ca^{2+} signaling in cerebellar granule neurons triggered by SDF-1. Left, percentage change in the ratio of fluorescence at 530 nm (Fluo-3) and 640 nm (Fura-red) at the soma before and after application of SDF-1 in normal medium and in medium supplemented with U73122 (50 nM) or Xest C (2 μM). Error bars represent mean ± s.e.m. Error bars for Xest C (similar to U73122) were omitted for clarity. Right, summary of the average percentage change (± s.e.m.) in the fluorescence ratio (F_{530}/F_{640}) 5–10 min after SDF-1 application under various experimental conditions: 8-Br-cGMP (100 μM), Cd^{2+} (50 μM), GF109203X (50 nM), Xest C (2 μM) or U73122 (50 nM). Numbers in parentheses refer to the number of cells examined.

The role of Ca^{2+} in SDF-1 signaling

Elevation of cytosolic Ca^{2+} may mediate either attractive or repulsive turning of the growth cone[26,27]. We found that when extracellular Ca^{2+} was reduced from 1 mM to 1 μM, or when Cd^{2+} was added to the medium (conditions that abolish turning of *Xenopus* growth cones in response to group I cues), the repulsion induced by baclofen or SDF-1 was unaffected (**Fig. 2**). The repulsion was completely abolished, however, when the internal Ca^{2+} stores were depleted by pre-incubation of the cells with thapsigargin (**Fig. 2**). Further fluorescence ratio imaging using the Ca^{2+}-sensitive fluorescence dyes Fluo-3 and Fura-red (Methods) showed that cytosolic Ca^{2+} was significantly elevated in the soma of cultured cerebellar granule cells within minutes after local application of SDF-1 (**Fig. 3**). This Ca^{2+} elevation was blocked by U73122, a specific inhibitor of PLC, and by Xestospongin C (Xest C), an inhibitor of IP_3 receptors, but was unaffected by extracellular addition of Cd^{2+} or 8-Br-cGMP (**Fig. 3**). Although we were unable to monitor SDF-1-induced Ca^{2+} signals at the growth cone, because of the limited amount of fluorescence dyes loaded into these small granule cell growth cones, our findings are consistent with the notion that Ca^{2+} influx is not involved in the SDF-1 and baclofen effects, whereas Ca^{2+} release from IP_3 -sensitive internal stores is required.

Dependence on PLC–PKC/IP_3 pathways

A major downstream effector of G_i in neurons is PLC[28], which is involved in the turning response induced by group I guidance cues[29]. Consistent with the involvement of PLC signaling in GPCR-mediated growth cone guidance, we found that bath application of the PLC inhibitor U73122 completely abolished both repulsive and attractive (in the presence of 8-Br-cGMP)

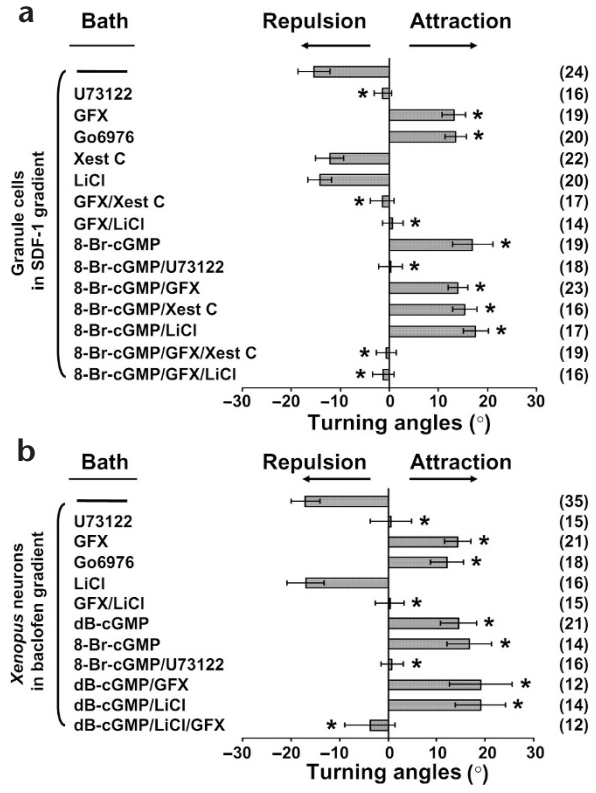

Fig. 4. Dependence of GPCR-mediated turning on PLC–PKC/IP_3 signaling pathways. (**a**) The average turning angles for growth cones of cerebellar granule cells exposed to a gradient of SDF-1 (20 μg/ml in the pipette) for 30 min. The bath contained normal medium with or without various drugs: U73122 (20 nM), GF109203X (GFX, 50 nM), Go6976 (20 nM), Xest C (1 μM), LiCl (50 mM) or 8-Br-cGMP (100 μM). (**b**) The average turning angles for *Xenopus* growth cones exposed to a gradient of baclofen (20 mM in the pipette) for 1 h. The bath was supplemented with drugs in (**a**), or U73122 (1 μM) or dB-cGMP (50 μM). *$P < 0.001$ as compared to control (exposed to the SDF-1 or baclofen gradient alone).

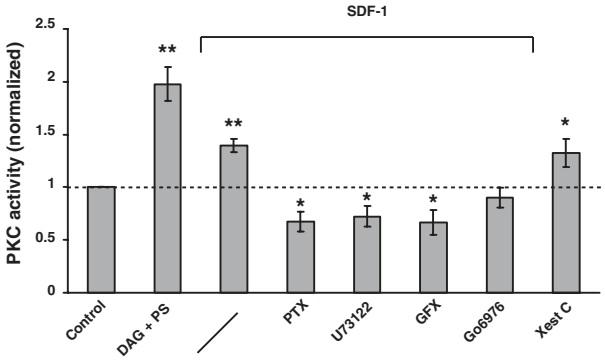

Fig. 5. PKC activation by SDF-1 in cultured cerebellar granule cells. The PKC activity was measured in various experimental conditions as indicated, and the scintillation counts (mean ± s.e.m.) were normalized to that of the control cells in the same experiment. PBS was used as control, and diacylglycerol (DAG, 0.032 mg/ml) + phosphatidylserine (PS, 0.32 mg/ml) were used as positive controls. For the PTX (1 μg/ml) experiment, cells were pretreated overnight. U73122 (20 nM), GF109203X (GFX, 50 nM), Go6976 (40 nM) or Xest C (1 μM) was added 30 min before SDF-1 (20 ng/ml) stimulation for 15 min. *$P < 0.05$, **$P < 0.001$.

turning induced by SDF-1 and baclofen (**Fig. 4a** and **b**). Activation of PLC leads to hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), producing two cytoplasmic second messengers[28]: diacylglycerol (DAG) and IP_3. Production of DAG, together with Ca^{2+} elevation due to IP_3-sensitive Ca^{2+} release from internal stores, further activates PKC, which in turn phosphorylates many target proteins. Indeed, we found that SDF-1-induced Ca^{2+} elevation in these cerebellar neurons was blocked by U73122 and Xest C, but unaffected by GF109203X, a specific membrane-permeable PKC inhibitor (**Fig. 3**). PKC activity has been implicated in nerve growth[30] and SDF-1-induced eukaryotic cell chemotaxis[31], so we examined the effects of inhibitors of PKC on the turning responses induced by SDF-1 and baclofen. We found that repulsion induced by SDF-1 and baclofen were converted to attraction when either GF109203X or Go6976, another PKC inhibitor, was added (**Fig. 4a** and **b**). This suggests that SDF-1 and baclofen induced repulsion by activating PKC, whereas attraction was mediated by a PKC-independent pathway. Treatment with PTX and U73122 did not result in turning (**Fig. 1g**; **Fig. 4a** and **b**), suggesting that the two pathways diverge at a point downstream of heterotrimeric G_i and PLC.

To test whether the IP_3 pathway mediates the attraction triggered by a SDF-1 or baclofen gradient in the presence of PKC inhibitor, we bath-applied Xest C, an inhibitor of the IP_3 receptor, or LiCl, which interferes with phosphatidylinositol turnover[32]. Although LiCl significantly reduced the growth rate of both types of neurons (data not shown, see also ref. 33), we found that neither Xest C nor LiCl had any effect on the repulsion induced by SDF-1 or baclofen (**Fig. 4a** and **b**). On the other hand, when GF109203X was applied together with Xest C or LiCl, the SDF-1 or baclofen gradient induced neither repulsion nor attraction (**Fig. 4a** and **b**). Thus we conclude that two divergent signaling cascades downstream of PLC activation—the PKC and IP_3 pathways—mediate the repulsive and attractive turning responses, respectively, of growth cones to GPCRs, and the PKC repulsive effect is dominant over that of the IP_3 pathway under normal culture conditions.

To further test this idea, we examined the involvement of PKC and IP_3 in the attraction induced by SDF-1 or baclofen in the presence of a cGMP analog (8-Br-cGMP or dB-cGMP). We found that application of GF109203X, Xest C or LiCl alone had no effect on the attractive turning, but the attraction induced by either SDF-1 or baclofen was completely abolished when GF109203X was applied together with Xest C or LiCl (**Fig. 4a** and **b**). In the presence of cGMP agonists, PKC pathway–induced repulsion was converted to attraction. The attractive signaling of the IP_3 pathway, on the other hand, seems to be unaffected by the level of cGMP.

SDF-1 increases PKC activity in cerebellar granule cells

If PKC indeed mediates SDF-1 signaling in cerebellar granule cells, SDF-1 should modulate PKC activity in these cells. We directly measured PKC activity in cultured cerebellar granule cells in response to bath-applied SDF-1. As a positive control, we applied known activators of PKC, diacylglycerol (DAG) and phosphatidylserine (PS), which significantly increased the PKC activity of these cells (**Fig. 5**). When cultured granule cells were pretreated with 20 ng/ml of SDF-1 for 15 minutes, we found that the PKC activity significantly increased (**Fig. 5**). This increase was prevented by stimulation of granule cells in the presence of inhibitors of PKC, PLC or G_i, but was relatively unaffected by an inhibitor of IP_3 receptors (**Fig. 5**). Furthermore, we found that the level of PKC activity was significantly lower than the control value when inhibitors of PKC, PLC or G_i were used (**Fig. 5**), suggesting that there is a basal level of activity of PKC in these neurons.

A PKC activity gradient induces turning

If PKC mediates the turning response induced by SDF-1 and baclofen gradients, we reasoned that a gradient of PKC activation (or inhibition) across the growth cone should induce turning itself. We found that the PKC activator phorbol 12-myristate 13-acetate (PMA), when applied extracellularly in a gradient across the *Xenopus* growth cone, indeed induced a repulsive turning response (**Fig. 6**). This PMA-induced repulsion was abolished by the PKC inhibitor GF109203X. Conversely, we found that a gradient of

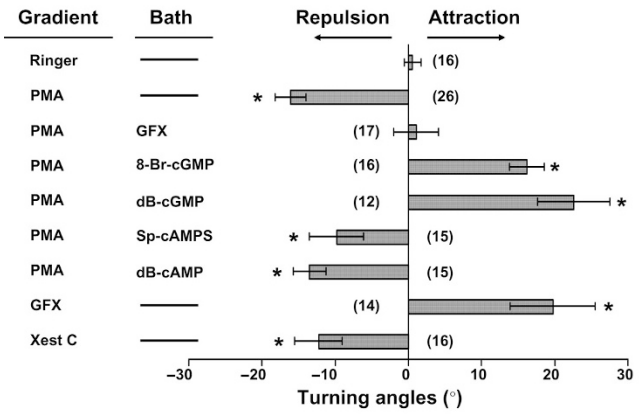

Fig. 6. Gradients of drugs that activate or inhibit PKC or inhibit IP_3 receptors are sufficient to induce growth cone turning. *Xenopus* spinal neurons were exposed to gradients of phorbol 12-myristate 13-acetate (PMA, 1 μM in the pipette), GF109203X (GFX, 25 μM) or Xest C (300 μM), using conditions of the standard turning assay (Methods). The bath contained normal medium or medium supplemented with GFX (50 nM), 8-Br-cGMP (100 μM), dB-cGMP (50 μM), Sp-cAMPS (20 μM) or dB-cAMP (50 μM). *Significantly different from the control group (Ringer only, $P < 0.001$).

GF109203X resulted in the attractive turning of the growth cone. Consistent with the finding that PMA can induce growth cone collapse[30], a gradient of PKC activity across the growth cone may result in a gradient of filopodial retraction (more retraction on the side of the higher PKC level), leading to repulsive turning, whereas a gradient of PKC inhibition induces the opposite pattern, leading to attractive turning. Furthermore, the repulsion induced by PMA became attraction when the cells were treated with 8-Br-cGMP or dB-cGMP, but not with Sp-cAMPS or dB-cAMP (**Fig. 6**). This supports the idea that cGMP activity switches the turning response by modulating the activity of PKC and/or its downstream effectors. Finally, consistent with the finding that blocking the IP_3 receptor inhibits neurite extension[33], we found that application of a gradient of Xest C resulted in repulsion of *Xenopus* growth cones. These results on the growth cone turning induced by gradients of inhibitors suggest the existence of a basal PKC and IP_3 activity, a gradient of which is sufficient to trigger growth cone turning.

Discussion

We have shown that a gradient of activation of GPCRs can induce a growth cone to turn. This response requires activation of PLC–PKC/IP_3 pathways and can be modulated by the cytosolic level of cGMP. Under normal conditions, the PKC pathway dominates over the IP_3 pathway, leading to the repulsive turning response. In the presence of cGMP analogs, the repulsion is converted to attraction. Further evidence for cGMP-modulated, PKC-dependent turning comes from the finding that a gradient of PKC activation induced by a PMA gradient induced either repulsive or attractive turning, depending on the cGMP level (**Fig. 6**). The cGMP-dependent signal seems to act on PKC and/or its downstream effectors, which in turn regulate cytoskeletal arrangements required for the turning responses. It would be of interest to determine whether the turning responses induced by semaphorin 3A and NT-3, which are also cGMP-dependent[22,25], signal through the PKC pathway as well.

SDF-1 and its receptor CXCR4 are widely expressed in both the immune and the nervous systems, including cerebellum, hippocampus and spinal cord[19,34]. Knockout of SDF-1 or CXCR4 results in defects not only in hematopoiesis, but also in neural development[9,10]. CXCR4-knockout mice show an aberrant laminar structure of the cerebellum, indicating that CXCR4-mediated signaling is required to prevent premature inward migration of proliferating granule cells from EGL[9]. A gradient of SDF-1 can attract migrating granule cells *in vitro*[16,17]. Neuronal migration and axon guidance may share common mechanisms[2,7,8] and these two processes are closely related in the development of cerebellar granule cells[35], leading us to suggest that SDF-1 signaling regulates axon guidance in developing cerebellum. In addition to CXCR4, many other GPCRs for chemotactic factors are expressed in the nervous system[36–38], including CXCR2, CXCR3, CCR1 and CCR5. It would be of interest to determine whether other chemokines are involved in neuronal migration and axon guidance.

Our results show that a gradient of GPCR activation across the growth cone is sufficient to trigger growth cone turning. Blocking the specific GPCRs for SDF-1 or baclofen blocked the turning responses, and a gradient of G protein activation (triggered by an extracellular gradient of mastoparan) was sufficient to induce repulsive turning of growth cones (**Fig. 1g**). Thus, signaling through GPCRs can result in the reorganization of the cytoskeleton and polarized cellular motility[39]. Studies of SDF-1 effects on cell motility in different systems strongly support the notion that chemotaxis of immune cells, neuronal migration and axon guidance all share similar molecular mechanisms.

In addition to mediating the guidance signals directly, the G protein signaling cascade may also modulate guidance signals mediated by receptor systems unrelated to GPCRs. Roundabout (Robo), a receptor for the repulsive neuronal guidance factor Slit, can functionally interact with the SDF–CXCR4 pathway to inhibit SDF-1-induced leukocyte chemotaxis[40]. In the nervous system, signaling through neuropilin, a receptor for semaphorin 3A, can be linked to heterotrimeric G protein via SEMCAP-1, a $G_{i\alpha}$-RGS-interacting protein[41]. Through reverse signaling affecting the G protein signaling cascade, ephrin-B can inhibit SDF-1-triggered granule cell migration[16]. Furthermore, we also found that attractive turning of the growth cone of cultured cerebellar granule cells induced by a gradient of BDNF, a well-characterized guidance cue for axon pathfinding[21] and cerebellar granule cell migration[42], was blocked by bath application of SDF-1 (Xiang, Y. *et al.*, unpub. data). Thus, substantial crosstalk may exist between signaling pathways for known axon guidance cues and those mediated through GPCRs.

Methods

Cell cultures. Cultures of rat cerebellar granular cells were prepared as described previously[43]. Briefly, we isolated cerebellum from postnatal days 0–2 rat (Sprague Dawley), removed the meninges directly covering the cerebellum, dissected EGL tissue from the anterior cerebellum using a tungsten needle, incubated the tissue in 0.125% trypsin (Sigma, St. Louis, Missouri) in PBS for 15 min at 37°C and then triturated to dissociate the cells. Dissociated cells were collected by centrifugation, resuspended and plated on a Matrigel (BD Biosciences, Bedford, Massachusetts) coated culture flask, and the medium was changed to NB (Neurobasal/B27, Gibco, New York, New York) 4 h later. The culture was used for experiments 20–40 h after plating. For antibody blocking experiments, anti-CXCR4 antibody (Santa Cruz, Santa Cruz, California) was added to the culture (at 4 μg/ml) 30 min before the experiment. Cultures of *Xenopus* spinal neurons were prepared from 1-d old *Xenopus* embryos by methods previously described[21]. The cells were used for experiments between 5–10 h after plating at room temperature (20—22°C). The use and care of animals in this study followed the guidelines of the Shanghai Institutes of Biological Sciences Animal Research Advisory Committee.

Growth cone turning assay. Microscopic gradients of chemicals were produced by methods previously described[21,22]. The turning angle was defined by the angle between the original direction of neurite extension and a straight line connecting the positions of the growth cone at the onset and the end of the turning assay. To assay growth cone turning, the pipette tip was placed 100 μm away from the center of the growth cone of an isolated neuron and at an angle of 45° with respect to the initial direction of neurite extension (indicated by the last 10-μm segment of the neurite). Only those growth cones with net extension >5 μm during the turning assay (30 min or 1 h for granule cells and *Xenopus* neurons, respectively) were included for analysis. Experiments with *Xenopus* neurons were carried out at room temperature in modified Ringer's solution (140 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, pH 7.40) and experiments with granule cells were done at 35°C using a heated chamber (Warner Inst. Corp., Hamden, Connecticut) in Hanks MEM (Gibco). For the standard gradient, the concentrations of SDF-1 and baclofen in the pipette were 20 μg/ml and 20 mM, respectively. For pharmacological treatments, chemicals were added to the culture medium 30 min before the assay, and were present throughout the experiments or otherwise as described. For the LiCl treatment, the medium compositions were readjusted for correct osmolarity. For PTX treatment, granule cells were treated overnight and *Xenopus* neurons were treated for 8 h before experiments. Data are presented as mean ± s.e.m. Statistical significance was analyzed by the Kolmogorov-Smirnov test.

Calcium imaging. Cultured granule cells were co-loaded with the cell-permeant, acetoxymethyl ester form of 3 μM Fluo-3 and 4 μM Fura-red (Molecular Probes, Eugene, Oregon) for 30 min at 37°C, and imaged with a Zeiss confocal microscope (LSM 510, Thornwood, New York). Hank's MEM was used to prevent PH changes during experiment. We applied various drugs 30 min before SDF-1 (20 μg/ml), which was

administrated to the neuron by a micropipette identical to that used in the turning assay, at a distance of 30 μm away from the cell body. The cells were illuminated with 488-nm light from an argon laser. Fluorescence images for the entire cell body were used for ratiometric Ca^{2+} measurements (at 530 ± 15 nm for Fluo-3, 640 ± 15 nm for Fura-red), collected at 30-s intervals. The Fluo-3/Fura-red ratio was calculated by dividing pixel values at 530 nm by those at 640 nm.

PKC assays. PKC assays were done using the SignaTECT PKC Assay System (Promega, Madison, Wisconsin). Serum-starved cultured cerebellar granule cells were stimulated by SDF-1 in the presence and absence of various drugs, scraped in a buffer and reacted (for 10 min at 30°C) with a mixture containing 20 mM Tris-HCl, 10 mM $MgCl_2$, 0.25 mM EGTA, 0.4 mM $CaCl_2$, 0.1 mg/ml BSA, 0.1 mM PKC biotinylated peptide substrate and [γ-^{32}P]ATP. The biotinylated, [γ-^{32}P]-labeled substrate was recovered from the reaction mixture with the SAM biotin capture membrane and the radioactivity measured by scintillation counting. For each condition, values for reactions lacking the substrate peptide were subtracted as blanks. For positive controls, cells were not treated with SDF-1, and phosphatidylserine (0.32 mg/ml) and diacylglycerol (0.032 mg/ml) were added to the mixture.

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Competing interests statement

The authors declare that they have no competing financial interests.

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