Nitric oxide/cyclic guanosine monophosphate-mediated growth cone collapse of dentate granule cells

Ryuji X. Yamada, Norio Matsuki and Yuji Ikegaya

Laboratory of Chemical Pharmacology, Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo, Japan

Correspondence and requests for reprints to Dr Yuji Ikegaya, Laboratory of Chemical Pharmacology, Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo 113-0033, Japan
Tel: +81 3 5841 4783; fax: +81 3 5841 4786; e-mail: ikegaya@mol.f.u-tokyo.ac.jp

Sponsorship: This work was supported in part by a Grant-in-Aid for Science Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (No. 17023015, No. 17650090, No. 17689004) and by the Sumitomo Foundation (No. 050038).

Received 9 December 2005; accepted 14 February 2006

Controlling axon and dendrite elongation is critical in developing precise neural circuits. Using isolated cultures of dentate granule neurons, we established an experimental system that can simultaneously monitor the behaviors of axonal and dendritic outgrowth. Our previous study shows that axons and dendrites respond differentially to manipulated cyclic adenosine monophosphate signaling, but we report here that cyclic guanosine monophosphate exerts similar effects on axons and dendrites; that is, both axonal and dendritic growth cones collapsed after activation of cyclic guanosine monophosphate signaling. In addition, nitric oxide donor-induced growth-cone collapse was prevented by the inhibition of cyclic guanosine monophosphate signaling, and this effect again did not differ between axons and dendrites. Thus, unlike cyclic adenosine monophosphate, cyclic guanosine monophosphate modulates extending axons and dendrites in a similar manner. *NeuroReport* 17:661–665 © 2006 Lippincott Williams & Wilkins.

Keywords: axon guidance, cyclic adenosine monophosphate, cyclic guanosine monophosphate, dendrite, dentate gyrus, hippocampus, network formation, nitric oxide synthase

Introduction

During neural development, neurites take precisely defined routes and come into contact with proper target nerve cells, thereby establishing the functional neural network. This process involves the guidance of neurites, that is, axons and dendrites. Axons and dendrites are both tipped with motile growth cones that decide the direction to extend by reading extracellular guidance signals [1,2]. To elucidate the mechanisms underlying the network formation, it is, therefore, essential to analyze the outgrowth of both axons and dendrites, yet most studies have investigated either axons or dendrites, simply because of the lack of appropriate experimental procedures to assess both simultaneously. Very little comprehensive information, therefore, is available to compare axonal and dendritic outgrowth.

We recently established an experimental system to cultivate isolated granule cells prepared from the dentate gyrus, which allows simultaneous monitoring of axons and dendrites [3]. Using this system, we disclosed that, within a single cell, the axon and dendrites differ in the regulation of cyclic adenosine monophosphate (cAMP) levels in growth cones as well as in the responsiveness to cAMP and also that cAMP differentially modulates growth cone responses to the repellents glutamate and Sema3F [3]. Such striking axo-dendritic disparity suggests that cAMP may be critical in shaping the axo-dendritic polarity of a granule cell.

In the present work, we focus on the cyclic guanosine monophosphate (cGMP) signaling cascade, which is activated by nitric oxide (NO). Immunoreactivity against NO synthase (NOS), an enzyme involved in the production of NO, is prominent in CA3 stratum lucidum and stratum oriens, the subfields where hippocampal mossy fibers, the axons of granule cells, terminate. The subgranular zone and the inner third of the molecular layer in the dentate gyrus are also NOS positive, and some types of hilar cells express NOS [4]. It appears plausible, therefore, that neurite outgrowth of dentate granule cells is subject to tight regulation by the diffusible molecule NO. We thus started this work by examining the effect of NO, an upstream of cGMP signaling, on axonal and dendritic growth cones.

Methods

Reagents

We used *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP, Dojindo Laboratories, Kumamoto, Japan), 8-bromo guanosine cyclic monophosphate sodium salt (8-Br-cGMP, Calbiochem, La Jolla, California, USA), 6-anilino-5,8-quinolinequinone

(LY83583, Calbiochem), and 3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole (YC-1, Sigma, St Louis, Missouri, USA). NOC13 was synthesized according to Hrabie *et al.* [5]. All reagents were bath-applied to cultures at 37°C in a humidified 5% CO_2 and 95% air atmosphere.

Primary cultures of dentate granule cells

Dispersed cultures of granule cells were prepared from postnatal day 3 Wistar/ST rats (SLC, Shizuoka, Japan) as previously described [3], according to the National Institutes of Health guidelines for laboratory animal care and safety. The hippocampal formation was dissected out from hypothermized animals, and the subicular complex and the Ammon's horn were removed in ice-cold Gey's balanced salt solution. The remaining dentate gyrus was trypsinized and triturated. Neurons were plated onto 13-mm-φ cover slips coated with poly-L-lysine (Sigma) at a cell density of 5.0×10^3 cells/cm^2 in 50% Neurobasal/B-27 (Life Technologies, Gaithersburg, Maryland, USA) and 50% astrocyte-conditioned medium at 37°C in a humidified 5% CO_2 and 95% air atmosphere. Culture medium was replaced 24 and 72 h after plating with astrocyte-conditioned medium-free Neurobasal/B-27, supplemented with 2 μM cytosine-D-arabino-furanoside (Sigma). Cultures were assayed on day 4 *in vitro*.

Growth-cone collapse assay

After a 96-h incubation, cultures were treated with the NO donors NOC13 and SNAP or the cGMP-relative reagents YC-1 and 8-Br-cGMP, and immediately fixed for 20 min at 37°C in buffer [60 mM piperazine-1,4-bis (2-ethanesulfonic acid), 25 mM *N*-2-hydroxyl piperazine-*N*′-2-ethane sulfonic acid, 10 mM ethylene glycol-bis (*b*-aminoethyl ether), 2 mM $MgCl_2$, pH 6.9] containing 4% paraformaldehyde, 0.25% glutaraldehyde (Nacalai Tesque, Kyoto, Japan), 0.1% Triton X-100, 10 μM Taxol (Sigma) and 1.3 μM phalloidin (Sigma) [6]. In some experiments, cells were pretreated with LY83583 for 30 min, and then SNAP was coapplied for another 30 min before fixation.

Cultures were triple-stained with rhodamine phalloidin and anti-tau-1 and anti-MAP-2 antibodies. Briefly, fixed cells were blocked with 2% goat serum for 60 min, followed by an overnight 4°C incubation with primary antibodies against tau-1 (1:2000, mouse monoclonal, MAB3420, Chemicon, Temecula, California, USA) and MAP-2 (1:1000, rabbit, AB5622, Chemicon). They were then incubated with rhodamine phalloidin (1:40, R-415, Molecular Probes, Eugene, Oregon, USA) and the fluorescein-conjugated secondary antibodies anti-mouse IgG Alexa-488 (1:400, A-11001, Molecular Probes) and anti-rabbit IgG Alexa-350 (1:400, A-11046, Molecular Probes) for 5 h at room temperature. Fluorescence signals were visualized with an ORCAII cooled CCD camera (Hamamatsu Photonics, Hamamatsu, Japan) equipped with a Nikon ECLIPSE TE300 inverted microscope and a 40× objective. Images were analyzed with a Hamamatsu AQUACOSMOS system.

We scored cells that did not come into contact with adjacent cells and had one longest, tau-1-positive, MAP2-negative axon and a few tau-1-negative, MAP2-positive dendrites. Owing to these strict criteria, about 50% of cells were discarded before data analysis. Growth-cone collapse was judged on the basis of phalloidin images (Fig. 1); that is, a growth cone was considered 'collapsed' if it had less than two filopodia and lamellipodia smaller than 10 μm^2 [3]. As for dendrites, we only analyzed terminals that were not overlapped onto neighboring protrusions within a cell of interest. Data are presented as the mean percentage (±SEM) of collapsed growth cones to the total number.

Results

As NOS is present in large amounts in the area where the axons and dendrites of dentate granule cells travel *in vivo* [4], it is feasible that NO regulates the network development of granule cells. Using primary cultures of granule cells, we first examined the response of axonal and dendritic growth

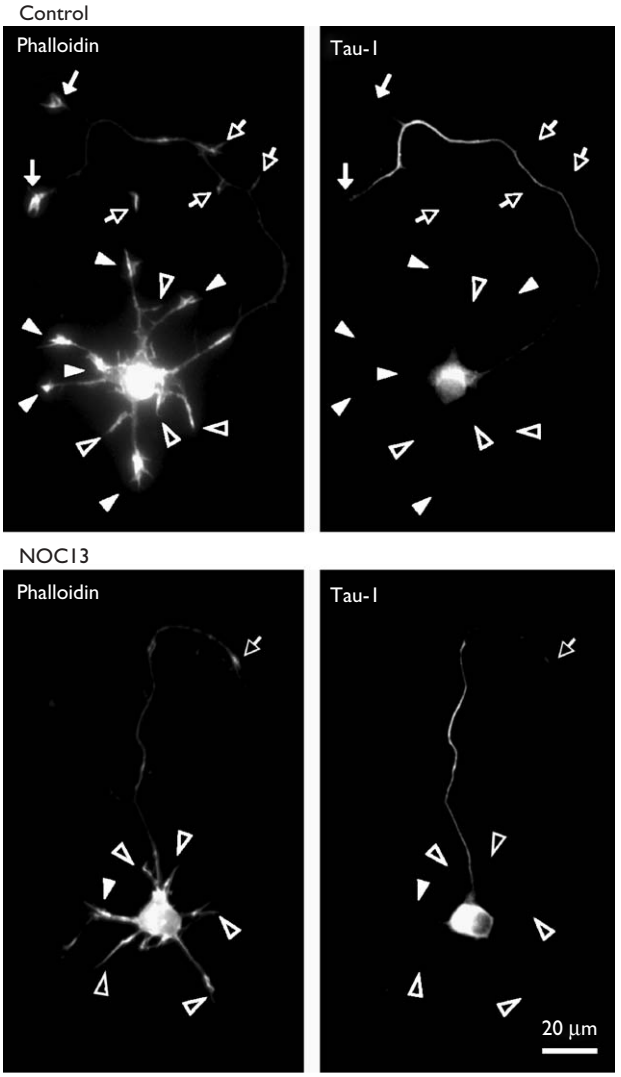

Fig. 1 Nitric oxide donor-induced collapse in axonal and dendritic growth cones of dentate granule cells. Typical epifluorescence images of isolated dentate granule cells costained with rhodamine-conjugated phalloidin (left) and anti-Tau-1 antibody (right) after 30 min treatment with (bottom) or without (top) 50 μM NOC13 on day 4 *in vitro*. The axon was defined as the longest, tau-1-positive process, and the other tau-1-negative neurites were regarded as dendrites. Closed arrows and arrowheads indicate intact (i.e. 'uncollapsed') growth cones of axons and dendrites, respectively. Open arrows and arrowheads indicate 'collapsed' axonal and dendritic terminals, respectively. Each right–left pair of images was obtained from the same microscopic field. Data are summarized in Fig. 2.

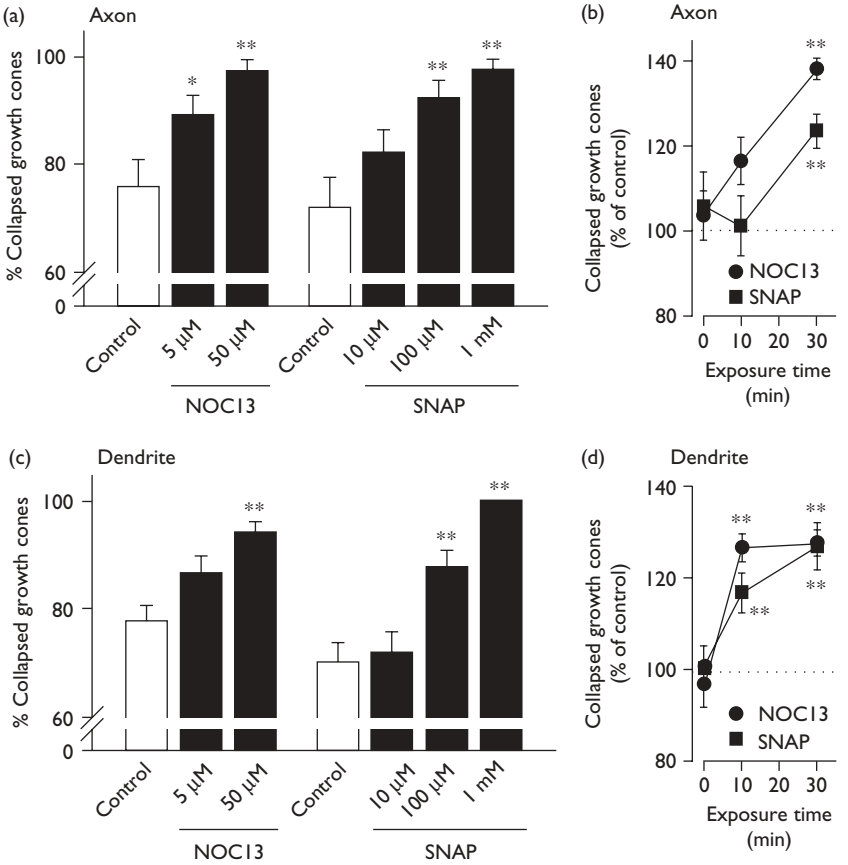

Fig. 2 Nitric oxide donor-induced collapse in axonal and dendritic growth cones of dentate granule cells. Dose dependence (a and c) and time course (b and d) of the effects of treatment with NOC13 and *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) on the number of collapsed terminals of axons (a and b) and dendrites (c and d). In the panels a and c, collapse was assessed after 30 min of treatment. The ordinates indicate the percentages of collapsed growth cones to the total. In the panels b and d, 50 μM NOC13 and 100 μM SNAP were applied at 0 min. The ordinates are expressed as a percentage relative to control at each time point. Data represent means ± SEM of 28–31 neurons (NOC13) and 28–38 neurons (SNAP) in three independent experiments. $*P < 0.05$, $**P < 0.01$ vs. control; Tukey's test after analysis of variance.

cones to NO-releasing reagents. Axons and dendrites were identifiable by tau-1 immunoreactivity; that is, the former was always tau-1-positive on day 4 *in vitro* (Fig. 1). Time-lapse analysis revealed that both axonal and dendritic growth cones were highly motile and spontaneously alternated between collapsed and uncollapsed states. On an average, 60–80% of growth cones were collapsed at any given time under naïve conditions in culture, and this value did not differ between axons and dendrites.

Treatment with NOC13 and SNAP for 30 min increased the percentage of collapsed growth cones, and the sensitivity to these reagents was almost equal between axons and dendrites (Figs 1 and 2a, c). NOC13 and SNAP have different chemical structures and different mechanisms for NO generation. We therefore believe that it is NO released by these reagents that induced the collapse of growth cones. We sought to examine the time course of growth cone responses to the NO donors (Fig. 2b and d). A significant collapse of axonal growth cones occurred 30 min after treatment with NOC13 and SNAP, whereas dendritic collapse was already evident at 10 min and maintained for another 20 min. In both axons and dendrites, the growth cone collapse was spontaneously reverted even in the prolonged presence (60 min) of NOC13 and SNAP (data not shown).

NO exerts its physiological effects dependent or independent of the activation of soluble guanylyl cyclase (sGC), an enzyme that produces cGMP [7,8]. To investigate whether NO-induced growth-cone collapse requires the cGMP signaling pathway, we applied 1 μM LY83583, an antagonist of sGC, for 30 min and, subsequently, together with SNAP for 30 min. LY83583 prevented SNAP-induced collapses of both axons and dendrites (Fig. 3a). Interestingly, the sGC activator YC-1 and the cGMP analog 8-Br-cGMP *per se* increased collapsed growth cones in a concentration-dependent manner (Fig. 3b). Therefore, activation of cGMP signaling is sufficient to induce growth cone collapse. The efficacy of YC-1 and 8-Br-cGMP did not differ between axons and dendrites (Fig. 3b).

Discussion

NOS is expressed in large amounts in the dentate gyrus [4] and, thus, NO naturally has multiple actions on dentate granule cells. For example, NO is involved in synaptic plasticity at perforant path-dentate granule cell synapses [9] and contributes to adult neurogenesis in dentate gyrus [10]. We have shown now that immature neurites of granule cells are also responsive to NO.

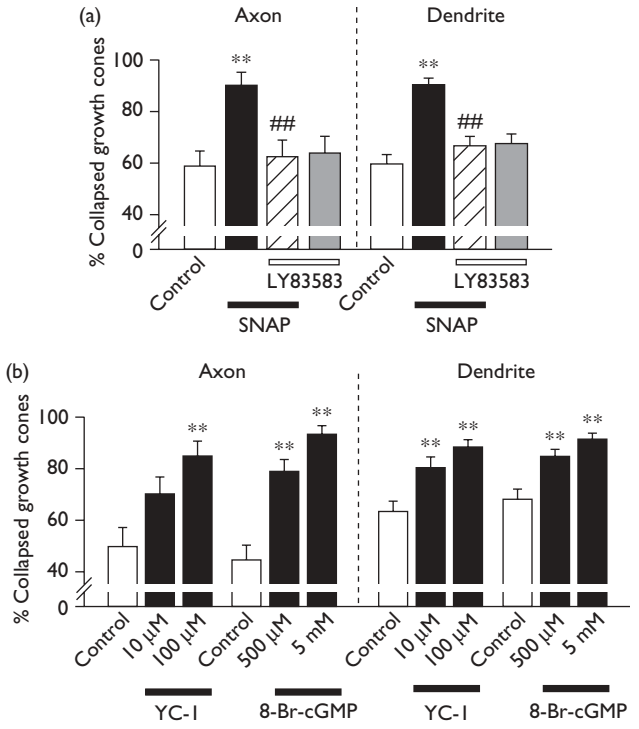

Fig. 3 Cyclic guanosine monophosphate (cGMP) mediates nitric oxide-induced growth-cone collapse. (a) Cells were pretreated with 1 μM LY83583, a soluble guanylyl cyclase inhibitor, for 30 min and then co-treated with 100 μM *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) for 30 min. (b) Neurons were treated for 30 min with 3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole (YC-1), a soluble guanylyl cyclase activator, or 8-bromo guanosine cyclic monophosphate sodium salt (8-Br-cGMP), a cGMP analog. Data represent means ± SEM of 27–48 neurons in three independent experiments. $^{**}P < 0.01$ vs. control, $^{\#\#}P < 0.01$ vs. SNAP; Tukey's test after analysis of variance.

Several reports indicate that NO affects growth-cone behaviors. The effect of NO is various, depending on the neuron types. NO slows down the axon extension of snail B5 neurons with filopodia elongation [11], induces collapse of axonal growth cones of *Xenopus* retinal ganglion cells [12] and abolishes chemorepellent-induced turning responses of *Xenopus* spinal growth cones [13]. NO is also involved in the neuritogenesis of rat pheochromocytoma PC12h cells [14] and retinal axon arborization in the *Xenopus* visual system [15]. We have shown that neurites of dentate granule cells collapse in response to exogenous NO. Importantly, we monitored both axons and dendrites in the same cells and found that the NO effect on axons and dendrites was almost equivalent in its potency as well as cGMP dependency.

We previously found that spontaneous or Sema3F-induced collapse of axonal and dendritic growth cones is enhanced and prevented, respectively, by the activation of cAMP signaling [3]. As for cGMP, it is reported that growth cones of chick dorsal root ganglion neurons are collapsed by increasing cellular cGMP levels [16], whereas apical dendrites of cortical pyramidal cells are attracted to Sema3A via cGMP signaling [17]. Therefore, it was possible that the cGMP modulation also differed between the axon and dendrites of a granule cell. We have shown that cGMP activation alone was capable of inducing neurite collapse, but found no evidence for the axo-dendritic difference.

Our previous study demonstrated that under blockade of cGMP signaling, the target-specific innervation of mossy fibers disappears and instead the fibers are sparsely distributed in the CA3 field in organotypic cultures of hippocampal slices [18,19]. Thus, the cGMP signaling pathway is likely to serve to constrain mossy fiber trajectories into the limited area, that is, stratum lucidum. We speculate that extracellular molecules that can activate cGMP signaling in the axons of granule cells may exist in CA3 subfields other than the stratum lucidum, thereby preventing developing mossy fibers from straying out of the stratum lucidum.

In conclusion, axonal and dendritic growth cones of dentate granule cells collapse when exposed to exogenous NO. Activation of cGMP signaling is likely to be necessary and sufficient for this NO effect. It is significant that, unlike cAMP, the NO/cGMP action did not show any marked contrast between axons and dendrites. Given that in *Xenopus* spinal neurons the ratio of cAMP to cGMP activities determines the turning behavior of extending axons [20], a combination of the biphasic cAMP action and the monotonic cGMP action on axonal and dendritic outgrowth of granule cells could embody a fine tuning of the dentate circuit development.

Acknowledgments

We are grateful to Dr Hirotatsu Kojima and Dr Tetsuo Nagano (Laboratory of Chemistry and Biology, Graduate School of Pharmaceutical Sciences, University of Tokyo) for providing NOC13.

References

1. Tessier-Lavigne M, Goodman CS. The molecular biology of axon guidance. *Science* 1996; **274**:1123–1133.
2. Huber AB, Kolodkin AL, Ginty DD, Cloutier JF. Signaling at the growth cone: ligand–receptor complexes and the control of axon growth and guidance. *Annu Rev Neurosci* 2003; **26**:509–563.
3. Yamada RX, Matsuki N, Ikegaya Y. cAMP differentially regulates axonal and dendritic development of dentate granule cells. *J Biol Chem* 2005; **280**:38020–38028.
4. Gotti S, Sica M, Viglietti-Panzica C, Panzica G. Distribution of nitric oxide synthase immunoreactivity in the mouse brain. *Microsc Res Tech* 2005; **68**:13–35.
5. Hrabie JA, Klose JR, Wink DA, Keefer LK. New nitric oxide-releasing zwitterions derived from polyamines. *J Org Chem* 1993; **58**: 1472–1476.
6. Dent EW, Kalil K. Axon branching requires interactions between dynamic microtubules and actin filaments. *J Neurosci* 2001; **21**:9757–9769.
7. Bicker G. STOP and GO with NO: nitric oxide as a regulator of cell motility in simple brains. *Bioessays* 2005; **27**:495–505.
8. Guix FX, Uribesalgo I, Coma M, Munoz FJ. The physiology and pathophysiology of nitric oxide in the brain. *Prog Neurobiol* 2005; **76**:126–152.
9. Wu J, Wang Y, Rowan MJ, Anwyl R. Evidence for involvement of the neuronal isoform of nitric oxide synthase during induction of long-term potentiation and long-term depression in the rat dentate gyrus *in vitro*. *Neuroscience* 1997; **78**:393–398.
10. Zhang R, Zhang L, Zhang Z, Wang Y, Lu M, Lapointe M, *et al.* A nitric oxide donor induces neurogenesis and reduces functional deficits after stroke in rats. *Ann Neurol* 2001; **50**:602–611.
11. Trimm KR, Rehder V. Nitric oxide acts as a slow-down and search signal in developing neurites. *Eur J Neurosci* 2004; **19**:809–818.
12. Renteria RC, Constantine-Paton M. Exogenous nitric oxide causes collapse of retinal ganglion cell axonal growth cones *in vitro*. *J Neurobiol* 1996; **29**:415–428.

13. Song H, Ming G, He Z, Lehmann M, McKerracher L, Tessier-Lavigne M, *et al*. Conversion of neuronal growth cone responses from repulsion to attraction by cyclic nucleotides. *Science* 1998; **281**: 1515–1518.
14. Yamazaki M, Chiba K, Mohri T. Fundamental role of nitric oxide in neuritogenesis of PC12h cells. *Br J Pharmacol* 2005; **146**:662–669.
15. Cogen J, Cohen-Cory S. Nitric oxide modulates retinal ganglion cell axon arbor remodeling *in vivo*. *J Neurobiol* 2000; **45**:120–133.
16. Dontchev VD, Letourneau PC. Nerve growth factor and semaphorin 3A signaling pathways interact in regulating sensory neuronal growth cone motility. *J Neurosci* 2002; **22**:6659–6669.
17. Polleux F, Morrow T, Ghosh A. Semaphorin 3A is a chemoattractant for cortical apical dendrites. *Nature* 2000; **404**:567–573.
18. Mizuhashi S, Nishiyama N, Matsuki N, Ikegaya Y. Cyclic nucleotide-mediated regulation of hippocampal mossy fiber development: a target-specific guidance. *J Neurosci* 2001; **21**:6181–6194.
19. Yamada RX, Matsuki N, Ikegaya Y. Soluble guanylyl cyclose inhibitor prevents Sema 3F-induced collapse of axonal and dendrite growth cones of dentate granule cells. *Biol Pharma Bull* (in press).
20. Nishiyama M, Hoshino A, Tsai L, Henley JR, Goshima Y, Tessier-Lavigne M, *et al*. Cyclic AMP/GMP-dependent modulation of Ca^{2+} channels sets the polarity of nerve growth-cone turning. *Nature* 2003; **424**:990–995.