

# A Subset of Signal Transduction Pathways is Required for Hippocampal Growth Cone Collapse Induced by Ephrin-A5

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**ABSTRACT:** The Eph family tyrosine kinase receptors and their ligands, ephrins, play key roles in a wide variety of physiological and pathological processes including tissue patterning, angiogenesis, bone development, carcinogenesis, axon guidance, and neural plasticity. However, the signaling mechanisms underlying these diverse functions of Eph receptors have not been well understood. In this study, effects of Eph receptor activation on several important signal transduction pathways are examined. In addition, the roles of these pathways in ephrin-A5-induced growth cone collapse were assessed with a combination of biochemical analyses, pharmacological inhibition, and overexpression of dominant-negative and constitutively active mutants. These analyses showed that ephrin-A5 inhibits Erk activity but activates c-Jun N-terminal kinase. However, regulation of these two pathways is not required for ephrin-A5-induced growth cone collapse in hippocampal neurons. Artificial Erk activation by

expression of constitutively active Mek1 and B-Raf failed to block ephrin-A5 effects on growth cones, and inhibitors of the Erk pathway also failed to inhibit collapse by ephrin-A5. Inhibition of JNK had no effects on ephrin-A5-induced growth cone collapse either. In addition, inhibitors to PKA and PI3-K showed no effects on ephrin-A5-induced growth cone collapse. However, pharmacological blockade of phosphotyrosine phosphatase activity, the Src family kinases, cGMP-dependent protein kinase, and myosin light chain kinase significantly inhibited ephrin-A5-induced growth cone collapse. These observations indicate that only a subset of signal transduction pathways is required for ephrin-A5-induced growth cone collapse. © 2008 Wiley Periodicals, Inc. *Develop Neurobiol* 00: 000–000, 2008

**Keywords:** growth cone collapse; Eph family; Ephrins; Erk; myosin light chain kinase; phosphotyrosine phosphatase; cGMP-dependent protein kinase

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## INTRODUCTION

The Eph family of receptors and ligands are widely expressed during development and in adult tissues, although individual members are only expressed in specific tissues or organs (Zhou, 1998). Consistent with the widespread expression, these molecules have been shown to regulate many important biological processes (Andres and Ziemiecki, 2003; Klein, 2004; Poliakov et al., 2004; Surawska et al., 2004; Wimmer-Kleikamp and Lackmann, 2005; Heroult et al., 2006; Ivanov and Romanovsky, 2006; Zhang and Hughes, 2006; Pasquale, 2008). For example, EphA4 and ephrin-B2 participate in the segmentation of the hindbrain (Wilkinson, 2000), and both Eph-A and Eph-B receptors and ligands are shown to regulate vascularization and angiogenesis (Wang et al., 1998; Adams et al., 1999; Zhang and Hughes, 2006). Another important function is to guide axons to their proper targets during nervous system development. Both EphA and EphB receptors and their ligands have been shown to regulate spatially organized axon projection maps in the brain (Flanagan and Vanderhaeghen, 1998; Zhou, 1998; McLaughlin and O'Leary, 2005). Recent studies have revealed additional functions in regulating neural plasticity (Klein, 2004; Pasquale, 2008), tumorigenesis (Wimmer-Kleikamp and Lackmann, 2005; Heroult et al., 2006), bone morphogenesis (Compagni et al., 2003; Davy et al., 2004), and inflammatory responses (Ivanov and Romanovsky, 2006). Mutations in ephrin-B1 have been associated with craniofrontonasal syndrome in humans (Wieacker and Wieland, 2005; Shotelersuk et al., 2006; Twigg et al., 2006; Vasudevan et al., 2006). With expression in many tissues and organs, it would not be surprising that novel functions of these receptors are continuously discovered in the future.

In spite of the clear importance of these molecules in physiological and pathological processes, the signaling mechanisms underlying their functions are not well understood. It has been shown that Eph receptors regulate Rho family small GTPases through interaction with Ephexin (Wahl et al., 2000; Shamah et al., 2001; Cheng et al., 2003; Ogita et al., 2003; Miao et al., 2005; Sahin et al., 2005; Fu et al., 2007) and Vav (Cowan et al., 2005) family guanine nucleotide exchange factors. In addition, many other signal transduction mediators including the Src family kinase, Nck, PI3-K, and LMW-PTP have been shown to physically associate with Eph receptors (Kullander and Klein, 2002). However, it is still largely unknown which molecules are required for what functional outputs. It is also not clear whether different functions utilize the same signaling pathways. In addition, since

the receptors are highly homologous, it is not known whether different Eph receptors activate similar downstream pathways. In this study, we have extensively surveyed roles of different signaling pathways in ephrin-A5-induced hippocampal growth cone collapse. Our analyses show that only a few selected ones are involved in this process.

## MATERIALS AND METHODS

### Materials

Ephrin-A5-Fc was purchased from R&D Systems (Minneapolis, MN); AffiniPure rabbit anti-human IgG Fc $\gamma$  fragment was from Jackson ImmunoResearch Laboratories (West Grove, PA). The following pharmacological reagents were from Calbiochem (La Jolla, CA): mitogen-activated protein (MAP) kinase kinase (MEK) inhibitors, U0126 and PD98059; c-Jun N-terminal kinase (JNK) inhibitor, SP600125; Src inhibitor PP2, and control PP3; phosphatidylinositol 3-kinase (PI3-K) inhibitors, Wortmannin and LY294002; broad-spectrum protein kinases inhibitor Staurosporine; protein kinase C (PKC) inhibitors, Go 6983, Bisindolylmaleimide I, Ro31-8220, and the control Bisindolylmaleimide V; protein kinase A (PKA) inhibitor KT5720, activator Sp-cAMPS; myosin light chain kinase (MLCK) inhibitor Peptide 18; Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (CaMKII) inhibitor KN-93; cGMP-dependent protein (PKG) inhibitor Rp-8-Br-cGMPs; the following reagents were purchased from Sigma (St. Louis, MO): protein tyrosine phosphatase (PTP) inhibitors, phenylarsine oxide (PAO), and sodium orthovanadate. The inhibitor concentrations used were based on IC<sub>50</sub>, previously published studies as well as potential inhibitory effects on growth cone morphology.

### Construction of Adenoviruses and Expression Vectors

cDNA sequences encoding the constitutively active MEK1 (CA-MEK1) and dominant negative MEK1 (DN-MEK1) mutant proteins (Jaaro et al., 1997) were inserted into the EGFP expressing shuttle plasmid pAdTrack-CMV of the adenoviral vector system AdEasy (QBiogene, Irvine, CA). Recombinant adenoviral constructs were generated by homologous recombination between the shuttle vectors and the viral backbone plasmid pAdEasy-1 in BJ5183 *E. coli* cells. Recombinant CA-MEK1 and DN-MEK1 adenoviruses, which also express EGFP, were generated by packaging in 293A cells. B-Raf constructs (WT and V599E) were kindly provided by Dr. Deborah K. Morrison (NCI-Frederick, Frederick, MD) and were cloned into the EGFP expressing shuttle vector pAdTrack-CMV. The EGFP-R-Ras constructs (WT and 38VY66F) were generously provided by Dr. Elena Pasquale (The Burnham Institute, La



Jolla, CA). All viruses and constructs coexpressed EGFP to allow identification of infected or transfected neurons.

### Neuron Culture and Gene Expression

Hippocampal explants were prepared from E18 Sprague-Dawley rat embryos and seeded onto glass chamber slides coated with poly-D-lysine (0.5  $\mu\text{g}/\mu\text{L}$ , Sigma) and laminin (20  $\mu\text{g}/\text{mL}$ , Sigma). Explants were maintained at 37°C in a humidified tissue culture incubator with 5% CO<sub>2</sub> in neurobasal medium supplemented with B27 and 2 mM L-glutamine (all obtained from Invitrogen, San Diego, CA). Dissociated hippocampal neurons were dissected from E18 rat embryo, digested with 0.1% trypsin for 15 min at 37°C, followed by trituration with Pasteur pipettes in the neurobasal medium and plated onto plastic dishes coated with poly-D-lysine (0.1  $\mu\text{g}/\mu\text{L}$ ) at a density of 10<sup>6</sup> neurons/35-mm dish.

To express constitutively active (CA) and dominant-negative (DN)-MEK1 mutant proteins, hippocampal neurons were infected with adenoviruses carrying these genes at a MOI (Multiplicity of Infection) of 100 on the day of plating. To express B-Raf and R-Ras mutants, hippocampal neurons were transfected using Amaxa Nucleofector (Amaxa Biosystems, Gaithersburg, MD). For each construct, 1 × 10<sup>6</sup> neurons were suspended in 100  $\mu\text{L}$  of rat neuron nucleofector solution with 3  $\mu\text{g}$  DNA and electroporated using program G-13. Transfected neurons were plated in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum on glass chamber slide coated with poly-D-lysine (0.5  $\mu\text{g}/\mu\text{L}$ ) and laminin (20  $\mu\text{g}/\text{mL}$ ). Two hours after plating, the medium was replaced with neurobasal medium containing B-27 supplement and glutamine. All the neurons were maintained for 4 days until neurites and growth cones were positive with GFP fluorescence, identifying the infected or transfected neurons and neurites. Dissociated neuron cultures (10<sup>6</sup>/35-mm dish) were used for western blot analysis to detect protein expression. Explants cultures were used to examine the effect of ephrins on growth cone. GFP-positive neurites were quantified on a Zeiss microscope equipped with epifluorescence (Axiovert 200M).

### Growth Cone Collapse Assay

The hippocampal explants were stimulated for 15 min with 0.2–2  $\mu\text{g}/\text{mL}$  ephrin-A5-Fc (preclustered for 2 h with rabbit anti-human IgG Fc $\gamma$  fragment at 37°C in an ephrin-A5-Fc: anti-IgG molar ratio of about 15:1, unless specified otherwise). Controls were incubated with preclustered IgG alone. Explants were fixed with 4% paraformaldehyde and 0.25% glutaraldehyde in a cacodylate buffer (0.1 M sodium cacodylate, 0.1 M sucrose, pH 7.4) for 30 min at 37°C as described previously (Guirland et al., 2003). The explants were stained for F-actin with Texas Red<sup>®</sup> X-phalloidin (Molecular Probes, Eugene, OR) and examined for growth cone morphology under a Zeiss microscope (Axiovert 200M).

### Western Blot Analysis

Primary cultures of dissociated rat hippocampal neurons were treated as indicated and washed with ice cold PBS and lysed in lysis buffer [150 mM NaCl, 50 mM Tris.Cl, 1%NP-40, with phosphatase inhibitors (Cocktail 2, Sigma), and protease inhibitors (Roche cocktail, Palo Alto, CA)]. Protein concentrations were determined using a Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA). Equal amounts (10  $\mu\text{g}$ ) of proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane (Bio-Rad). The membranes were blocked with 5% BSA for 1 h at room temperature and then incubated with primary antibodies in 1% BSA at 4°C overnight before detection with horseradish peroxidase (HRP)-conjugated secondary antibodies. Chemiluminescence was detected with a reaction kit from Roche. After initial blot, membranes were stripped by using a membrane stripping kit Western Re-Probe (Genotech, St. Louis, MO) and then reprobbed with different antibodies. Antibodies used are the following: anti-phospho-Erk1/2, anti-Erk1/2, anti-phospho-JNK, anti-JNK, anti-phospho-Akt, and anti-Akt (Cell Signaling, Danvers, MA), anti-phosphotyrosine, clone 4G10 (Upstate Biotechnology, Lake Placid, NY), and anti-EphA3 (Santa Cruz, Santa Cruz, CA).

### PKA Activity Assay

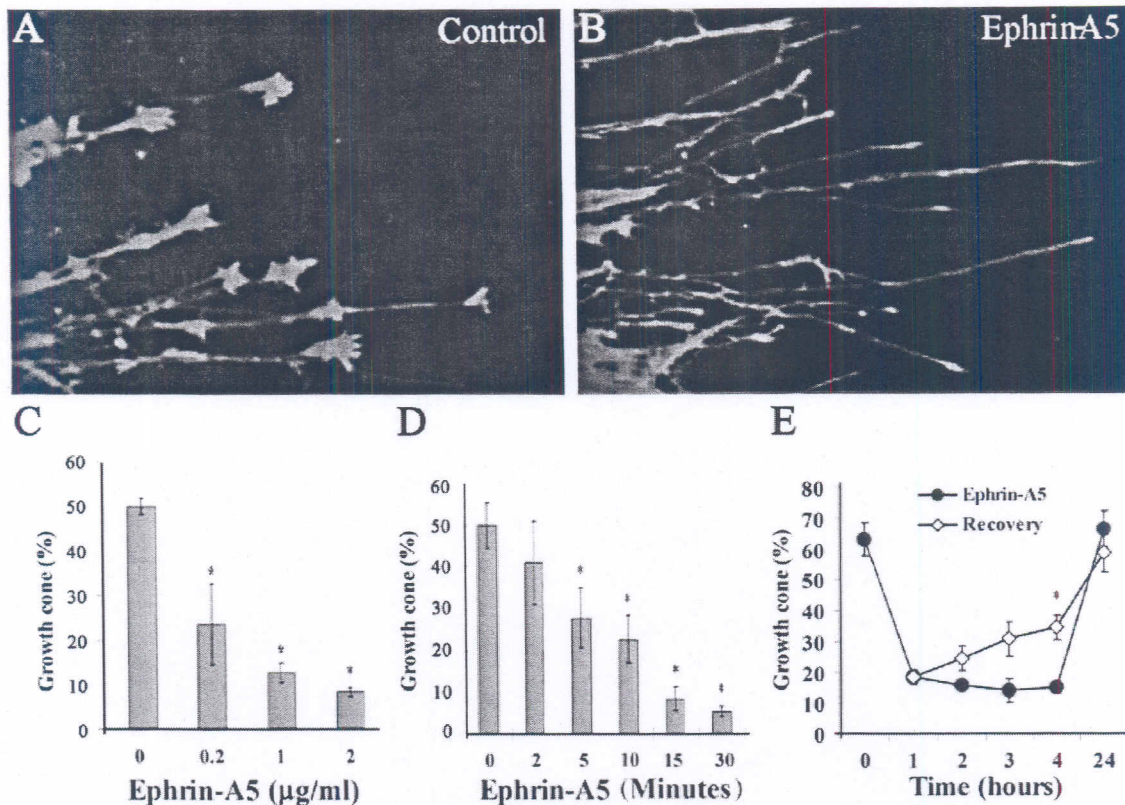
PKA activity assay was performed with a PKA assay kit from Promega (Madison, WI) according to manufacturer's instructions. Briefly, 1 × 10<sup>7</sup> treated neurons were suspended in 0.5 mL PKA extraction buffer and homogenized with a Dounce homogenizer on ice. About 10  $\mu\text{L}$  lysate were incubated with PKA specific substrate Kemptide for 30 min at 30°C in a final volume of 25  $\mu\text{L}$ . The reaction was stopped by heating at 95°C for 10 min. Phosphorylated Kemptide was separated from nonphosphorylated on 0.8% agarose at 100 V for 20 min, by moving toward the anode, and the gel was photographed on a UV transilluminator.

## RESULTS

### Ephrin-A5 Induces Hippocampal Growth Cones Collapse

To study signaling mechanisms underlying axon guidance functions of ephrin-A5, we examined effects of ephrin-A5 on hippocampal growth cones. E18 rat hippocampi were dissected and cut into small explants. The explants were then seeded in chamber slides coated with poly-D-lysine and laminin in neural basal medium supplemented with B27. After 24 h of culture, the explants extend neurites with prominent growth cones. The cultures were then treated with various concentrations of ephrin-A5 for different time periods (see Fig. 1). These studies showed that ephrin-A5 induced collapse of about 50% of the





**Figure 1** Ephrin-A5 induces hippocampal growth cone collapse. Hippocampal explants from rat E18 embryos were cultured in neurobasal media for 24 h. The cultures were treated with preclustered ephrin-A5 for indicated time and concentration. Equal amounts of anti-Fc antibody alone were used as control. (A and B) Hippocampal explants treated with control or 1  $\mu\text{g}/\text{mL}$  ephrin-A5, respectively, for 15 min. The explants were stained for F-actin with Texas Red<sup>®</sup> X-phalloidin and examined for growth cone morphology under a Zeiss fluorescence microscope. (C) Higher concentrations of ephrin-A5 increase the percent of collapsed growth cones. Explants were treated with various concentrations of ephrin-A5 for 15 min. (D) The time course of ephrin-A5 (0.2  $\mu\text{g}/\text{mL}$ )-induced growth cone collapse. Error bars indicate SEM. \* $p < 0.05$  for ephrin-A5 treated versus control by t-test ( $n \geq 3$  independent experiments). (E) Growth cone recovery after the ephrin-A5 treatment. In each experiment,  $\geq 200$  growth cones were quantified. "Growth cones (%)" on the Y-axis in (C-E) (and in the following figures unless indicated otherwise) represents percent of hippocampal neurites with growth cones. Ephrin-A5 causes a significant reduction in the percent of neurites with growth cones. [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]

growth cones at 0.2  $\mu\text{g}/\text{mL}$  [Fig. 1(C)]. By 2  $\mu\text{g}/\text{mL}$  ephrin-A5, over 80% of the growth cones were collapsed compared with the control explants. In the presence of 0.2  $\mu\text{g}/\text{mL}$  ephrin-A5, growth cones started to collapse at about 5 min, and the process was complete by about 15 min [Fig. 1(D)].

To address a potential concern that growth cone collapse by ephrin-A5 is a toxic effect, we performed two different recovery experiments. In these experiments, the axons were treated for 1 h to allow the full effects of any potential toxicity. In the first experiment, the ligand was removed after 1 h, and replaced

with fresh culture medium. At different times, the culture was fixed and the percent of axons with growth cone quantified. This analysis showed that significant number of growth cones had recovered by 4 h after ephrin-A5 removal [Fig. 1(E)]. By 24 h, growth cones were fully restored [Fig. 1(E)]. In the second experiment, the ligand was left in the medium and growth cone recovery was monitored over time. In the presence of the ligand, no recovery was observed by 4 h. However, full recovery was achieved in 24 h [Fig. 1(E)]. These experiments indicate that ephrin-A5 does not cause nonspecific toxicity



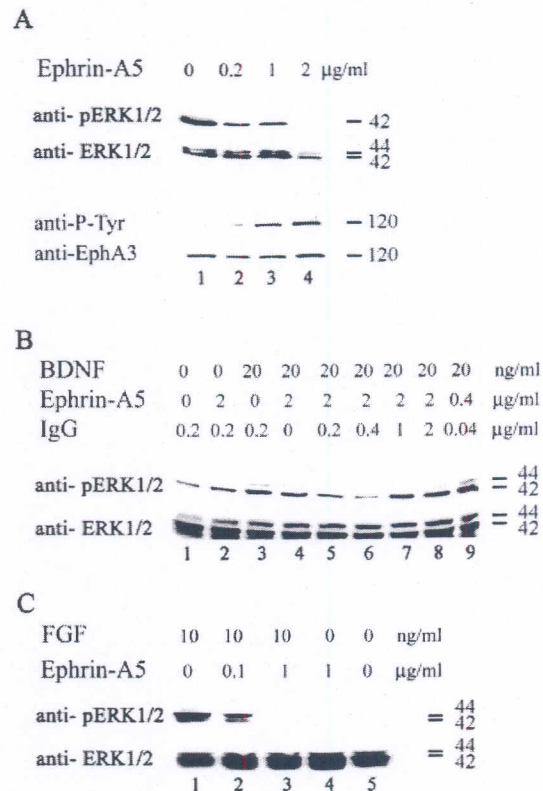
in neurons, since changes in growth cone morphology are reversible.

### Ephrin-A5 Inhibits Erk Activity in Primary Hippocampal Neurons

It has been shown previously that ephrins inhibit Erk activity in cultured cells (Elowe et al., 2001; Miao et al., 2001; Tong et al., 2003). To examine whether ephrin-A5 regulated Erk activity in primary neurons, cultured embryonic day (E) 18 rat hippocampal neurons were treated with ephrin-A5. Basal Erk activity was significantly inhibited by ephrin-A5 even at the concentration of 0.2  $\mu\text{g}/\text{mL}$ , correlating with the appearance of phospho-tyrosine containing proteins that correspond to Eph receptors [Fig. 2(A)]. To examine whether ephrin-A5 also inhibited Erk activation by growth factors, the cultured neurons were treated with BDNF in the presence or absence of ephrin-A5 [Fig. 2(B)]. Since it has been reported that ephrins need to be artificially cross-linked to be active (Davis et al., 1994; Stein et al., 1998; Huynh-Do et al., 1999; Pabbisetty et al., 2007), we also investigated how cross-linking affects regulation of Erk activity. Cultured hippocampal neurons were treated with BDNF with or without ephrin-A5 (with different cross-linking ratio with anti-Fc IgG). This analysis showed that 20 ng/mL of BDNF activated Erk activity in the hippocampal neurons, in the absence of ephrin-A5 [Fig. 2(B), Lane 3]. In the absence of cross-linking IgG, ephrin-A5 only weakly inhibited Erk activity [Fig. 2(B), Lane 4]. The most effective cross-linking ratio for ephrin-A5 is 2  $\mu\text{g}$  ephrin-A5 to 0.4  $\mu\text{g}$  anti-Fc IgG, which corresponded to a molar ratio of about 15:1 [Fig. 2(B), Lane 6]. A lower ratio also reduced the effectiveness of ephrin-A5 to inhibit Erk activity, possibly due to overabundance of the cross-linking antibody resulting in smaller ephrin-A5 aggregates. This analysis demonstrated that ephrin-A5 aggregation enhanced its activity and that the ligand also inhibited Erk activation by BDNF. To examine whether ephrin-A5 also inhibited Erk activation by other growth factors, we tested the ability of FGF to activate Erk in the presence of clustered ephrin-A5 [Fig. 2(C)]. FGF completely failed to activate Erk in the presence of 1  $\mu\text{g}/\text{mL}$  clustered ephrin-A5 [Fig. 2(C)].

### Inhibition of Erk Activity is not Required for Ephrin-A5-Induced Hippocampal Growth Cone Collapse

To directly test whether regulation of Erk activity was critical to growth cone collapse induced by



**Figure 2** Ephrin-A5 inhibits Erk1/2 activity. (A) Dissociated hippocampal neurons were exposed to different concentration of clustered ephrin-A5 or anti-Fc control for 15 min. Lysate were analyzed with Western blot using anti-phospho-Erk1/2 and anti-phospho-tyrosine antibodies. The membranes were stripped and reprobred for total Erk and Eph-A3 protein levels. Decreased Erk activity correlated with increased Eph receptor activation. (B) Hippocampal neurons were serum-starved for 2 h and then incubated with BDNF and clustered ephrin-A5 as indicated. BDNF alone activated Erk (Line 3). Ephrin-A5 was preclustered with anti-Fc antibody at different ratio (Lane 3–8). Without anti-Fc antibody, ephrin-A5 failed to reduce Erk activity (Lane 4). With cross-linking, ephrin-A5 was able to inhibit Erk activity and this effect maximized at 2  $\mu\text{g}$ :0.4  $\mu\text{g}$  ratio (molar ratio about 15:1) (Lane 6). (C) Erk activity induced by FGF was also inhibited by ephrin-A5. Numbers at the right of each panel indicate size of the proteins detected (kDa).

ephrin-A5, effects of constitutive Erk activation were examined. Adenoviruses carrying either a CA MEK1 (CA-MEK1) or a DN MEK1 (DN-MEK1) were used to activate or inhibit Erk in hippocampal neurons. A mock infection with no viruses and an infection with adenoviruses carrying only EGFP were used as controls. These studies showed that the CA-MEK1 virus induced significantly higher Erk activity even in the



absence of any growth factors, and that cross-linked ephrin-A5 showed no inhibition of the activity [Fig. 3(A), Lanes 7 and 8], consistent with previous findings that ephrins inhibit Erk activity upstream of Mek1 (Elowe et al., 2001; Tong et al., 2003). In contrast, no significant effects on basal Erk activity were observed with viruses carrying either EGFP or the DN-MEK1 [Fig. 3(A), Lane 3–6]. When infected

neurons were treated with cross-linked ephrin-A5, neither virus significantly prevented growth cone collapse induced by the ligand [Fig. 3(B)].

To further clarify the roles of Erk activity in ephrin-A5-induced growth cone collapse, we also examined effects of Erk activity by expression of CA B-Raf gene. Both the wild type B-Raf and the CA mutant V599E-B-Raf were cloned into an expression

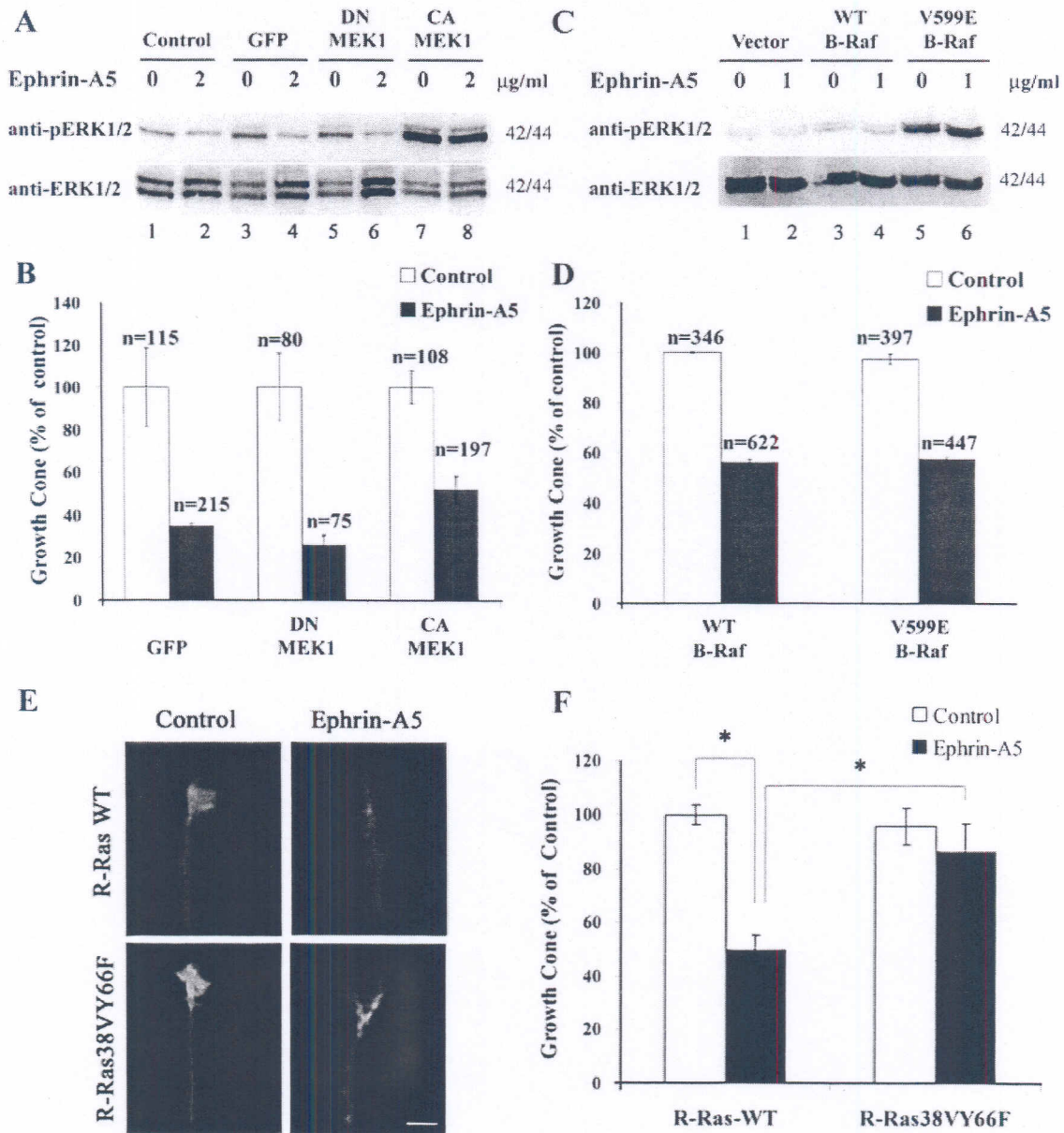


Figure 3



vector that also carries an EGFP gene (see Materials and Methods for details). Similar to CA-MEK1, V599E-B-Raf induced high levels of Erk activity that could not be inhibited by ephrin-A5 [Fig. 3(C), Lanes 5 and 6]. Collapse assay of transfected neurons showed that V599E-B-Raf did not block growth cone collapse induced by ephrin-A5 [Fig. 3(D)]. To validate the transfection assays used here, a CA R-Ras mutant (R-Ras38VY66F) which has been shown to block ephrin-A1 and B1-induced hippocampal growth cone collapse (Dail et al., 2006) was used as a positive control. Consistent with the previous study, the R-Ras mutant prevented ephrin-A5-induced growth cone collapse, while wild type R-Ras showed no effect, as expected [Fig. 3(E,F)]. These data strongly suggest that inhibition of Erk activity is not necessary for ephrin-induced growth cone collapse in hippocampal neurons.

### Erk Activation is also not Required for Ephrin-A5-Induced Growth Cone Collapse

It has been shown previously that Erk activation is required for Semaphorin 3A-induced growth cone collapse (Campbell and Holt, 2003). Although ephrin-A5 inhibits Erk activity, it is still possible that transient activation of Erk is required for growth cone collapse by ephrin-A5. Therefore we also determined effects of inhibition of the Erk pathway on ephrin-induced growth cone collapse. Hippocampal

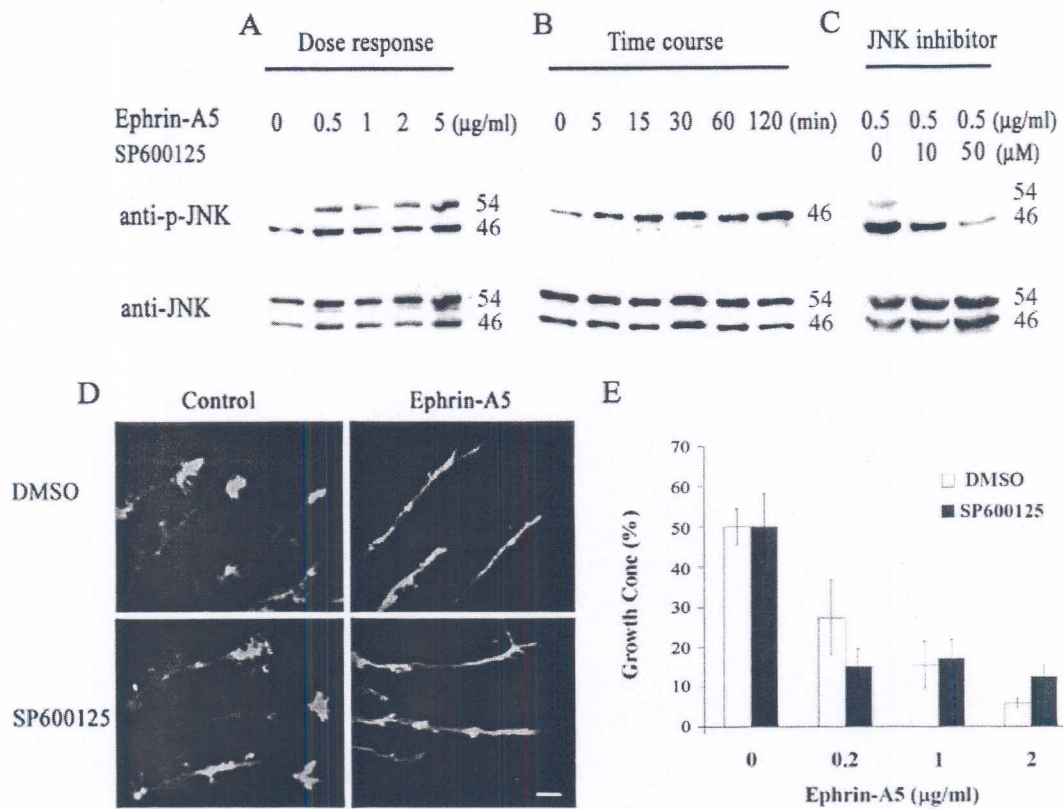
neurons were preincubated with MEK1/2 inhibitor U0126 or MEK1 inhibitor PD98059 before ephrin-A5 treatment. Both inhibitors inhibited the enzyme by a noncompetitive mechanism (Favata et al., 1998). While both inhibitors inhibited Erk activity, neither inhibitor blocked growth cone collapse induced by ephrin-A5, even at 20  $\mu\text{M}$  of U0126 or 40  $\mu\text{M}$  of PD98059 (Supplementary Fig. 1). These results suggest that Erk activation is also not required for ephrin-A5-induced growth cone collapse.

### Ephrin-A5 Activates JNK but does not Require the Activity for Growth Cone Collapse

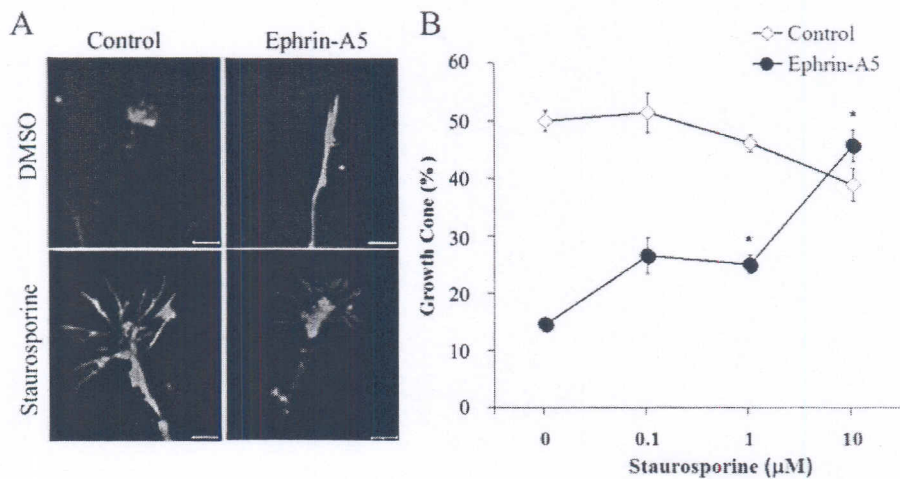
A previous report showed that JNK was activated by ephrin-B1 (Stein et al., 1998), we tested whether ephrin-A5 also activated JNK and whether JNK activity was required for the growth cone collapse function. Ephrin-A5 indeed activated JNK in a dose and time-dependent manner. At 0.5  $\mu\text{g}/\text{mL}$ , ephrin-A5 significantly activated JNK [Fig. 4(A)]. The increase in JNK activity was apparent by 5 min and continued up to 2 h, the latest time point analyzed [Fig. 4(B)]. To assess whether JNK activation mediated ephrin-A5-induced growth cone collapse, effects of a JNK inhibitor SP600125 was examined. SP600125 inhibits JNK by competing with ATP binding ( $\text{IC}_{50} = 40 \text{ nM}$ ) (Bennett et al., 2001; Han et al., 2001). At 50  $\mu\text{M}$ , the compound severely inhibited JNK activation [Fig. 4(C)]. However, the inhibitor showed no significant

**Figure 3** Inhibition of Erk activity is not required for ephrin-A5-induced growth cone collapse. (A, B) Expression of CA MEK1 (CA-MEK1) does not prevent ephrin-A5-induced growth cone collapse in hippocampal neurons. Neurons were infected with indicated adenoviruses (control: no infection; GFP: adenoviruses expressing only EGFP; DN-MEK1: adenoviruses expressing dominant negative-MEK1 and EGFP; CA-MEK1: adenoviruses expressing constitutively-active MEK1 and EGFP) for 4 days. Infected neurons were then stimulated with or without 2  $\mu\text{g}/\text{mL}$  of cross-linked ephrin-A5 for 15 min when 90% of neurons expressed EGFP as an infection marker. (A) CA-MEK1 infected neurons showed high level of Erk activity, which was not inhibited by ephrin-A5 (compare Lanes 7 and 8). (B) Infected explants were stained for F-actin and quantified for growth cone. Ephrin-A5 treatment caused significant collapse in neurons infected with adenoviruses expressing control EGFP, and DN-MEK1 and CA-MEK1. A slightly higher percent of growth cones were observed in explants infected with CA-MEK1 viruses, but the difference did not reach statistical significance. (C–F) Rat hippocampal neurons were transfected with the indicated B-Raf or R-Ras constructs that also expressed EGFP and stimulated with preclustered 1  $\mu\text{g}/\text{mL}$  ephrin-A5 for 15 min. Neurons were stained for F-actin for quantification. Only GFP-positive growth cones were quantified. (C) Neurons transfected with V599E-B-Raf expressed high Erk activity and the activity was not reduced by ephrin-A5 treatment (compare Lanes 5 and 6). (D) Ephrin-A5-induced growth cone collapse in both WT and V599E-B-Raf transfected hippocampal neurons. (E) Expression of R-Ras38VY66F but not the wild type prevents growth cone collapse by ephrin-A5. (F) Quantification of effects of wild type and mutant R-Ras on ephrin-A5 activity. Student *t* test, \**p* < 0.05. [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]





**Figure 4** Ephrin-A5 activates JNK but does not require the activity for growth cone collapse. (A) Hippocampal neurons were treated with various concentrations of clustered ephrin-A5 for 15 min and analyzed for levels of phospho-JNK. Total JNK levels were assessed by stripping and re-probing the blot with an anti-total JNK antibody. (B) Hippocampal neurons were treated with 0.5  $\mu\text{g/mL}$  clustered ephrin-A5 for different time periods. (C) Ephrin-A5 failed to activate JNK in the presence of a JNK inhibitor SP600125. Hippocampal neurons were pretreated with SP600125 for 15 min at indicated concentrations before the addition of 0.5  $\mu\text{g/mL}$  clustered ephrin-A5 for 15 min. (D and E) Inhibition of JNK activity does not prevent ephrin-A5-induced growth cone collapse. Hippocampal explants were pretreated with DMSO or 25  $\mu\text{M}$  SP600125 for 15 min before addition of ephrin-A5. Error bars represent the standard errors from three experiments. Ephrin-A5 treatment caused significant collapse, with or without SP600125. [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]



**Figure 5**

effects on the percent of growth cones that are collapsed by ephrin-A5 [Fig. 4(D,E)], indicating that JNK activity was not required.

### Staurosporine-Sensitive Kinases Mediate Ephrin-A5-Induced Growth Cone Collapse

To identify pathways that do mediate ephrin-A5-induced growth cone collapse, we tested the effects of a cell-permeable, broad-spectrum inhibitor of protein kinases, Staurosporine. Staurosporine is an alkaloid isolated from the culture broth of *Streptomyces staurosporesa*. The compound inhibits kinases by preventing ATP binding (Ruegg and Burgess, 1989). We showed that it effectively inhibited ephrin-A5-induced growth cone collapse and enhanced the size and complexity of the growth cones [Fig. 5 and Suppl. Fig. 2]. In the presence of Staurosporine, the growth cones had very long filopodia [Fig. 5(A) and Suppl. Fig. 2]. With increasing concentrations of Staurosporine, growth cones were gradually protected from the collapsing effects of ephrin-A5 [Fig. 5(A,B)]. By 10  $\mu$ M, ephrin-A5 failed to collapse hippocampal growth cones completely [Fig. 5(B)]. It is possible that Staurosporine inhibits multiple kinases, which lead to a freezing of growth cones, thereby preventing growth cone collapse. To test for this possibility, we observed growth cone dynamics in the presence of the inhibitor using time-lapse video microscopy. The growth cones maintained dynamic changes in the presence of 10  $\mu$ M Staurosporine, and continued to grow even when ephrin-A5 was added to the culture (Supplementary Fig. 2), indicating that the inhibitor blocked specific biochemical processes leading to growth cone collapse. In contrast, in the absence of Staurosporine, ephrin-A5 induced both collapse of the growth cone and a retraction of the neurites (Supplementary Fig. 2).

Staurosporine is known to inhibit several protein kinases including protein kinase A (PKA) ( $IC_{50} = 7$  nM), protein kinase C (PKC) ( $IC_{50} = 0.7$  nM) (Tamaoki et al., 1986; Matsumoto and Sasaki, 1989), myosin light chain kinase (MLCK) ( $IC_{50} = 1.3$  nM), protein kinase G (PKG) ( $IC_{50} = 8.5$  nM), and  $Ca^{2+}$ /calmodulin-dependent protein kinase II (CaMKII)

( $IC_{50} = 20$  nM) (Yanagihara et al., 1991). First we sought to determine whether inhibition of PKA was responsible for blocking ephrin-A5 activity. The growth cone collapse assay was performed in the presence of a PKA inhibitor KT5720, which prevents cAMP binding to PKA (Kohda and Gemba, 2001). Hippocampal explants were pretreated with KT5720 for 20 min at indicated concentrations, and then treated with preclustered ephrin-A5 (0.5  $\mu$ g/mL) or IgG for 15 min. DMSO was used as solvent controls. This analysis showed that KT5720 had no effect on ephrin-A5-induced growth cone collapse over a wide range of concentrations, although the inhibitor clearly inhibited PKA activity in the neurons (Supplementary Fig. 3), suggesting that inhibition of PKA activity was probably not responsible for the Staurosporine effects. Since high concentration of cAMP levels have been shown to favor attractive turning (Lohof et al., 1992; Ming et al., 1997; Nishiyama et al., 2003; Henley and Poo, 2004) and promote neurite outgrowth (Kao et al., 2002), we determined that whether PKA activation would counteract the effects of ephrin-A5. Explants were pretreated with the PKA activator Sp-cAMPS, a cAMP analog (Dostmann et al., 1990; Takuma and Ichida, 1991) before addition of ephrin-A5. No significant effects were observed in the presence of Sp-cAMPS (Supplementary Fig. 3), indicating that regulation of PKA pathway was not essential for ephrin-A5-induced growth cone collapse.

### MLCK and PKG Mediate Ephrin-A5-Induced Growth Cone Collapse

We next tested effects of MLCK, an enzyme that was also inhibited by Staurosporine. A MLCK inhibitor, peptide 18, has been shown to specifically inhibit MLCK by competing with substrate binding (Lukas et al., 1999). In the presence of inhibitor peptide 18, significant inhibition to ephrin-A5-induced growth cone collapse was observed [Fig. 6(A,B)]. With increasing concentration of the peptide inhibitor, nearly 90% of ephrin-A5 effect could be blocked [Fig. 6(B)]. The inhibitor peptide alone slightly increased the percentage of growth cones [Fig. 6(B,C)], consistent with a stabilizing effect on growth

**Figure 5** Staurosporine prevents ephrin-A5-induced growth cone collapse. (A) Fluorescence images of hippocampal explants pretreated with control or 1  $\mu$ M Staurosporine for 20 min before ephrin-A5 treatment (0.5  $\mu$ g/mL). Bars = 10  $\mu$ m. (B) Staurosporine prevents ephrin-A5-induced collapse in a dose-dependent manner. Error bars represent SEM. \* indicates significant difference from samples without Staurosporine treatment ( $p < 0.05$ ;  $t$  test). [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]



cones. A preincubation of the peptide inhibitor with hippocampal neurons for about 10 min had the best effect [Fig. 6(C)]. Prolonged incubation before ephrin-A5 treatment resulted in the loss of inhibitory activity, possibly due to peptide degradation, although the exact mechanism was not known at present. Taken together, these results show that Staurosporine-sensitive kinases such as MLCK may be responsible for the inhibitory effects of Staurosporine.

Previous studies have implicated the cGMP-regulated kinase PKG, also inhibited by Staurosporine, in mediating retinal growth cone responses to ephrin-B1 (Mann et al., 2003) and Semaphorin 3A (Dontchev and Letourneau, 2002). To examine the role of PKG in ephrin-A5-induced collapse, PKG-specific inhibitor Rp-8-Br-cGMPS was bath-applied 20 min before the addition of the ephrin-A5. Rp-8-Br-cGMPS blocks cGMP binding to PKG (Butt et al., 1990). Pre-

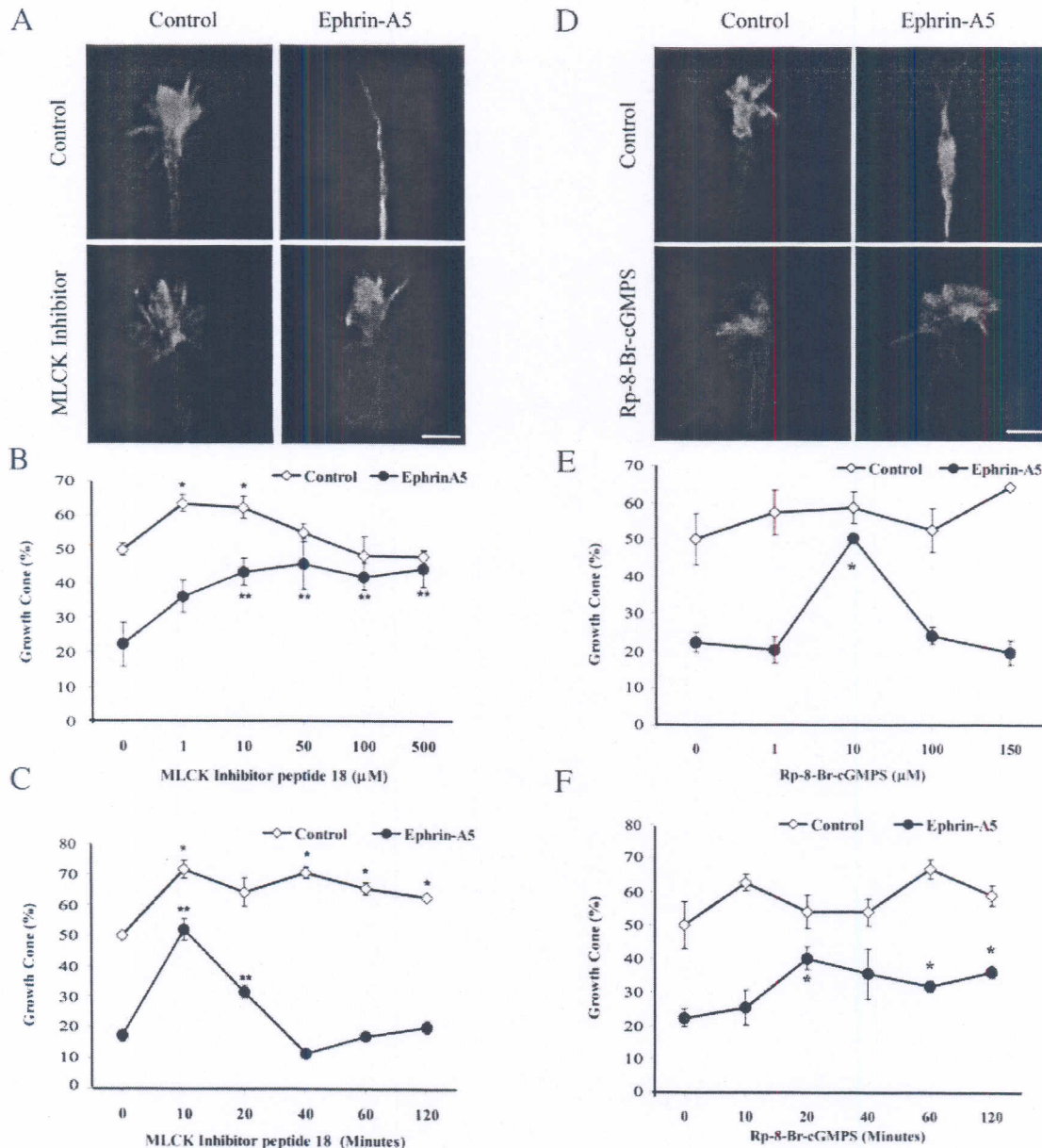


Figure 6

incubation of 10  $\mu\text{M}$  Rp-8-Br-cGMPS abolished the collapse response of hippocampal growth cones exposed for 15 min to 1  $\mu\text{g}/\text{mL}$  of ephrin-A5 [Fig. 6(D–F)]. Higher concentrations led to a loss of inhibition, possibly due to nonspecific inhibition of other kinases. Preincubation longer than 20 min in 10  $\mu\text{M}$  inhibitor did not significantly enhance the inhibitory effects, indicating that a 20-min incubation was sufficient to allow enough inhibitor to enter the cells and inhibit most of PKG activity (Dontchev and Letourneau, 2002; Mann et al., 2003). These results suggest that PKG activity is also required for ephrin-A5 collapsing effects. Inhibitors for the other Staurosporine-sensitive kinases (PKC, CaMKII) did not yield any specific effects (data not shown), although further studies are necessary to assess their contribution to ephrin-A5 signaling. Taken all these data together, effects of Staurosporine may be exerted, at least in part by inhibiting MLCK and PKG activity.

### Protein Tyrosine Phosphatase Activity Participates in the Regulation of Growth Cone Morphology by Ephrin-A5

Eph receptors are phosphorylated upon activation by ephrin-A5, and the inactivation of the receptors likely requires dephosphorylation of the receptors. In addition, downstream mediators are also likely regulated by tyrosine phosphorylation. We therefore tested whether inhibitors to PTP regulate growth cone collapse by ephrin-A5. Two PTP inhibitors, sodium vanadate and PAO, were used to assess roles of PTP. Sodium vanadate is a competitive inhibitor of phosphatases and PAO inactivates PTPs by chemically reacting with the enzymes (Seargeant and Stinson, 1979). Both compounds inhibited growth cone

collapse by ephrin-A5 [see Fig. 7]. In the presence of sodium vanadate, the growth cones continued to extend, indicating that the phosphatase inhibitors did not cause “freezing” of the cellular machinery required for collapse (Suppl. Fig. 2). These observations strongly suggest that PTP activity is required for ephrin-A5-induced growth collapse.

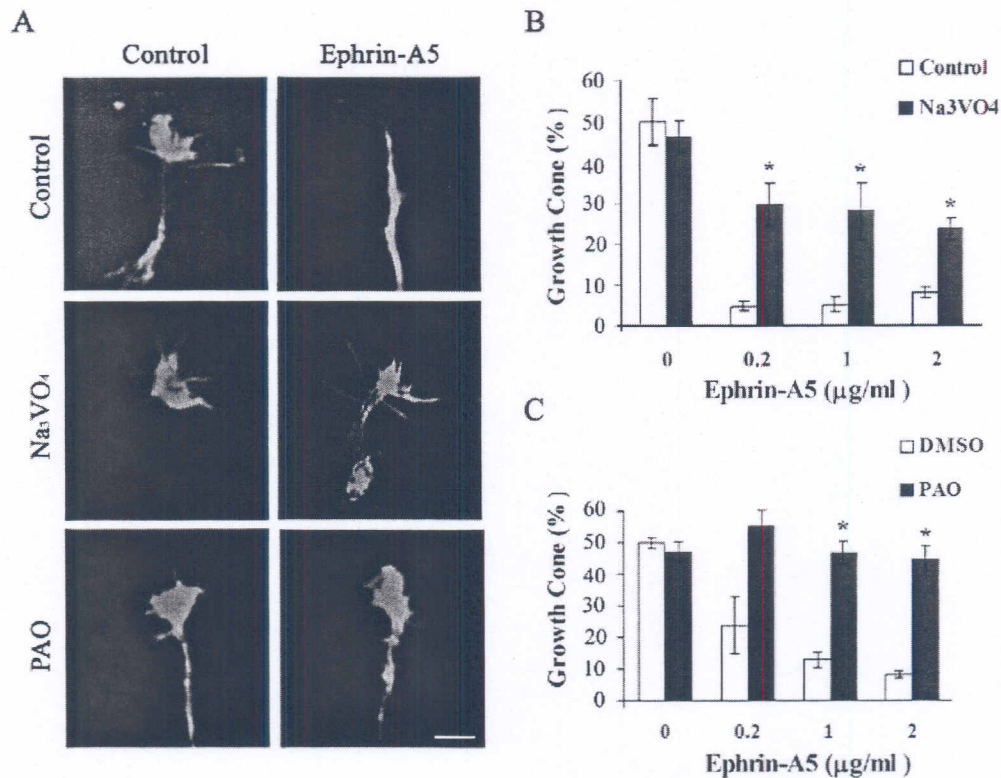
### Src Kinases are Required for Ephrin-A5-Induced Growth Collapse

It has been shown that Src kinases associate with Eph receptors, and are required for repulsive guidance of retinal growth cones by ephrin-A5 (Knoll and Drescher, 2004; Wong et al., 2004). We tested whether Src kinases are also required for hippocampal growth cone collapse by ephrin-A5. The ability of ephrin-A5 to induce growth cone collapse was significantly inhibited by the Src kinase inhibitor PP2 (see Fig. 8). PP2 is a potent and selective inhibitor of the Src family kinases, acting either as a competitive or noncompetitive inhibitor for different members of the family (Karni et al., 2003). In contrast, a negative control compound, PP3, which did not inhibit the Src kinases, showed no effect on ephrin-A5-induced collapse (see Fig. 8).

We also examined whether the phosphoinositide-3 (PI-3) kinase was involved in mediating ephrin-A5 activity, since the activity appeared to be required for ephrin-A5 induced retinal growth cone collapse (Wong et al., 2004). Activation of PI3 kinase led to the phosphorylation of AK mouse transforming (Akt) kinase, which could be detected using an anti-phospho-Akt antibody (Dhawan et al., 2002). Treatment of hippocampal neurons with ephrin-A5 did not lead to the activation of Akt [Supplementary Fig. 4(A)], indicating that ephrin-A5 function did not require

**Figure 6** MLCK and PKG inhibitors prevent ephrin-A5-induced growth cone collapse. (A) Hippocampal explants were pre-incubated with 50  $\mu\text{M}$  MLCK inhibitor peptide 18 for 10 min before treated with 0.5  $\mu\text{g}/\text{mL}$  clustered ephrin-A5 for 15 min. Explants were fixed and visualized with rhodamine-phalloidin staining. (B) Dose-response effect of MLCK inhibitor peptide 18. (C) Time-dependent effect of MLCK inhibitor peptide 18 (50  $\mu\text{M}$ ). Error bars represent SEM. In the absence of ephrin-A5, the inhibitor caused a significant increase in the number of growth cones compared to the DMSO controls ( $*p < 0.05$ ,  $t$  test). The inhibitor also significantly inhibited ephrin-A5 activity in inducing collapse, as compared with no inhibitor controls ( $***p < 0.05$ ,  $t$  test). (D) Hippocampal explants were pre-incubated with 10  $\mu\text{M}$  PKG inhibitor Rp-8-Br-cGMPS for 20 min before treatment with 1  $\mu\text{g}/\text{mL}$  clustered ephrin-A5 for 15 min. Ephrin-A5 failed to induce growth cones collapse in the presence of PKG inhibitor. (E) Dose-response effects of Rp-8-Br-cGMPS. Hippocampal explants were pretreated with various concentrations of Rp-8-Br-cGMPS for 20 min then stimulated with or without 1  $\mu\text{g}/\text{mL}$  ephrin-A5 for 15 min. Error bars refer to SEM. \*significant differences from the samples without pretreatment with Rp-8-Br-cGMPS. (F) Time-dependent effect of Rp-8-Br-cGMPS (10  $\mu\text{M}$ ). \*Significantly different from the samples without pretreatment with Rp-8-Br-cGMPS. ( $p < 0.05$ ;  $t$  test). [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]





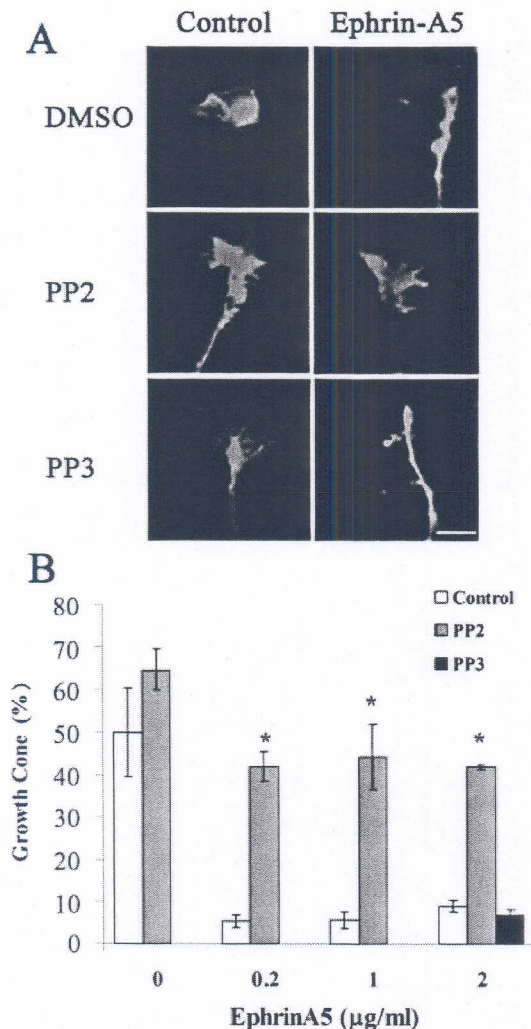
**Figure 7** PTP activity is required for ephrin-A5-induced growth cone collapse. (A) Hippocampal explants were pretreated with 250  $\mu\text{M}$   $\text{Na}_3\text{VO}_4$  or 50  $\mu\text{M}$  PAO for 30 min before treatment with 0.2  $\mu\text{g}/\text{mL}$  clustered ephrin-A5. (B and C) Percent of neurites with growth cones remained in explants treated with ephrin-A5 in the presence or absence of inhibitors pretreatment (250  $\mu\text{M}$   $\text{Na}_3\text{VO}_4$  or 50  $\mu\text{M}$  PAO for 30 min). \*Significantly different from samples without inhibitors ( $p < 0.05$ ;  $t$  test). [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]

activation of the PI-3 kinase/Akt pathway. To further test the roles of PI-3 kinase, effects of two inhibitors, wortmannin ( $K_d = 5 \text{ nM}$ ), which irreversibly blocks the catalytic site by covalent bonding (Wipf and Halter, 2005), and LY294002 ( $KD = 1.4 \mu\text{M}$ ) that competitively blocks the ATP-binding (Vlahos et al., 1994), were examined. At concentrations that were effective in retinal neurons (Wong et al., 2004), neither inhibitor showed any effects on ephrin-A5-induced hippocampal growth cone collapse [Supplementary Fig. 4(B)].

## DISCUSSION

In this study, we showed that ephrin-A5 induced efficient collapse of hippocampal growth cones. Under the conditions in our studies, the cultured E18 rat embryonic hippocampal explants grew dense neurites in radial fashion, and about half of these neurites had growth cones. The reason why not every neurite has a

growth cone is not known. It may reflect the dynamic nature of growth cones, which expand and contract continuously (Gallo and Pollack, 1997). It is also possible that a subpopulation of the neurons grow neurites without growth cones. Thus, a combination of different factors may contribute to the lack of growth cones on some neurites. Ephrin-A5 stimulation leads to a loss of most growth cones, leaving only about 5–15% of neurites with growth cones, depending on the concentration of the ligand and exposure time. Using this convenient assay, we demonstrate that ephrin-A5 inhibits Erk but activates JNK in primary hippocampal neurons. However, the regulation of these two biochemical activities is not critical for growth cone collapse by ephrin-A5. In addition, we have determined that several enzymes, MLCK, PKG, PTP, as well as the Src family kinases, are required for ephrin-A5-induced hippocampal growth cone collapse. Furthermore, this study reveals that PKA and PI-3 kinase pathways are not central to this process.



**Figure 8** Src inhibitor PP2 prevents ephrin-A5-induced growth cone collapse. (A) Fluorescence images of hippocampal growth cones pretreated with DMSO control, 1  $\mu$ M PP2 or 1  $\mu$ M negative control PP3 for 30 min before treatment with 0.2  $\mu$ g/mL ephrin-A5 for 15 min. PP2, but not PP3, preserved growth cone from ephrin-A5-induced collapse. (B) Percent of neurites with growth cones in the explants treated with ephrin-A5 with pretreatment of DMSO control, PP2, and PP3. \*Significantly different from the control treatment ( $p < 0.05$ ;  $t$  test). [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]

### Roles of Erk and JNK-MAP Kinases in Ephrin-Induced Growth Cone Collapse in Hippocampal Neurons

We show here that ephrin-A5 inhibits the basal Erk activity and activity induced by both BDNF and FGF. Several earlier studies have reported that ephrin-A1

and ephrin-B1 both inhibit Erk activity in established cell lines (Elowe et al., 2001; Miao et al., 2001; Tong et al., 2003; Ojima et al., 2006), consistent with our present studies. However, several other reports showed that ephrin-A1 activated Erk in several cell lines (Pratt and Kinch, 2002; Tang et al., 2007), and ephrin-B2 stimulated Erk activity in EphB1-overexpressing CHO cells (Vindis et al., 2003; Vihanto et al., 2006). While it is not clear what leads to these contradicting findings, it is possible that established cell lines may have accumulated as yet undefined molecular changes that alter responses to ephrins. Differences are also possibly due to cell type-specific expression of different signaling molecules that regulate Erk activity. These observations suggest that ephrins could exert opposite biochemical effects depending on specific cellular environment, and also raise the critical question of what are the physiological consequences of ephrin stimulation. Using primary hippocampal neurons, we showed that ephrin-A5 consistently inhibits Erk activity, indicating that Erk inhibition is likely to be physiologically relevant, since primary cells are less likely to accumulate artifactual molecular changes during cell culture process.

Studies using NG108 cells showed that down-regulation of Erk activity by ephrin-B1 is mediated through interaction of the receptor EphB2 with Ras-GAP, an enzyme which activates Ras GTPase (Elowe et al., 2001; Tong et al., 2003). It is likely that Ras-GAP also mediates ephrin-A5-induced Erk inhibition in hippocampal neurons. What biological functions of ephrins are regulated by Erk activity changes is not clear at present. Previous studies in NG108 cells showed that blocking down-regulation of Erk activity with a DN RasGAP also blocked retraction of cellular processes induced by ephrin-B1 (Elowe et al., 2001; Tong et al., 2003), suggesting that Erk down-regulation was necessary for ephrin-B1-induced process retraction. However, an alternative explanation is that RasGAP also regulates other small GTPases such as R-Ras that are critical for ephrin-induced growth cone collapse. Consistent with this notion, a recent study showed that R-Ras activity was inhibited by ephrin-A5, and this inhibition was required for the collapsing activity, since expression of a CA R-Ras blocked ephrin-A5-induced collapse (Dail et al., 2006). In contrast, a CA H-Ras, which activates Erk but not the R-Ras pathway, failed to prevent ephrin-A5-induced collapse (Dail et al., 2006), consistent with our observations in this study that both CA MEK1 and B-Raf which activate Erk activity failed to block ephrin-A5-induced collapse. These observations together suggest that at least in the hippocampal



neurons, down regulation of Erk activity is not critical for ephrin-A5-induced growth cone collapse.

Previous studies showed that Semaphorin 3A and Slit2 required Erk activation to induce retinal growth cones collapse (Campbell and Holt, 2003; Piper et al., 2006). Our studies show that Erk activation is not an integral part of the pathway leading to growth cone collapse by ephrin-A5 in the hippocampal neurons. Thus, it is likely that different guidance molecules or different neurons use distinct pathways to induce growth cone collapse.

Since Erk activity is regulated by ephrins, but this regulation is not required for growth cone collapse in the hippocampal neurons, what is the function of Erk regulation? The answer to this question is currently unknown. However, it is possible that Erk activity participates in subtle modulation of axon guidance not detected in the collapse assay. Another possibility is that the inhibition of Erk activity by ephrins may be responsible for the anti-oncogenic activity of EphA receptors in non-neural tissues (Miao et al., 2001; Bardelli et al., 2003). It is also possible that Erk regulation is important for biological functions yet to be defined. Another interesting issue is whether regulation of Erk activity occurs in the cell soma and the growth cones, and whether different pools are regulated differentially. Future studies are needed to address these issues.

We also showed in this study that ephrin-A5 activates JNK. Several studies reported that ephrin-B1 activates JNK, through interaction with Nck (Stein et al., 1998; Becker et al., 2000). However, JNK activity is not required for ephrin-A5-induced hippocampal growth cone collapse, since an inhibitor, SP600125, at a concentration which successfully inhibited JNK activity in these neurons, showed no effects on ephrin-A5-induced collapse, suggesting that JNK activation may be important for functions other than growth cone collapse.

### Signaling Molecules and Pathways that are Required for Ephrin-A5-Induced Hippocampal Growth Cone Collapse

In contrast to inhibitors of the Erk and JNK pathways, Staurosporine prevented ephrin-A5-induced growth cone collapse. In addition, Staurosporine allowed growth of filopodia even in the presence of ephrin-A5, indicating that the inhibitor blocks kinases required for growth cone collapse. Since Staurosporine is a broad-spectrum inhibitor, we examined effects of inhibiting specific kinases. These analyses identify several kinases that are required for the ephrin-A5-induced collapse. A specific MLCK Inhibitor Peptide 18 (Lukas et al., 1999) inhibits collapse by

ephrin-A5. It has been shown that Eph receptor activation modulates the activity of Ephexin leading to Cdc42/Rac1 and PAK1 inhibition (Wahl et al., 2000; Shamah et al., 2001). MLCK is negatively regulated by Cdc42/Rac1 activity via PAK1 (Sanders et al., 1999). Thus, it is likely that Eph receptor activation inhibits Cdc42/Rac1 and PAK1, leading to the activation of MLCK, which in turn regulates growth cone collapse. The function of MLCK is likely to be mediated through the phosphorylation of myosin regulatory light chain (MRLC) leading to myosin activation (Luo, 2002). This is consistent with several previous studies which have demonstrated that activation of RhoA and its downstream target Rho-associated kinase (ROCK) is also required for growth cone collapse induced by ephrins and semaphorins (Sanders et al., 1999; Wahl et al., 2000; Shamah et al., 2001; Dontchev and Letourneau, 2002; Gallo et al., 2002; Luo, 2002; Swiercz et al., 2002), since ROCK can also phosphorylate MRLC (Amano et al., 1996; Ueda et al., 2002). In addition, ROCK may promote MRLC activation by inhibiting activation of MRLC phosphatase (Kaibuchi et al., 1999). Our observations reported here provide additional evidence that signaling mechanisms leading to myosin activation play key roles in repulsive axon guidance.

Our results also show that cGMP-activated PKG is involved in ephrin-A5-induced growth cone collapse. The PKG inhibitor Rp-8-Br-cGMPS has the strongest effect on ephrin-A5-induced growth cone collapse at 10  $\mu$ M [Fig. 6(E)]. Higher concentrations had no effects. The mechanisms underlying these differential effects are currently unknown. It is possible that at higher concentrations, the inhibitor may exert nonspecific inhibition on targets required to maintain growth cone morphology in the presence of ephrin-A5. The observation that inhibiting PKG activity blocks ephrin-A5-induced growth cone collapse is consistent with the previous finding that PKG activity is required in ephrin-B1-induced *Xenopus* retina and Semaphorin 3A-induced DRG growth cone collapse (Dontchev and Letourneau, 2002; Mann et al., 2003). Increases in intracellular concentration of cGMP has been shown to switch axon repulsion to attraction by Semaphorin 3D, the chemokine SDF-1, and a GABA(B) receptor agonist baclofen (Song et al., 1998; Xiang et al., 2002). In addition, cGMP signaling has been shown to be required for growth cone repulsion mediated by netrin-DCC-UNC5 interaction in *Xenopus* spinal cord neurons (Nishiyama et al., 2003). Thus, activation of PKG could lead to either attraction or repulsion, depending possibly on the interplay of intracellular signaling pathways (Nishiyama et al., 2003). PKG may affect ion channels,  $Ca^{2+}$  release, and myosin contractility to regulate growth



cone motility (Silveira et al., 1998; Hofmann et al., 2000). Our findings are consistent with the idea that PKG signaling is important in growth cone guidance (Ming et al., 1997; Song et al., 1998). It also indicates that different repulsive regulators, ephrin-A5, ephrin-B1, netrin-1, and Semaphorin 3A may share common downstream signaling pathways. Since Staurosporine exhibits potent inhibition of ephrin-A5-induced growth cone collapse and also inhibits MLCK and PKG, we conclude that effects of Staurosporine may be in part due to MLCK and PKG inhibition. However, Staurosporine generates enlarged and complex growth cone morphology (see Fig. 5), but neither MLCK nor PKG inhibitor produces this effect. A combination of these two inhibitors also failed to generate this morphology (data not shown). Thus, it is possible that the complex morphology is generated by Staurosporine inhibition of other unidentified targets.

Two inhibitors of PTP activity, showed profound inhibition of growth cone collapse by ephrin-A5, indicating that PTP activity plays key roles in Eph receptor signal transduction. The identity of this activity is currently not clear. Previous studies have identified small molecular weight phosphatases and a phosphotyrosine phosphatase receptor, PTPRO, that associates with Eph receptors and serve to terminate receptor activation (Parri et al., 2005; Shintani et al., 2006). These phosphatases are unlikely to be the activity needed for growth cone collapse, since inhibition of these phosphatases should promote, rather than inhibit the collapse. A different PTP, the SH2-domain containing phosphotyrosine phosphatase Shp2, has been shown to associate with EphA2 in PC3 cells derived from a prostate cancer (Miao et al., 2000), and in hippocampal neurons (Shi and Zhou, unpublished observations). However, whether Shp2 activity is required for ephrin-A5-induced growth cone collapse is currently under investigation.

Previous studies have shown that Src family kinases are associated with Eph receptors and mediate retinal axon repulsion by ephrin-A5 (Knoll and Drescher, 2004; Wong et al., 2004). We show here that the Src family kinases are also required for ephrin-A5-induced hippocampal growth cone collapse. This observation suggests that certain downstream pathways and molecules are shared in different types of neurons in ephrin-induced growth cone collapse.

### Other Pathways that are not Required for Ephrin-A5-Induced Growth Cone Collapse

Inhibition of two other pathways analyzed does not seem to interfere with ephrin-A5 signal transduction

leading to growth cone collapse. PKA and PI3-K have been implicated in regulating axon migration (Igarashi and Komiya, 1991; Lohof et al., 1992; Zheng et al., 1994; Ming et al., 1997; Song et al., 1997; Xiang et al., 2002; Mikule et al., 2003; Nishiyama et al., 2003; Henley and Poo, 2004; Wong et al., 2004; Wen and Zheng, 2006). Our studies show that both inhibitors and activators of PKA lack influence on growth cone collapse by ephrin-A5, and inhibitors to PI3-K also have no effects, suggesting differences in mechanisms underlying guidance in different types of neurons or by different guidance cues. What is interesting is that ephrin-A5 stimulation appears to down-regulate PKA activity [Supplementary Fig. 3(C)], suggesting that PKA regulation may play a role in other neuronal functions.

Our studies here indicate that ephrin-A5 regulates multiple signal transduction pathways, implicating functions in many different biological and pathological processes. In addition, we have shown that activation of only a subset of these pathways, namely Src, but not Erk, JNK, PKA, or PI-3 kinase, is required for hippocampal growth cone collapse. This study further demonstrates that phosphotyrosine phosphatase, PKG, and MLCK activities are required for ephrin-A5-induced growth cone collapse. Future studies are necessary to determine whether these activities are regulated by ephrin-A5.

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