EphA activation overrides the presynaptic actions of BDNF

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Bi C, Yue X, Zhou R, Plummer MR. EphA activation overrides the presynaptic actions of BDNF. J Neurophysiol 105: 2364–2374, 2011. First published March 16, 2011; doi:10.1152/jn.00564.2010.—The adult pattern of neural connectivity is shaped by repulsive and attractive factors, many of which are modulated by activity. Although much is known about the actions of these factors when studied in isolation, little is known about how they interact. To address this question, we examined the effects of sequential or coapplication of brain-derived neurotrophic factor (BDNF) and Fc-conjugated ephrin-A5 or EphA5 in cultured embryonic hippocampal neurons. BDNF promotes neurite outgrowth and synapse formation, and when applied acutely, it elicits an increase in ongoing synaptic activity. Members of the ephrin family of ligands and receptors can be repulsive and prevent formation of synaptic contacts. Acute exposure to either ephrin-A5-Fc or EphA5-Fc transiently enhanced synaptic activity when applied alone, but when applied prior to BDNF, they dramatically reduced the electrophysiological effects of the neurotrophin. Conversely, BDNF had no effect on subsequently applied ephrin-A5-Fc or EphA5-Fc. Consistent with this, ephrin-A5-Fc also prevented BDNF-induced activation of p42/44 MAPK. The effect of ephrin-A5-Fc appears to be presynaptic, as it prevented the BDNF-induced increase in spontaneous miniature postsynaptic current frequency, whereas EphA5-Fc did not. These results suggest that these factors can be categorized differently, with the contact-mediated activation of EphA receptors by ephrin-A5 overriding the diffusion-mediated effect of BDNF.

synaptogenesis; synaptic plasticity; synaptic transmission; neurotrophin; ephrin

DURING DEVELOPMENT of the nervous system, growing neurites are guided to their appropriate targets by a variety of cues. Chief among these are the neurotrophins and the ephrins, which are responsible for both attractive and repulsive interactions. The neurotrophin family is comprised of four diffusible ligands that bind to three high-affinity cognate receptors and one low-affinity receptor (for review, see Chao 2003; Huang and Reichardt 2003; Teng and Hempstead 2004). The neurotrophins are multifunctional molecules that influence both neuronal survival and outgrowth. One of the neurotrophins, brain-derived neurotrophic factor (BDNF), has also been implicated in activity-dependent refinement of neural connections (for review, see Frost 2001; Nagappan and Lu 2005; Poo 2001). BDNF release from neurons can be elicited by depolarization, a property that has been shown to give BDNF a role in both short- and long-term forms of synaptic plasticity (for review, see McAllister et al. 1999; Rose et al. 2004; Stoop and Poo 1996; Tyler et al. 2002; Waterhouse and Xu 2009).

Unlike the neurotrophins, ephrins are not diffusible molecules but require cell-to-cell contact to exert their effects. Ephrins are divided into two types (A and B ephrins) based on whether they have a glycosylphosphatidylinositol anchor or transmembrane domain, respectively (for review, see Flanagan and Vanderhaeghen 1998). Like the neurotrophin receptors (trkA–C), the receptors for the A and B ephrins (EphA and EphB) are receptor tyrosine kinases. Unlike the neurotrophins, however, ephrin signaling can be bidirectional with both the membrane-bound ligand and the membrane-bound receptor able to initiate intracellular signaling cascades upon binding (Bruckner et al. 1997; Holland et al. 1996).

The functional roles of ephrins and Eph receptors are diverse, but involvement in synaptic plasticity and the formation of topographic maps are prominent features in the nervous system (for review, see Knoll and Drescher 2002; Lai and Ip 2009; McLaughlin and O’Leary 2005). The effect of ephrin-A/EphA binding is generally repulsive, and complementary distributions of the ligand and receptors are consistent with connectivity patterns in a variety of brain structures.

Although a great deal is now known about the actions of neurotrophins and ephrins on synaptic connectivity, information about interaction between these factors is limited (Fitzgerald et al. 2008; Marler et al. 2008; Nie et al. 2010). The generally opposite nature of their effects (attraction vs. repulsion) and their different modes of action (diffusion vs. contact) suggest that one may take precedence over the other. The goal of this work was to examine neurotrophin-ephrin interactions, focusing on the effects of BDNF and ephrin-A5 on synaptic activity in cultured embryonic hippocampal neurons. We found that application of either soluble ephrin-A5-Fc or EphA5-Fc elicited a transient increase in ongoing synaptic activity. When tested in combination with BDNF, pre-exposure to ephrin-A5-Fc prevented the effect of BDNF, which, however, had no influence on the response to subsequently applied ephrin-A5-Fc. An analysis of spontaneous transmitter release suggested that the action of ephrin-A5 was presynaptic. This finding was supported by experiments in which interrupting EphA receptor signaling presynaptically rescued the response to BDNF. EphA5-Fc also reduced the effect of subsequently applied BDNF, but our experiments suggest that the mechanism is not presynaptic. Taken together, our data indicate that the contact-dependent action of ephrin-A5 supercedes the diffusion-based effect of BDNF.

METHODS


Primary hippocampal cell cultures. Embryonic day 18 fetuses of time-mated pregnant Sprague-Dawley rats were removed in accordance with protocols approved by the Institutional Animal Care and Use Committee of Rutgers University. Fetal hippocampi were dissected and dissociated by trypsinization, followed by trituration
through fire-polished Pasteur pipettes. Cells were plated in poly-
d-lysine-coated 35-mm Nunc dishes at 4 × 10^5 cells/dish. Cultures were maintained in serum-free medium (SFM) composed of a 1:1 mixture of Ham’s F-12 and Eagle’s MEM supplemented with 25 
µg/ml insulin, 100 µg/ml transferrin, 60 µM putrescine, 20 nM 
progesterone, 30 nM selenium, and 6 mg/ml glucose. Cells were 
grown at 37°C in 5% CO₂.

Electrophysiological recordings. Whole-cell patch-clamp recordings were performed after 12–14 days in vitro (DIV). Currents were recorded with an Axopatch 200 amplifier, digitized at 2.9 kHz, and filtered at 5 kHz. Pyramidal-type cells were recorded in voltage clamp mode withVm set to −50 mV. The external bath solution [neuron 
recording solution (NRS)] was (in mM) 1.67 CaCl₂, 1 MgCl₂, 5.36 
KCl, 137 NaCl, 17 glucose, 10 HEPES, and 13.15 sucrose. The 
pipette solution contained (in mM) 105 Cs-methanesulfonate, 17.5 
CsCl, 10 HEPES, 0.2 EGTA, 8 NaCl, 2 Mg-ATP, 2 Na₂-ATP, 0.3 
Na₂-GTP, and 20 phosphocreatine. The pH of the internal solution 
was set to 7.3 with CsOH. The typical range of pipette resistance was 
3–5 MΩ. BDNF (20 ng/ml), 2 µg/ml ephrin-A5-Fc, and 2 µg/ml 
EphA5-Fc solutions for perfusion were diluted in NRS. The concen-
tration of BDNF was chosen to favor selective activation of the 
high-affinity trkB receptor (Chao and Hemphstead 1995). The concen-
tration of the ephrin/Eph fusion proteins was chosen to match what 
has been used in other studies (e.g., Elowe et al. 2001; Tong et al. 
2003). Vehicle control refers to a 10⁻⁶ BSA/PBS solution, which was 
used to prepare BDNF and Ephrin-A5-Fc/EphA5-Fc stock solutions. 
For control recordings, 2 µg/ml human IgG Fc fragment (Jackson 
ImmunoResearch, West Grove, PA) was prepared in vehicle solution. 
To record spontaneous miniature postsynaptic currents (mPSCs), 1 
µM tetrodotoxin was added to the NRS, BDNF, Ephrin-A5-Fc, and 
EphA5-Fc solutions, which were applied with a microperfusion sys-
tem (Ogata and Tatebayashi 1991). Perfusion of test solutions was 
as indicated in the figures. When no test solution is indicated, NRS 
was applied to the cells.

Data analysis. Data were analyzed by integrating the synaptic 
currents for each sweep (synaptic charge) and then grouping into 
1-min bins. The baseline is defined as the average charge during the 
2 min prior to switching the perfusion solution from NRS to the first 
experimental condition. Percent increases were calculated by dividing 
the binned charge during experimental exposure by the baseline value.
Magnitudes of secondary responses superimposed on initial responses 
(e.g., the peak response to BDNF subsequent to application of ephrin-
A5-Fc) were calculated by taking the percent baseline at the peak 
response (average percent baseline at 8–9 min) and subtracting from 
that the percent baseline at the onset of the second perfusion and an 
offset value used to compensate for the decline in the initial response.
Student’s t-test was used for statistical comparisons (two-tailed, P < 
0.05 indicating significance).

Neuronal transfection. To interrupt signaling by ephrin-A5-Fc, 
cells were transfected with a dominant-negative, kinase-deficient EphA DNA 
construct linked with green fluorescent protein [EphA5(K—)−GFP or 
EphA3(K—)−GFP] (Yue et al. 2002). The neurons were transfected 
with this construct using the calcium-phosphate (Ca-Pi) method at 5 
DIV and were recorded 9 days later. The plasmid-Ca-Pi coexpression 
was carried out on dissociated hippocampal neuronal cultures. 
Briefly, for each 35 mm dish, 3 µg of DNA (at 0.5 µg/µl) was mixed with 
2 M CaCl₂ and 20 µl sterile water and then added with 30 µl 2X 
HBSS by drop while vortexing. HBSS (2X) contained 50 mM 
HEPES, 1.5 mM NaCl, 10 mM KCl, and 15 mM glucose (all 
from Sigma, St. Louis, MO). After keeping the DNA mixture for 20 
min precipitation in the dark, cells were washed with DMEM (Sigma) 
three times; 80% of the medium exchanged each time. DMEM (1 ml) 
was left in the dishes after the final round of washing. The DNA-Ca-Pi 
mixture (60 µl) was added to each dish. Cells were incubated with the 
DNA for 40 min in the incubator. The medium was then discarded and 
the dish washed with SFM three times and incubated for 1 h at 37°C in 
5% CO₂ for thorough clearing of the remaining DNA-Ca-Pi re-
agents. Finally, the medium was replaced with the original condi-
tioned medium taken from the original dishes, and the cells were 
returned into the incubator.

Biochemistry. Dissociated hippocampal neurons were treated with 
20 ng/ml BDNF, with or without 2 µg/ml ephrin-A5-Fc for varying 
time periods. Western blot analysis was used to assess the extent of 
p42/44 MAPK phosphorylation using a phosphorylation-specific an-
tibody. The blots were then reprobed for total p42/44 MAPK to 
evaluate relative protein levels. Intensity was quantified with ImageJ.

RESULTS

Ephrin-A5-Fc and EphA5-Fc increase synaptic activity in 
embryonic hippocampal neurons. To examine ephrin-BDNF 
interactions, we first measured the effects of ephrin-A5-Fc or 
EphA5-Fc alone on synaptic activity in cultured embryonic 
hippocampal neurons. When placed in tissue culture, hip-
 pocampal neurons form networks that exhibit synaptically 
driven, ongoing activity. Whole-cell patch-clamp recording 
was used to measure baseline activity for several minutes until 
it became stable, following which, soluble ephrin-A5-Fc or 
EphA5-Fc (2 µg/ml) was perfused continuously for 20 min (Fig. 1). Application of either molecule elicited a transient 
increase in synaptic activity at short latency. Synaptic charge 
increased by ~50% within 1 min of ephrin-A5-Fc application 
(152 ± 17% of baseline, n = 8) and returned to baseline within 
10 min, despite maintained exposure to the ligand (Fig. 1, A 
and D). Similarly, application of EphA5-Fc produced a rapid 
doubling of synaptic activity (220 ± 22% of baseline, n = 6), 
which also decayed to baseline within 10 min (Fig. 1, B and E). 
In both cases, the elevated activity declined despite the main-
tained presence of ephrin-A5-Fc or EphA5-Fc. Application of 
2 µg/ml Fc fragment alone in vehicle solution had no effect on 
its own (98.9 ± 11% of baseline, n = 8) or on the response to 
subsequently applied BDNF (195 ± 20% of baseline, n = 8). 
This effect was limited to evoked release of transmitter. Mea-
surement of spontaneous miniature currents showed no effect 
of exogenously applied ephrin-A5-Fc or EphA5-Fc, either on 
event frequency or average current amplitude (Fig. 1, C and F).

Ephrin-A5-Fc inhibits the synaptic actions of BDNF. To 
study ephrin-BDNF interactions, we used a protocol in which 
a stable baseline was established for 3 min, followed by a 
5-min application of either vehicle or a test molecule, followed 
by a 15-min application of a second test molecule. Our initial 
recordings tested the effect of ephrin-A5-Fc on the already 
well-characterized response to BDNF (Levine et al. 1995). 
Application of vehicle for 5 min produced no change in synaptic 
activity, and subsequent application of 20 ng/ml BDNF 
causd a doubling of synaptic activity (average at 8–9 
min: 255 ± 20%, n = 8), which began within 1 min of BDNF 
application, declined slowly, yet remained elevated during the 
15 min of exposure to BDNF (Fig. 2A). This response to 
BDNF, however, was virtually eliminated by a 5-min preap-
lication of ephrin-A5-Fc (Fig. 2B). Instead of the expected 
doubling of activity, only a slight increase was seen (Fig. 2C; 
107 ± 10%, n = 20). The solid gray line in Fig. 2B shows the 
time course predicted if the response to BDNF were added to 
the response to ephrin-A5-Fc. To ensure that the inhibition of 
the BDNF response was not exclusive to the specific 
concentrations that we used or the sequential application pro-
cedure, we repeated the experiments with different conditions. 
First, we used coapplication of ephrin-A5-Fc and BDNF (Fig.
and F change significantly the response to ephrin-A5-Fc (Fig. 2, of ephrin-A5-Fc and BDNF. In this case, BDNF still failed to application. BDNF was preapplied, followed by coapplication from washout of BDNF during application of the ephrin, we BDNF on the response to ephrin-A5-Fc could have resulted (163 ± 40%, n = 10). Because the absence of an effect of BDNF on the response to ephrin-A5-Fc could have resulted from washout of BDNF during application of the ephrin, we also used a coapplication procedure in addition to sequential application. BDNF was preapplied, followed by coapplication of ephrin-A5-Fc and BDNF. In this case, BDNF still failed to change significantly the response to ephrin-A5-Fc (Fig. 2, E and F; 172 ± 38%, n = 5). EphA5-Fc reduces the synaptic actions of BDNF. It is known that ephrin signaling is bidirectional, with both the ligand and receptor able to engage intracellular signaling pathways. We therefore conducted parallel experiments with soluble EphA5-Fc to determine whether binding to ephrin-A ligand could also modulate the synaptic responses to BDNF (Fig. 3). As with ephrin-A5-Fc, pre-exposure to EphA5-Fc reduced the synaptic response to subsequently applied BDNF (Fig. 3A; 153 ± 11%, n = 9; compare with response following vehicle in Fig. 2A). For EphA5-Fc, however, the reduction in the BDNF response was less complete and most dramatic toward the end of the 15-min application period. To ensure that the incomplete inhibition was not due to washout of EphA5-Fc during the perfusion of BDNF, we also tested coapplication of BDNF and EphA5-Fc after 5-min exposure to EphA5-Fc (Fig. 3B). The response was very similar to that observed with sequential application (178 ± 18%, n = 7). In both cases, the peak response to BDNF was significantly lower following EphA5-Fc application (Fig. 3C), although the largest inhibitory effect was observed 12 min after the beginning of BDNF application.

To determine whether the response to receptor activation by EphA5 would be inhibited by BDNF, we reversed the order of application. As with ephrin-A5-Fc, however, BDNF did not have a significant effect on the response to EphA5-Fc when applied either sequentially (199 ± 20%, n = 8, vs. 169 ± 18%, 2C, bar labeled “ephr-A5 (+B)”)] and found a block of the BDNF effect comparable with that with sequential application (99.9 ± 5.7%, n = 5). In addition, we tested 2 μg/ml ephrin-A5-Fc with 50 ng/ml BDNF and 0.5 μg ephrin-A5-Fc with 20 ng/ml BDNF. For these additional concentrations, the responses were consistent with our initial observations. Despite using the higher concentration of BDNF, the normal enhancement of synaptic activity was virtually eliminated by ephrin-A5-Fc (103 ± 6.8%, n = 6). Even 0.5 μg/ml ephrin-A5-Fc was able to reduce the response to BDNF, although not as completely as 2 μg/ml ephrin-A5-Fc (124 ± 12%, n = 8).

The effect of ephrin-A5-Fc on the response to BDNF was not mirrored by reverse application. When a 15-min exposure to ephrin-A5-Fc was preceded by vehicle, the expected transient increase in activity was observed (Fig. 2D; 184 ± 11%, n = 10). When the exposure to ephrin-A5-Fc was preceded by a 5-min application of BDNF, synaptic activity still increased to a level predicted by application of ephrin-A5-Fc alone (163 ± 40%, n = 10). Because the absence of an effect of BDNF on the response to ephrin-A5-Fc could have resulted from washout of BDNF during application of the ephrin, we also used a coapplication procedure in addition to sequential application. BDNF was preapplied, followed by coapplication of ephrin-A5-Fc and BDNF. In this case, BDNF still failed to change significantly the response to ephrin-A5-Fc (Fig. 2, E and F; 172 ± 38%, n = 5).
n = 5) or coapplied (Fig. 3, D–F; 199 ± 20%, n = 8, vs. 167 ± 29%, n = 9).

Inhibitory and excitatory ephrin actions are independent. The experiments described above show that both ephrin-A5-Fc and EphA5-Fc elicit an increase in synaptic activity and reduce the response to BDNF. To determine whether these two processes are separable, we increased the interval between addition of ephrin/Eph and exposure to BDNF, such that the transient excitatory response to the former had ended before application of the latter. As shown in Fig. 1, the increased synaptic activity produced by ephrin-A5-Fc or EphA5-Fc lasted ~10 min, so in these experiments, BDNF was not applied until 15 min after the onset of ephrin/Eph. To establish a baseline for comparison, cells were exposed to vehicle solution for 15 min prior to adding BDNF (Fig. 4A), and the magnitude of the BDNF effect was quantified at 3–4 min after the start of BDNF perfusion (18–19 min into the recording).

As expected, synaptic charge increased during the BDNF perfusion (198 ± 16%, n = 10). When ephrin-A5-Fc was applied 15 min before the BDNF, the response to BDNF was virtually eliminated (86 ± 3.8%, n = 6), even though the excitatory action of ephrin-A5-Fc had decayed completely (Fig. 4B). EphA5-Fc also reduced the response to BDNF, even though its excitatory effect was over (Fig. 4C; 145 ± 6.4%, n = 10), although the inhibitory effect was not as pronounced with the longer delay between EphA5-Fc exposure and application of BDNF.

The effect of ephrin-A5-Fc is presynaptic. We have shown previously (Alder et al. 2005) that BDNF enhances postsynaptic N-methyl-D-aspartate (NMDA) receptor activity independently of its presynaptic modulation of transmitter release (for review, see Lessmann 1998), suggesting that ephrin-A5-Fc and EphA5-Fc may also act at different loci. To gain some understanding of the locus at which ephrin-A5-Fc exerted its effects, we recorded and quantified mPSC frequency. As already shown, when applied for 20 min, neither ephrin-A5-Fc nor EphA5-Fc had a significant effect on mPSC frequency (Fig. 1, C and F). As expected, however, a 15-min application of

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**Fig. 2.** Application of ephrin-A5-Fc prevented the increase in activity normally elicited by brain-derived neurotrophic factor (BDNF). A: time course of synaptic activity during 5-min application of ephrin vehicle (Veh), followed by 15-min application of 20 ng/ml BDNF. Note the lack of response to vehicle and the large and relatively long-lasting response to BDNF (n = 8). B: time course of response to 15-min application of 20 ng/ml BDNF when preceded by 5-min application of 2 μg/ml ephrin-A5-Fc (n = 20). Note the almost complete absence of a response to BDNF after ephrin-A5-Fc. The gray line represents the summed response expected if BDNF had increased synaptic activity to the same level as shown in A and if the response to BDNF adds linearly onto the response produced by ephrin-A5-Fc. For experiments such as this, in which 2 conditions were tested and the responses superimposed, the percent baseline at the onset of the second perfusion and an offset factor to compensate for the decline in the response to ephrin-A5-Fc were subtracted from the peak response to calculate the magnitude of response to BDNF. C: comparison of the peak response (average of 8–9 min after beginning of application) with BDNF when preceded by vehicle solution, preceded by ephrin-A5-Fc (ephr-A5) or coapplied with ephrin-A5-Fc (ephr-A5 (+B)). The response to BDNF was significantly lower (**P < 0.01) when either preceded by or coapplied with ephrin-A5-Fc. D: time course of response to 5-min application of BDNF vehicle, followed by 15-min application of 2 μg/ml ephrin-A5-Fc. Unlike vehicle solution, ephrin-A5-Fc produced a transient increase in synaptic charge (n = 10). E: time course of response to 15-min coapplication of ephrin-A5-Fc and BDNF preceded by 5-min application of BDNF (n = 5). Note that the actual activity closely resembled the predicted response (gray line). F: comparison of the peak response with ephrin-A5 (average of 7–8 min after the beginning of application) when preceded by vehicle or by BDNF. The responses did not differ significantly between the 2 conditions (P > 0.7).

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BDNF produced an elevation of mPSC frequency (Fig. 5A; 200 ± 16%, n = 7), whereas the preceding application of vehicle had no effect. When applied in tandem with BDNF, an initial 5-min application of ephrin-A5-Fc produced no change in mPSC frequency. Surprisingly, subsequent application of BDNF failed to increase mPSC frequency (Fig. 5B; 99.3 ± 8%, n = 6). This result suggests that ephrin-A5-Fc prevented the presynaptic effect of BDNF on mPSC frequency but had little overt effect of its own.

We also tested for potential presynaptic effects of EphA5-Fc and found none. Application of EphA5-Fc had no effect of its own on mPSC frequency and did not alter the response to BDNF (Fig. 5B, C; 213 ± 16%, n = 8). None of the conditions tested produced an effect on mPSC amplitude (Fig. 5D–F).

**Fig. 3. Application of EphA5-Fc reduces the increase in activity normally elicited by BDNF.** A: time course of response to 15-min application of 20 ng/ml BDNF when preceded by 5-min application of 2 μg/ml EphA5-Fc (n = 9). The gray line represents the summed response expected if BDNF had increased synaptic activity to the same level as shown in Fig. 2A and added onto the response produced by EphA5-Fc. Note that although BDNF elicited an increase in synaptic activity, the increase wasn’t as large as in control nor did it last as long. B: time course of response to 15-min coapplication of BDNF and EphA5-Fc preceded by 5-min application of EphA5-Fc (n = 7). The response profile was similar to that observed when EphA5-Fc and BDNF were applied sequentially instead of together. C: comparison of the peak response (average of 8-9 min) to BDNF when preceded by vehicle solution, preceded by EphA5-Fc but applied on its own (EphA5) or preceded by and then coapplied with EphA5-Fc [EphA5 (+B)]. The response to BDNF was significantly lower in the latter 2 cases than when applied alone (**P < 0.01; **P < 0.001). D: time course of response to 5-min application of BDNF vehicle, followed by 15-min application of 2 μg/ml EphA5-Fc. Vehicle caused no change in baseline activity; EphA5-Fc produced a transient increase in synaptic charge (n = 8). E: time course of response to 15-min coapplication of EphA5-Fc and BDNF preceded by 5-min application of BDNF (n = 9). Note that the actual activity closely resembled the predicted response (gray line). F: responses to EphA5-Fc (average of 7–8 min after the beginning of application) did not differ significantly when preceded by vehicle or by BDNF (P > 0.3).

Presynaptic transfection of an EphA dominant-negative receptor restores the response to BDNF.** The experiments described above suggest that application of exogenous ephrin-A5-Fc interferes with the presynaptic response to BDNF. To test this more directly, we transfected cells with EphA3(K–)-GFP or EphA5(K–)-GFP constructs so that transfected cells could be visualized (Yue et al. 2002). It has been shown previously that these constructs act promiscuously, inhibiting, at a minimum, endogenous EphA5 and EphA3 (Yue et al. 2002) and likely preventing activation of multiple members of the endogenous EphA family.

To examine our prediction regarding the presynaptic locus of ephrin-A5-Fc inhibition of the BDNF response, we first recorded from untransfected cells in the vicinity of other transfected cells. The rationale for these experiments is that the cell that is being recorded would show normal EphA activation, whereas EphA receptors in the presynaptic terminals from nearby transfected cells would be inhibited. If the inhibition of the BDNF response by ephrin-A5-Fc is presynaptic, then the net result should be restoration of the response to BDNF due to an absence of inhibition by ephrin-A5-Fc. This is what was observed (Fig. 6A). Following the initial, direct effect of ephrin-A5-Fc, synaptic activity increased upon subsequent application of BDNF. Comparison of the responses to earlier recordings (Fig. 2B) shows clearly that there was a substantial recovery of the BDNF-induced increase in synaptic charge. This suggests that signaling by presynaptic EphA receptors was prevented, thus interrupting presynaptic inhibition by ephrin-A5-Fc and enabling BDNF activation of trkB to pro-
presynaptic cells.

presumably via normal EphA signaling in the untransfected

tors were inhibited, BDNF modulation was still prevented,

did not occur, presumably because postsynaptic EphA recep-

To examine the possibility that the postsynaptic response
involves NMDA receptors, we applied ephrin-A5-Fc in the
presence of the NMDA receptor antagonist 2-amino-5-phos-
phonopentanoic acid (AP5). This procedure prevented the
direct effect of ephrin-A5-Fc, providing further support for the
idea that this action is either triggered or manifested postsyn-
aptically (Fig. 6C).

**Ephrin-A5-Fc prevents BDNF-induced phosphorylation of p42/44 MAPK.** MAPK has been implicated as a downstream effector for some of the synaptic actions of BDNF (Alonso et al. 2004; Dijkstra and Ghosh 2005; Gooney et al. 2004; Gottschalk et al. 1999; Matsumoto et al. 2006; Slack et al. 2004; Ying et al. 2002), suggesting that phosphorylation of MAPK induced by exposure to BDNF could be reduced by ephrin-A5. To begin to characterize potential signaling pathways that could mediate the clear presynaptic effect of ephrin-A5-Fc on the response to BDNF, a Western blot analysis using a phospho-specific anti-MAPK antibody was performed. These experiments showed that treatment with 20 ng/ml BDNF alone increased the level of p42/44 MAPK phosphorylation in hippocampal neurons (Fig. 7A) compared with the constitutive phosphorylation level in the absence of any treatment (Fig. 7A). As predicted from our electrophysiological recordings, coapplication of 2 μg/ml ephrin-A5-Fc reduced this phosphorylation (Fig. 7A). Quantification from four separate experiments showed the reproducibility of this effect (Fig. 7B).

**DISCUSSION**

The central finding of this study is that with regard to effects on synaptic transmission in the embryonic hippocampus, the contact-triggered action of ephrin-A prevents the diffusion-mediated effect of BDNF. Similarly, exposure to EphA-Fc reduces the action of subsequently applied BDNF, although lack of effect on spontaneous mPSCs suggests that the mechanism is different than that of ephrin-A-Fc. Conversely, BDNF has little if any effect on the transient increase in synaptic activity produced by subsequently applied ephrin-A-Fc or EphA-Fc. Such an interaction is consistent with aspects of the interplay between BDNF and ephrin-A in other systems. In purified cultures of retinal ganglion cells, for example, application of ephrin-A5 completely prevented the enhancement of branching and terminal arborization elicited by BDNF, whereas BDNF had relatively little effect on the growth cone collapse induced by ephrin-A5 (Fitzgerald et al. 2008). Interestingly, in that same study, more reciprocity between BDNF and ephrin-A was observed in retinal explants, suggesting that in some instances, exposure to BDNF can overcome the effects of ephrin-A.

Ephrin-A5-Fc and EphA5-Fc appear to interfere with the response to BDNF at different loci. Previous work from our laboratory and others has shown that BDNF acts presynaptically and postsynaptically (for review, see Lessmann 1998; Lu and Gottschalk 2000), with the postsynaptic actions involving a variety of ion channels and neurotransmitter receptors (for

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**Fig. 4.** The excitatory and inhibitory effects of ephrin-A5-Fc and EphA5-Fc are independent. A: time course showing synaptic charge during a 25-min recording. Vehicle solution was perfused for 15 min, followed by 10 min of 20 ng/ml BDNF. Exposure to BDNF still produced an approximate doubling of synaptic charge (n = 10). B: application of 2 μg/ml ephrin-A5-Fc elicited a rapid increase in synaptic charge, but delayed application of 20 ng/ml BDNF had no effect (n = 6). Note that the increased synaptic activity produced by ephrin-A5-Fc had declined back to baseline prior to application of BDNF. The gray line shows the control response to BDNF. C: the partial inhibitory effect of EphA5-Fc on the response to BDNF is also independent of the direct excitatory effect (n = 10). The gray line shows the control response to BDNF. D: comparison of the response to BDNF when preceded by application of vehicle, ephrin-A5-Fc, or EphA5-Fc. Both ephrin-A5-Fc and EphA5-Fc significantly reduced the response to BDNF, although the inhibition produced by the former is virtually complete compared with the latter (*P < 0.05; **P < 0.01 relative to vehicle).
review, see Rose et al. 2004) including NMDA receptors (Arvanian and Mendell 2001; Crozier et al. 1999, 2008; Jarvis et al. 1997; Kolb et al. 2005; Levine et al. 1998; Levine and Kolb 2000; Lin et al. 1998, 1999; Slack et al. 2004; Song et al. 1998). Our analysis of knockout mice lacking the synaptic vesicle protein Rab3A showed that the pre- and postsynaptic actions of BDNF occur independently and can be studied in isolation (Alder et al. 2005). To relate these findings to the current study, we examined the frequency of spontaneous mPSCs, which can be an indicator of presynaptic release probability (Bouron 2001), although not necessarily in all cases (Atasoy et al. 2008; Sara et al. 2005). Application of ephrin-A5-Fc did not change mPSC frequency, but it did eliminate the effect of subsequently applied BDNF, indicating that ephrin-A5-Fc works presynaptically to prevent the BDNF-induced upregulation of synaptic activity. By using a dominant-negative EphA construct, we were able to confirm this observation by rescuing the response to BDNF. Transfection of presynaptic...
neurons disabled the ephrin inhibition and allowed the BDNF response to occur. Conversely, postsynaptic transfection had no apparent impact on the ephrin inhibition of the BDNF response. Application of EphA5-Fc had no effect on mPSC frequency, and the response to subsequently applied BDNF was normal, suggesting that EphA5-Fc may be acting postsynaptically, but we have not yet characterized this effect in detail.

A surprising finding in this study was that exogenously applied ephrin-A5-Fc and EphA5-Fc produced a transient increase in ongoing synaptic activity. At present, the mechanism of this effect is unknown. It could result from changes in synaptic properties, intrinsic membrane currents that regulate action-potential firing, or even changes in transmitter reuptake. Glial cells contain ephrin ligands and receptors (Murai et al. 2003), and negative effects on spines of astrocytic ephrin-A3 binding to dendritic EphA4 have been found (Murai et al. 2003). EphA4/ephrin-A3 interactions have also been shown to alter levels of glutamate transporters, consequently impacting induction of long-term potentiation (LTP) (Filosa et al. 2009). Moreover, EphA4 is located in presynaptic vesicles (Bouvier et al. 2010) and could potentially be involved in vesicle trafficking. Application of ephrin-A3 has even been shown to reduce the probability of transmitter release from astrocytes (Nestor et al. 2007). While it is unknown at present whether these same properties are shared by ephrin-A5 or EphA5, the potential exists that the transient change in synaptic activity could arise from very indirect causes.

To date, we have obtained three pieces of information regarding the direct excitatory response produced by application of ephrin-A5-Fc. First, the lack of effect on mPSC frequency does not support a change in presynaptic release probability, although a caveat is the recent demonstration that evoked and spontaneous vesicle release can draw from different vesicle pools (Atasoy et al. 2008; Sara et al. 2005), so this conclusion is not definitive. Second, postsynaptic transfection of the kinase-deficient EphA construct eliminates the direct effect of ephrin-A5-Fc. This could be consistent with changes in intrinsic membrane characteristics or trafficking of neurotransmitter receptors. Third, the direct effect is blocked by application of AP5, suggesting perhaps that the response is triggered postsynaptically by an influx of calcium and may be activity dependent, thus explaining the lack of effect on spontaneous mPSCs but the presence of an effect on evoked activity. Nonetheless, it is clear that additional work needs to be done to distinguish these possibilities. Moreover, because of the possibility of promiscuous binding of ephrins and their receptors, we cannot be certain that the same ligand/receptor combination responsible for the inhibition of the BDNF effect is also responsible for the enhancement of synaptic activity.

Based on the electrophysiological and biochemical results, a model can be proposed that includes our findings to date. Activity-dependent release of BDNF provides a general impetus for neurite outgrowth and synapse elaboration. When cells expressing presynaptic EphA contact potential postsynaptic neurons containing ephrin-A, they would lose responsiveness to BDNF, which could contribute to a net repulsive interaction, particularly once the transient, excitatory direct effect of the ephrin/Eph pairing has decayed. When cells that possess ephrin-A presynaptically contact potential targets containing EphA, they would experience no reduction in their responsiveness to BDNF and could therefore establish synaptic connections. An important corollary is that there must still be sufficient presynaptic EphA to mediate the inhibitory effects of exogenously applied ephrin-A5-Fc on the response to BDNF. Normally, a reduced BDNF response would only occur when presynaptic EphA was directly apposed to postsynaptic ephrin-A. A synapse containing presynaptic EphA without matching ephrin-A would show a reduced effect of BDNF in response to exogenously applied ephrin-A5-Fc but would not necessarily be eliminated in culture, because it would not be in contact with endogenous ephrin-A.

Interestingly, BDNF-induced modulation of synaptic transmission by EphAs and ephrin-As may also be present in the adult nervous system. EphA and ephrin-As are present postnatally as well as in the developing hippocampus (for review, see Zhou 1998). The expression of EphAs and ephrin-As in the adult brain suggests that the interplay between ephrins and BDNF may persist into adulthood and potentially have an impact on synaptic plasticity and cognitive function.

We chose to study ephrin-As because we were interested in antagonistic interactions, and ephrin-Bs have been associated with enhancement of NMDA receptor function (Armstrong et al. 2006; Dalva et al. 2000; Henderson et al. 2001; Takasu et al. 2002), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor trafficking (Essmann et al. 2008), promotion of LTP (Armstrong et al. 2006; Contractor et al. 2002; Gruwold et al. 2001, 2004; Henderson et al. 2001), increased synapse formation (Kayser et al. 2008; McClelland et al. 2009, 2010; Shi et al. 2003, 2004; Henderson et al. 2001), and negative effects on spine and astrocyte morphology (Nestor et al. 2007). While it is unknown at present whether these same properties are shared by ephrin-A5 or EphA5, the potential exists that the transient change in synaptic activity could arise from very indirect causes.
al. 2009), and potentiation of BDNF effects on local protein synthesis (Miyata et al. 2005), making them likely to act synergistically with BDNF rather than antagonistically. Indeed, the excitatory actions of ephrin-A5-Fc may be mediated by EphB2, since it is capable of binding to this receptor, even though it is a member of the B ephrin group (Himanen et al. 2004).

Our specific interest in EphA5/ephrin-A5 was based on their distribution in the hippocampus and effects on synaptic plasticity (for review, see Martinez and Soriano 2005). Application of EphA5-IgG to hippocampal slices has been shown to impair LTP induction without affecting baseline transmission (Gao et al. 1998), which is consistent with inhibiting the postsynaptic effects of BDNF (for review, see Rose et al. 2004). The same group, however, also observed a sustained, presynaptic enhancement of synaptic transmission by ephrin-A5-IgG, for which we found no evidence, although it is interesting that the presynaptic/postsynaptic relationship for ephrin-A5-IgG and EphA5-IgG was reminiscent of what we found in this study. In addition, deficits in hippocampal synapse formation and improper pathway formation have been identified in mice engineered to constitutively express EphA5-Fc and ephrin-A5 knockout mice (Martinez et al. 2005; Otal et al. 2006; Yue et al. 2002).

The inhibitory effects of ephrin-A5 on BDNF-induced neural activity may be mediated by the downregulation of p42/44 MAPK activity. In the presence of ephrin-A5, BDNF failed to induce the rise of p42/44 MAPK activity. Ephrins have been shown to inhibit MAPK activation by a number of extracellular ligands, including PDGF, EGF, and glutamate (Elowe et al. 2001; Grunwald et al. 2001; Miao et al. 2001). Similar to the current study, opposite regulation of ERK1/2 has also been described by other investigators (Nie et al. 2010). There is evidence that the inhibition is mediated by Ras GTPase-activating protein (RasGAP) (Elowe et al. 2001). RasGAP is physically associated with Eph receptors, and a dominant-negative mutant of RasGAP prevented MAPK downregulation by ephrin-B2 (Elowe et al. 2001). How signals from trk and Eph receptors are integrated is not known. One candidate, the ankyrin repeat-rich membrane-spanning protein, appears to bind to both trk and Eph receptors (Kong et al. 2001; Luo et al. 2005) and may be a key to signal integration between different receptors. Together, these observations suggest that Eph receptors converge onto the Erk/MAPK pathway to modulate trk receptor activity. The mechanism by which this occurs is likely to be complex, with direct interaction of EphA/ephrin-A5s with trkB potentially being involved (Fitzgerald et al. 2008; Marler et al. 2008).

There are other studies that have examined antagonistic relationships between BDNF and other factors. It has been shown in the auditory system that BDNF and neurotrophin 3 (NT-3) can elicit opposite effects on neuronal phenotype (Adamson et al. 2002), and it has been shown that chronic treatment with NT-3 can prevent the presynaptic effects of BDNF (Pual et al. 2001). An important difference between those studies and this, however, is that the effects that we observe are nonreciprocal. Our working hypothesis is that contact-mediated interactions overrule diffusion-mediated ones. Although the activity-dependent release of BDNF provides some spatial specificity, it cannot do so with the precision of cell-to-cell interaction. Such an attraction, followed by a pruning model, has already been suggested (Gao et al. 1999); this could provide an important mechanism that limits the action of released BDNF to appropriate targets.

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DISCLOSURES

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