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Cumulative Activation of Akt and Consequent Inhibition of Glycogen Synthase Kinase-3 by Brain-Derived Neurotrophic Factor and Insulin-Like Growth Factor-1 in Cultured Hippocampal Neurons

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ABSTRACT

Brain-derived neurotrophic factor (BDNF) and insulin-like growth factor-1 (IGF-1) seem to play key roles in mediating neuronal plasticity in the hippocampus. In the current studies, we have used cultured hippocampal neurons to study possible interactions between the two growth factors in modulating neuronal signaling pathways. BDNF and IGF-1 were found to each effectively activate the neuroprotective Akt pathway, with the magnitude of activation being at least additive when cultures were simultaneously treated with supramaximal concentrations of peptides. Likewise, a cumulative inhibitory Akt-dependent phosphorylation of proapoptotic glycogen synthase kinase-3 was observed. Immunofluorescent studies demonstrated that a single population of neurons responded to BDNF and IGF-1. In contrast, the magnitude of BDNF-stimulated extracellular signal-regulated kinase (ERK) activation was found to be much greater than that of IGF-1-stimulated ERK, such that the difference in magnitude stimulated by BDNF in the presence and absence of IGF-1 did not reach statistical significance. Consistent with the observed agonist-stimulated activation of Akt, BDNF and IGF-1 were both found to act as neurotrophins, enhancing neuronal survival under low-insulin culture conditions. Maximal survival was achieved when both growth factors were present. These findings provide insight into the significance of multiple growth factors stimulating activation of ERK and Akt in the central nervous system. In some cases, the magnitude of activation required to elicit biological responses may be achieved only with a combination of compounds.

The hippocampus represents a useful region for studying neuroplasticity. Neurons are exposed to multiple growth factors, stimulating such diverse responses as long-term potentiation, neuroprotection, and neurogenesis. Although the mechanisms underlying these processes have not been completely delineated, evidence supports roles for insulin-like growth factor-I (IGF-1) and brain-derived neurotrophic factor (BDNF). For example, peripheral infusion of IGF-1 has been found to induce hippocampal neurogenesis (Aberg et al., 2000) and the peptide has been shown to directly act on hippocampal progenitor cells to stimulate proliferation (Aberg et al., 2003). The growth factor has also been shown in vitro to inhibit induction of apoptosis in hippocampal neurons (Nitta et al., 2004; Zheng and Quirion, 2004). Likewise, BDNF has been shown to inhibit in at least one model system, apoptosis in hippocampal neurons, although reportedly less effectively than IGF-1 (Zheng and Quirion, 2004).

Extracellular-regulated kinase (ERK) mitogen-activated protein kinase and Akt (protein kinase B) are thought to mediate relevant signaling pathways, because activation of the kinases has been found to confer neuroprotection in several models of apoptosis (Tamatani et al., 1998; Hetman et al., 1999; Matsuzaki et al., 1999; Yamaguchi et al., 2001; Zheng and Quirion, 2004). Both have similarly been reported to play a role in regulating hippocampal neurogenesis (Aberg et al., 2003). Moreover, BDNF and IGF-1 have been reported to activate ERK and Akt in various cell types (Yamada et al., 2001; Qiao et al., 2004; Shelton et al., 2004) and have been hypothesized to play important roles in maintaining neuronal survival and mediating plasticity. Although much is known about the cellular effects elicited by treatment with

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ABBREVIATIONS: IGF-1, insulin-like growth factor-1; BDNF, brain-derived neurotrophic factor; ERK, extracellular signal-regulated kinase; GSK-3, glycogen synthase kinase; MAP-2, microtubule-associated protein-2; PBS, phosphate-buffered saline; DAPI, 4,6-diamidino-2-phenylindole; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; PI 3-kinase, phosphatidylinositol 3-kinase.

either IGF-1 or BDNF, little is known about possible positive or negative interactions between the peptides resulting from simultaneous treatment. Identifying such interactions would be useful in understanding complex processes such as depression and aging. Antidepressants, for example, have been reported to increase hippocampal levels of both IGF-1 and BDNF (Duman et al., 1997; Khawaja et al., 2004), and the two factors have been postulated to positively interact in protecting against aging (Mattson et al., 2004). Our current studies directly compared the coupling of receptors for IGF-1 and BDNF to activation of Akt and ERK. Moreover, our studies were designed to examine possible interactions of the two peptides in activating these two important kinases.

Materials and Methods

Materials. Recombinant human BDNF was purchased from Alomone Labs (Jerusalem, Israel), and recombinant human insulin-like growth factor-1 was from Sigma-Aldrich (St. Louis, MO). Wortmannin was obtained from Calbiochem (La Jolla, CA).

Cell Culture. Hippocampal neuronal cultures were prepared as described previously (Cowen et al., 2005), with some modification. Hippocampi were isolated from embryonic day 18 (E18) Sprague-Dawley rats obtained from Charles River Laboratories (Raleigh, NC), and 10^6 cells were plated per poly(D-lysine)-coated 35-mm Petri dish. Cells were maintained in serum-free medium consisting of neurobasal media (Invitrogen, Carlsbad, CA) supplemented with 25 μg/ml insulin, 100 μg/ml transferrin, 60 μM putrescine, 20 nM progesterone, 30 nM selenium, 6 mg/ml glucose, 1 mg/ml bovine serum albumin (Fraction V), fatty acid-free (Calbiochem), and 7.5 U of penicillin-7.5 μg of streptomycin/ml at 37°C (95% air, 5% CO_2). Cytosine arabinoside (0.6 μM) was additionally included to prevent proliferation of non-neuronal cells. Cells remained in culture for 5 to 7 days before use, with the medium aspirated the day before use and replaced with culture medium containing no cytosine arabinoside and reduced concentrations of insulin (0.1 μg/ml) and albumin (0.1 mg/ml). Cultures contained primarily neurons, with greater than 90% of cells staining positive for the neuronal marker microtubule-associated protein (MAP)-2 (Fig. 4).

Immunoblots. Monoclonal anti-phospho-ERK1/ERK2 (Thr202/Tyr204), rabbit polyclonal anti-phospho-Akt (Ser473), rabbit polyclonal anti-phospho-GSK-3α/β (Ser21/9), and rabbit polyclonal anti-total Akt were obtained from Cell Signaling (Beverly, MA). Rabbit polyclonal anti-total ERK1/ERK2 and horseradish peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-total GSK-3α/β was obtained from Calbiochem. Cells were treated with the addition of reagents directly to the culture medium. Cells were then washed with phosphate-buffered saline and routinely lysed with a 26-gauge needle in 25 mM HEPES, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM β-glycerol phosphate, 50 mM NaF, 5 mM EDTA, 1 mM sodium orthovanadate, 250 μM 4-(2-aminoethyl)-benzene-sulfonylfluoride hydrochloride, 0.1% aprotinin, and 10 μg/ml leupeptin. After 30 min on ice, the lysate was centrifuged at 10,000*g* for 10 min at 4°C. Supernatant proteins were separated on 10% resolving gels (Cambrex Bio Science Rockland, Inc., Rockland ME) and transferred to 0.45-μm Immobolin-P polyvinylidene difluoride membranes (Millipore Corporation, Bedford, MA). Bound antibodies were visualized using Enhanced Luminol Chemiluminescence Reagent (PerkinElmer Life Sciences, Boston, MA) and direct exposure to a Kodak Image Station 440CF with a cooled full-frame-capture CCD camera (Eastman Kodak, Rochester, NY). Net intensity of bands was calculated directly from stored images using Kodak Digital Science 1D Image Analysis Software (version 3.5) on defined regions of interest.

Statistics. Results are expressed as the means ± S.E.M. of three or more experiments performed in duplicate. Experimental groups were compared by ANOVA followed by Bonferroni post-tests.

Immunofluorescence. Cells were plated, as described above, in poly(D-lysine)-coated 35-mm Petri dishes. Cultures were washed with phosphate-buffered saline (PBS) and fixed in methanol for 6 min at −20°C. Cultures were incubated for 30 min at room temperature (22°C) in PBS-blocking buffer containing 10% goat serum (Vector Laboratories, Burlingame, CA). Cultures were then incubated at room temperature (22°C) for 1 h with rabbit polyclonal anti-MAP-2 antibody (1:100) or for 2 h with polyclonal anti-phospho-(Ser473)-Akt (1:50), polyclonal anti-TrkB (1:50), or polyclonal anti-IGF-1 receptor β subunit (1:50) (all from Santa Cruz Biotechnology) in PBS containing 1.5% goat serum. Cultures were washed with PBS and incubated for 45 min at room temperature (22°C), with Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:1000) obtained from Invitrogen in PBS containing 1.5% goat serum. Cultures were then washed and coverslipped with Ultra Cruz Mounting Media (Santa Cruz Biotechnology) containing DAPI. Stained cultures were viewed under fluorescence illumination, and images were digitally captured using MetaVue 6.1 image analysis software (Molecular Devices, Sunnyvale, CA).

Cell Viability. Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in which the yellow tetrazolium base compound is reduced by viable cells to a blue formazan product. Cells were incubated at 37°C in humidified air with 5% CO_2 for 40 min with 0.5 mg/ml MTT to allow reduction of MTT. The MTT-containing media were subsequently discarded, and cells containing formazan product dissolved in 0.9 ml of dimethyl sulfoxide. Absorbance at a wavelength of 570 nm (O.D. 570) was measured using a spectrophotometer.

Results

Neurons were routinely cultured in medium containing 25 μg/ml insulin to optimize survival. At this concentration, insulin is known to act at both insulin and IGF-1 receptors (Rosenfeld and Hintz, 1980; Steele-Perkins et al., 1988). Therefore, to avoid baseline occupancy of IGF-1 receptors during our studies, we routinely replaced culture medium the day before use with medium containing a concentration of insulin (0.1 μg/ml) 20-fold lower than the 2 μg/ml K_i of insulin for IGF-1 receptors (Rosenfeld and Hintz, 1980). Neuronal viability was not compromised by the low concentration of insulin during this short-term period of culture. Immunofluorescent studies demonstrated that greater than 90% of cells stained positively for the neuronal marker MAP-2, the TrkB receptor for BDNF, and the β subunit of the IGF-1 receptor (Fig. 4A). Nearly all cells similarly stained positively for the α subunit of the IGF-1 receptor (data not shown).

IGF-1 and BDNF-Stimulated Activation of Akt and ERK. Treatment of hippocampal neurons with BDNF was found to stimulate an approximate 5-fold maximal increase in the level of activated (phosphorylated) Akt and ERK (Fig. 1A), with maximal stimulation observed at 5 ng/ml BDNF. Activation of both kinases was observed within 2 min of treatment and was maximal at 5 min (Fig. 1B). The level of activated ERK and Akt exhibited a time-dependent decrease but remained somewhat elevated even at 1 h.

IGF-1 was found to be similar to BDNF in activating Akt and ERK, with maximal activation occurring at 100 ng/ml IGF-1 (Fig. 2A). However, the magnitude of maximal activation of both pathways was smaller than that stimulated by BDNF. This was particularly striking with respect to ERK activation where the magnitude of activation was minor com-

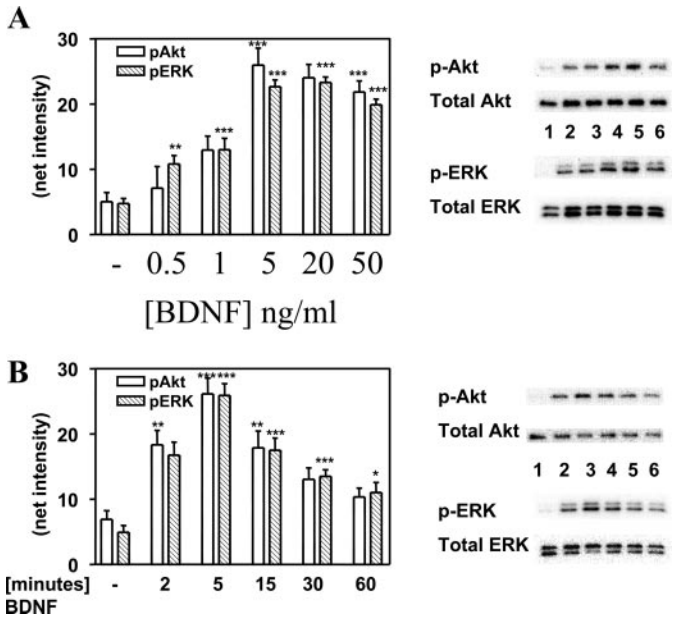

Fig. 1. BDNF stimulates activation of Akt and ERK in cultured hippocampal neurons. A, hippocampal neurons were incubated for 5 min with the indicated concentrations of BDNF and lysed. B, hippocampal neurons were incubated with 20 ng/ml BDNF for the indicated times and lysed. Supernatant was analyzed by immunoblotting with antibody to phospho-Akt (p-Akt), phospho-ERK (p-ERK), total Akt, or total ERK. Results are expressed as the means ± S.E.M. of five (A) or three (B) experiments performed in duplicate. Experimental groups were compared by ANOVA followed by Bonferroni post-tests.*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ versus control. Representative immunoblots are shown.

pared with that of BDNF (Fig. 3B). The kinetics of activation was also somewhat different from that observed for BDNF. Although activation of Akt was detected between 2 and 5 min, it continued to increase for the first 15 min of treatment and then remained stable for the remainder of the 60-min period of study (Fig. 2B).

We next examined the effect of treating hippocampal neurons simultaneously with BDNF and IGF-1. Initial studies examined cotreatment with maximal concentrations of both peptides to determine whether treatment with BDNF was sufficient to maximally activate neuronal ERK and Akt, such that addition of IGF-1 would have no effect. Five-minute time points were chosen for these studies to achieve maximal BDNF activity. The 5-fold activation of Akt stimulated by BDNF was found to not be the maximum achievable in the cultured neurons. To the contrary, the activation of Akt stimulated by simultaneous treatment with 100 ng/ml IGF-1 and 20 ng/ml BDNF was found to be statistically greater than that achieved by either individual peptide and seemed to be at least additive (Fig. 3). There was a trend toward a similar cumulative increase in ERK activity. However, the interaction was difficult to quantify, in that the magnitude of ERK activation stimulated by BDNF was much larger than that stimulated by IGF-1. Therefore, the difference in activation of ERK stimulated by maximal concentrations of BDNF in the presence versus absence of maximal concentrations of IGF-1 did not reach statistical significance.

The demonstration that greater than 90% of cultured cells expressed TrkB and IGF-1 receptors (Fig. 4A) suggested that a single population of neurons was responding to both BDNF

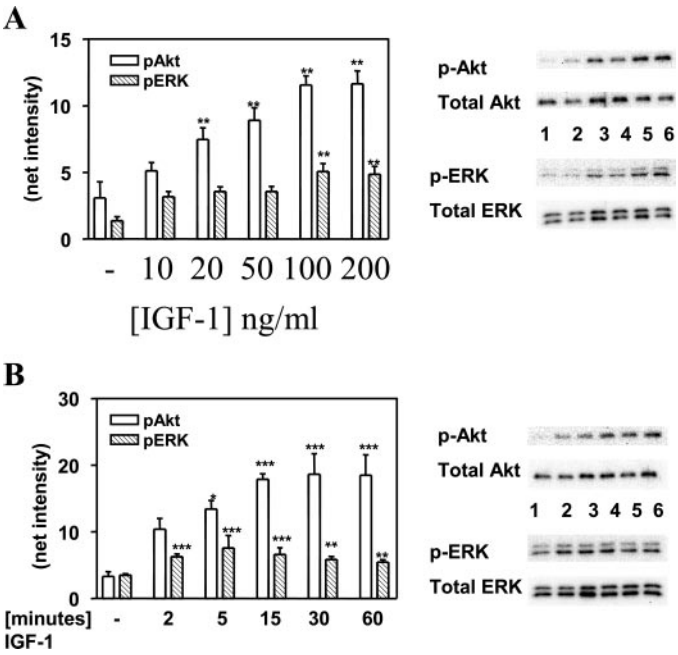

Fig. 2. IGF-1 more effectively stimulates activation of Akt than ERK in cultured hippocampal neurons. A, hippocampal neurons were incubated for 5 min with the indicated concentrations of IGF-1 and lysed. B, hippocampal neurons were incubated with 100 ng/ml IGF-1 for the indicated times and lysed. Supernatant was analyzed by immunoblotting with antibody to phospho-Akt (p-Akt), phospho-ERK (p-ERK), total Akt, or total ERK. Results are expressed as the means ± S.E.M. of three experiments performed in duplicate. Experimental groups were compared by ANOVA followed by Bonferroni post-tests. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ versus control. Representative immunoblots are shown.

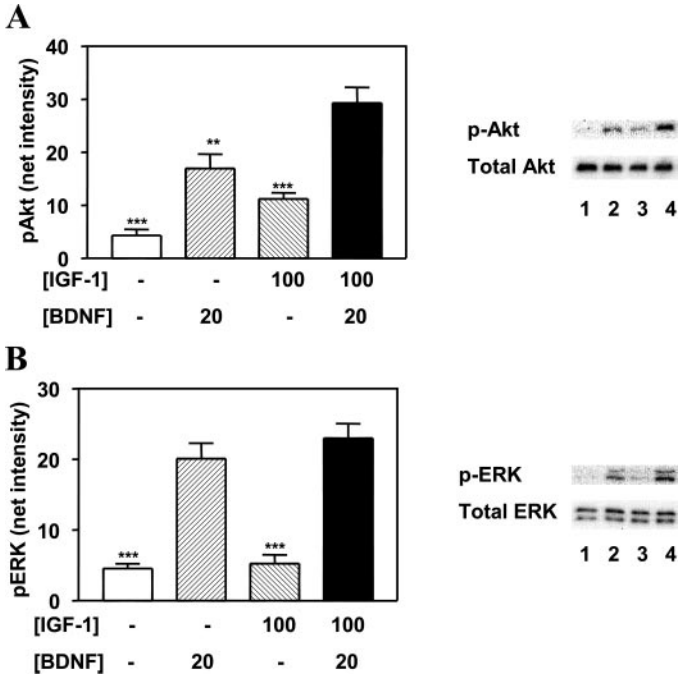

Fig. 3. Maximal concentrations of BDNF and IGF-1 cumulatively activate Akt. Hippocampal neurons were incubated for 5 min with 20 ng/ml BDNF and/or 100 ng/ml IGF-1 and lysed. Supernatant was analyzed by immunoblotting with antibody to phospho-Akt (p-Akt) and total Akt (A) or phospho-ERK (p-ERK) and total ERK (B). Results are expressed as the means ± S.E.M. of four experiments performed in duplicate. Experimental groups were compared by ANOVA followed by Bonferroni post-tests. **, $p < 0.01$; ***, $p < 0.001$ versus IGF-1 plus BDNF. Representative immunoblots are shown.

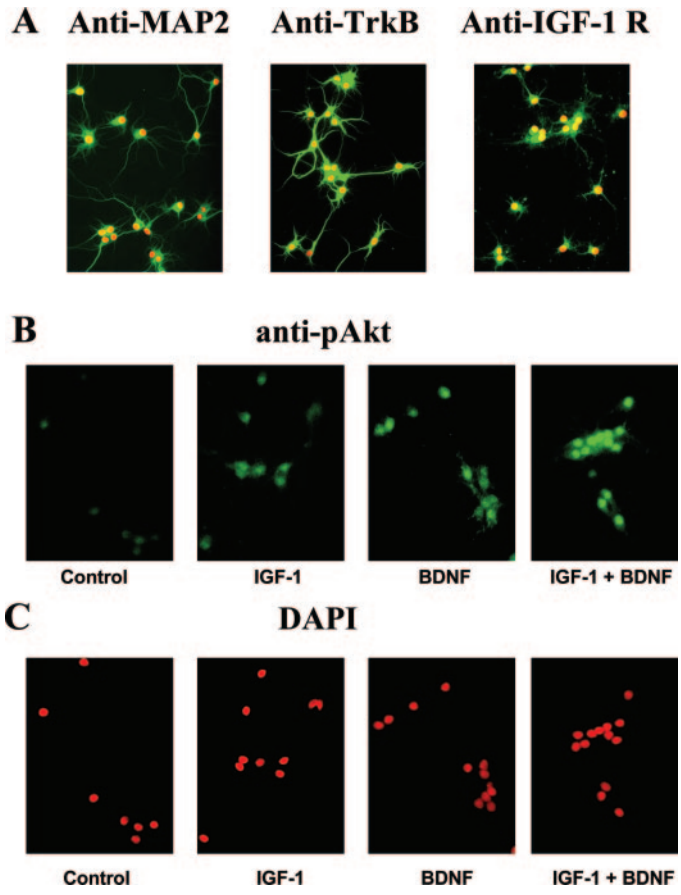

Fig. 4. Neurons responding to IGF-1 and BDNF represent a single cell population. A, hippocampal neurons were stained with anti-MAP-2, anti-TrkB, or anti-IGF-1 receptor antibodies, and nuclei were visualized with the nuclear dye DAPI. B, hippocampal neurons were incubated for 5 min with 20 ng/ml BDNF and/or 100 ng/ml IGF-1 before fixation and permeabilization. Activation of Akt was analyzed by immunoblotting with antibody to phospho-Akt (p-Akt). C, the nuclear dye DAPI was used to visualize all nuclei. Magnification was 20×.

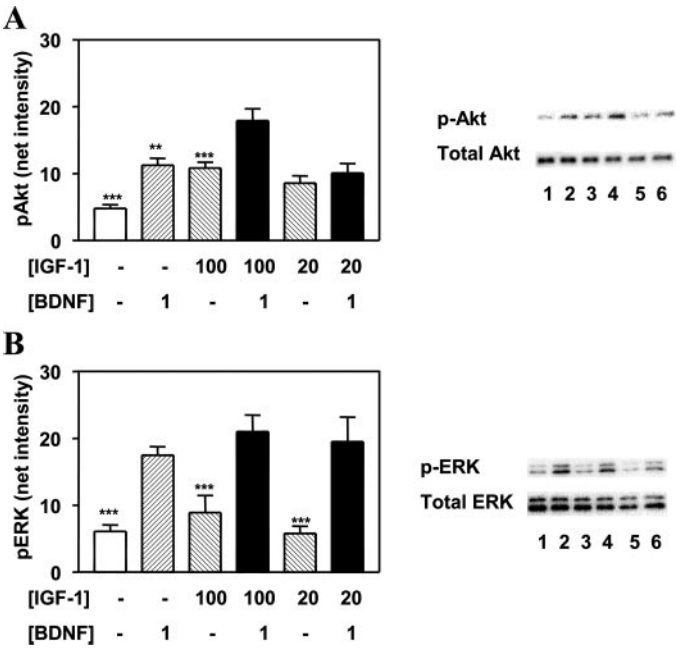

Fig. 5. IGF-1 and BDNF do not exhibit synergist activation of Akt and ERK. Hippocampal neurons were incubated for 5 min with 1 ng/ml BDNF and/or the indicated concentrations of IGF-1 (100 or 20 ng/ml) and lysed. Supernatant was analyzed by immunoblotting with antibody to phospho-Akt (p-Akt) and total Akt (A) or phospho-ERK (p-ERK) and total ERK (B). Results are expressed as the means ± S.E.M. of five experiments performed in duplicate. Experimental groups were compared by ANOVA followed by Bonferroni post-tests. **, $p < 0.01$; ***, $p < 0.001$ versus BDNF plus the relevant concentration of IGF-1. Representative immunoblots are shown.

and IGF-1. However, the possibility existed that a subpopulation of neurons might express receptors at a density below that required to couple effectively to activation of Akt. Therefore, we stained cells with antibody directed at the activated form of Akt to directly confirm that nearly all cells responded to both growth factors. Not surprisingly, we found that untreated (control) neurons exhibited a range of baseline Akt activity (Fig. 4B). However, treatment with either 20 ng/ml BDNF or 100 ng/ml IGF-1 resulted in clear activation, with nearly all neurons exhibiting bright staining for phosphorylated Akt. Therefore, the at least additive activation of Akt found in the immunoblots studies (Fig. 3) resulted from individual neurons simultaneously responding to both compounds.

We next studied cotreatment with submaximal concentrations of peptides to determine whether IGF-1- and BDNF receptor-mediated cell signaling exhibited positive or negative interactions. Simultaneous treatment with a submaximal concentration of BDNF (1 ng/ml) and a maximal concentration of IGF-1 (100 ng/ml) was found to be approximately additive (Fig. 5). In contrast, simultaneous treatment with submaximal concentrations of both compounds was found to cause a magnitude of Akt stimulation no greater than that induced by each separately, suggesting a small, negative interaction.

We next looked at possible heterologous desensitization by studying the effect of pretreating neurons with one of the peptides before treatment with the second peptide. Akt and ERK remained active 30 min after treatment with maximal (20 ng/ml) concentrations of BDNF. Treatment with maximal concentrations (100 ng/ml) concentrations of IGF-1 for an additional 5 min was found to result in a further increase in Akt activity, which seemed to be approximately additive (Fig. 6). Similar results were observed when neurons were pretreated for 30 min with maximal (100 ng/ml) concentrations of IGF-1 before 5 min of treatment with maximal concentrations (20 ng/ml) of BDNF (Fig. 7). Cells pretreated with IGF-1 exhibited an additional increase in Akt activity when treated with BDNF. Total activation was approximately additive. In addition, the activation of ERK stimulated by a combination of IGF-1 and BDNF was greater than that stimulated by BDNF alone. Therefore, we can conclude that pretreatment with one peptide caused no significant inhibition of the receptor-mediated signaling stimulated by the other peptide. In addition, the combined activation seemed to be additive rather than synergistic.

Akt-Dependent Inhibitory Phosphorylation of GSK-3. Proapoptotic GSK-3 is known to be an important downstream target for Akt, with Akt catalyzing an inhibitory serine phosphorylation (Cross et al., 1995). Therefore, we sought to determine whether the cumulative increase in pAkt activity stimulated by IGF-1 and BDNF translated into increased phosphorylation of GSK-3. Both IGF-1 and BDNF were found to stimulate serine phosphorylation of the kinase.

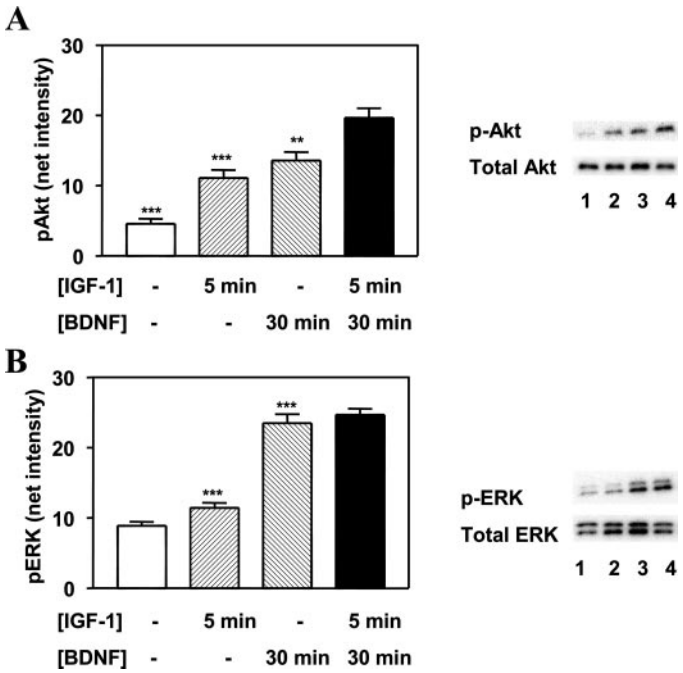

Fig. 6. Pretreatment with BDNF does not inhibit the activity of IGF-1. Hippocampal neurons were pretreated with 20 ng/ml BDNF for 30 min before treatment for 5 min with 100 ng/ml IGF-1. Supernatant was analyzed by immunoblotting with antibody to phospho-Akt (p-Akt) and total Akt (A) or phospho-ERK (p-ERK) and total ERK (B). Results are expressed as the means ± S.E.M. of five experiments performed in duplicate. Experimental groups were compared by ANOVA followed by Bonferroni post-tests. **, $p < 0.01$; ***, $p < 0.001$ versus BDNF plus IGF-1. Representative immunoblots are shown.

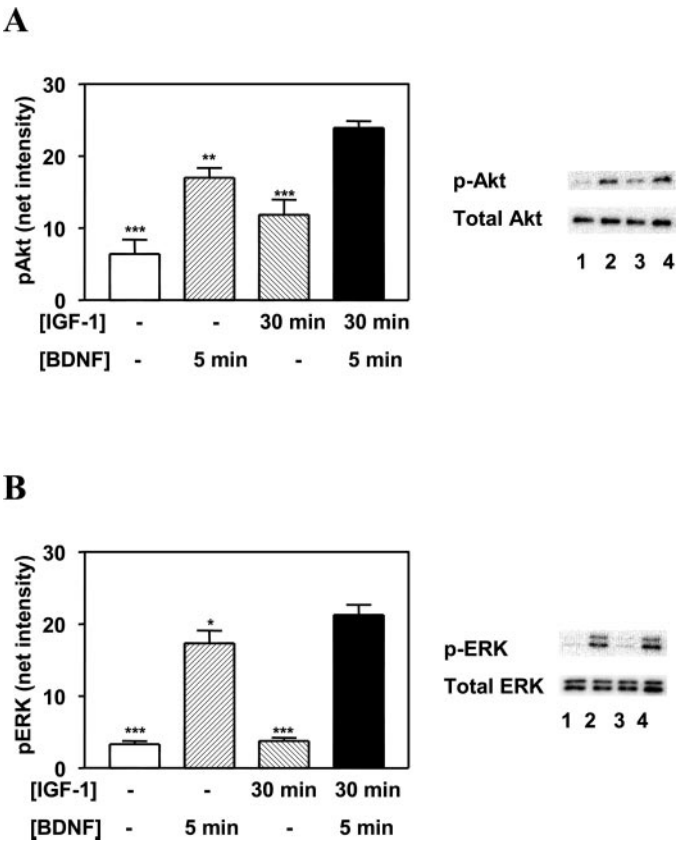

Fig. 7. Pretreatment with IGF-1 does not inhibit the activity of BDNF. Hippocampal neurons were pretreated with 100 ng/ml IGF-1 for 30 min before treatment for 5 min with 20 ng/ml BDNF. Supernatant was analyzed by immunoblotting with antibody to phospho-Akt (p-Akt) and total Akt (A) or phospho-ERK (p-ERK) and total ERK (B). Results are expressed as the means ± S.E.M. of four experiments performed in duplicate. Experimental groups were compared by ANOVA followed by Bonferroni post-tests. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ versus BDNF plus IGF-1. Representative immunoblots are shown.

This was true of the α form, as well as the β form, which was the predominate form of GSK-3 expressed in our cultures. Phosphorylation could be detected within 5 min, although the magnitude was quite variable. In contrast, phosphorylation was consistently observed after 15 min of treatment (Fig. 8A). Combined treatment with maximal concentrations of IGF-1 and BDNF was found to stimulate increases in serine-phosphorylated α and β forms of GSK-3 greater than that stimulated by either individual compound and seemed to be at least additive.

Pretreatment with the phosphatidylinositol 3-kinase (PI 3-kinase) inhibitor wortmannin confirmed that the increased phosphorylation of GSK-3 was predominantly secondary to Akt activation. Wortmannin completely inhibited BDNF- and IGF-1- stimulated activation of Akt, which was of similar magnitude at 15 min (Fig. 8B). The PI 3-kinase inhibitor also inhibited agonist-induced serine phosphorylation of GSK-3 to below basal levels. However, a small increase in the level of phosphorylated Akt and GSK-3 was still observed after treatment with wortmannin in neurons treated with BDNF and IGF-1 compared with neurons treated only with wortmannin. It is not clear whether this represented a minor pathway through which Akt can be activated independent of PI 3-kinase or was secondary to incomplete inhibition of PI 3-kinase.

IGF-1 and BDNF as Neurotrophic Factors. To directly study the neurotrophic actions of BDNF and IGF-1, we used a paradigm in which hippocampal neurons were cultured for the first 24 h under routine conditions in high-insulin-containing media to enhance survival after dissection and harvesting. The culture conditions were subsequently changed to low-insulin conditions for the next 72 h to study the effect of peptides on survival. Cell viability was assessed using the MTT assay in which the yellow tetrazolium base compound is reduced by viable cells to a blue formazan product. Neurons survived and formed processes under the low-insulin culture conditions (Fig. 9). However, a single treatment of 100 ng/ml IGF-1 at the beginning of the 72-h period increased survival by 15% and seemed to have a small positive effect on process formation. Treatment with 20 ng/ml BDNF enhanced survival by approximately 20% and had a more significant impact on process formation. Similar to our findings from the Akt studies, simultaneous treatment with 100 ng/ml IGF-1 and 20 ng/ml BDNF had the largest trophic effect, stimulating a 43% increase in survival ($p < 0.001$ for IGF-1 + BDNF versus either growth factor alone). The combination of growth factors also seemed to promote the most extensive arborization of processes.

Discussion

Regulation of neuroplasticity has been hypothesized to be mediated by multiple growth factors. Many of these factors have been reported to exhibit similar patterns of coupling to cellular signaling pathways. Therefore, it has been unclear whether the presence of multiple growth factors in the hip-

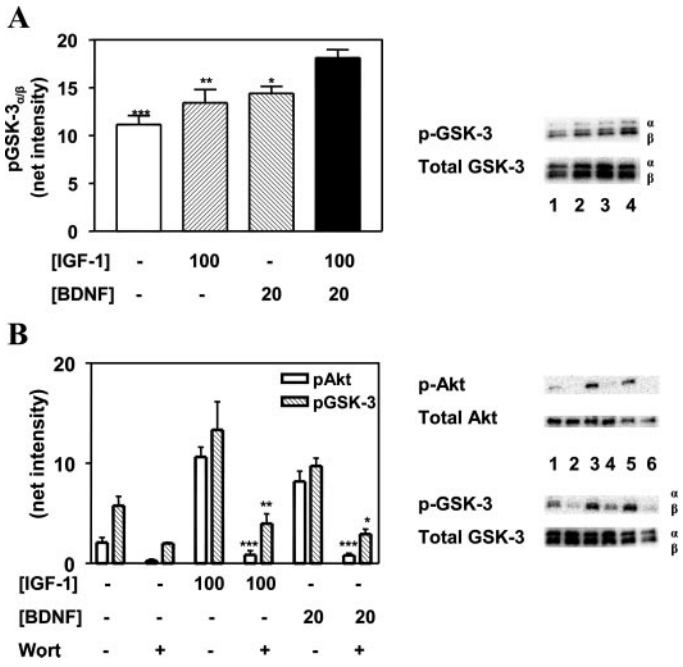

Fig. 8. Maximal concentrations of BDNF and IGF-1 cumulatively stimulate Akt-dependent inhibitory phosphorylation of GSK-3. A, hippocampal neurons were incubated for 15 min with 20 ng/ml BDNF and/or 100 ng/ml IGF-1 and lysed. B, cultures were pretreated for 15 min, where indicated, with 30 nM wortmannin before treatment with BDNF and/or IGF-1. Cultures not treated with wortmannin were pretreated with vehicle (0.2% dimethyl sulfoxide). Supernatant was analyzed by immunoblotting with antibody to phospho-Akt (p-Akt), phospho-GSK-3α/β (p-GSK-3), total Akt, or total GSK-3α/β. Results are expressed as the means ± S.E.M. of five (A) or three (B) experiments performed in duplicate. Experimental groups were compared by ANOVA followed by Bonferroni post-tests. A, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ versus IGF-1 plus BDNF. B, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ versus the relevant treatment in the absence of wortmannin. Representative immunoblots are shown with the α and β forms of kinase indicated.

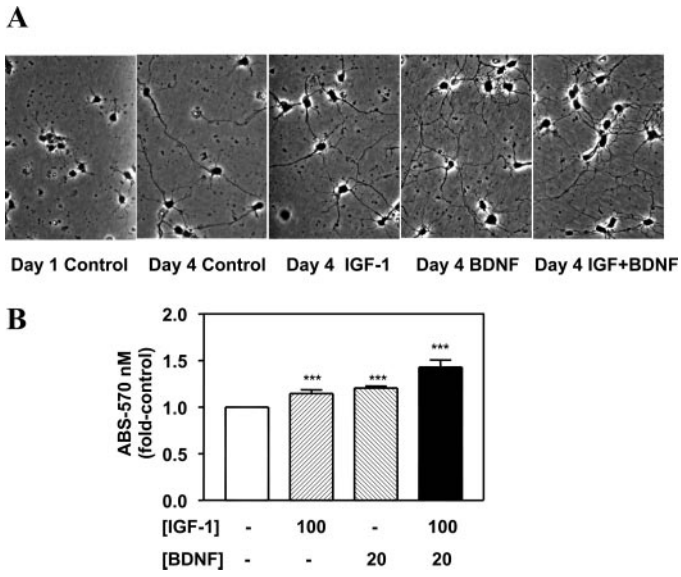

Fig. 9. Cotreatment with maximal concentrations of BDNF and IGF-1 maximally enhances neuronal survival. After the first 24 h of routine culture, neurons were cultured for the subsequent 72 h under low-insulin conditions in the presence or absence of 100 ng/ml IGF-1 and/or 20 ng/ml BDNF. A, phase-contrast photographs (20×) taken, as specified, on either day 1 of culture or on day 4 of culture (72 h after media change). B, cell viability was assessed on day 4 of culture by MTT assay with formation of formazon product detected at a wavelength of 570 nm (O.D. 570). Results were normalized in each experiment by dividing the mean absorbance of treated cultures by the mean absorbance of nontreated cultures to obtain fold-control values and are expressed as the means ± S.E.M. of three experiments.

pocampus represents duplicity or rather reflects a combinatorial approach to regulating highly complex systems. Our findings of BDNF and IGF-1 cumulatively activating Akt and enhancing neuronal survival support the latter hypothesis, as does our finding of differential activation of ERK by the two growth factors.

Although BDNF was found to be much more effective in stimulating activation of ERK than was IGF-1, it should be noted that the efficacy of IGF-1 receptor coupling to ERK may be cell-dependent. The growth factor has been reported to effectively activate both ERK and Akt in endothelial and hematopoietic cells (Qiao et al., 2004; Shelton et al., 2004). In contrast, our finding of relatively poor coupling in hippocampal neurons is consistent with previous reports of poor coupling in hippocampal and cortical neurons (Yamada et al., 2001; Zheng and Quirion, 2004). The very small magnitude of ERK activation stimulated by IGF-1, relative to that stimulated by BDNF, made it difficult to determine in our studies whether IGF-1 and BDNF cumulatively activated ERK. The trend in data were suggestive of this but did not reach statistical significance.

Interestingly, in contrast to our studies, Zheng and Quirion (2004) reported at 5 min of treatment IGF-1 activated Akt in hippocampal cultures more effectively than did BDNF. Our neurons were cultured under somewhat different conditions, possibly resulting in differences in the level of expression of TrkB receptors relative to IGF-1 receptors. Unlike the B27 supplement used in their studies, our cultures did not contain thyroid hormone (T3), which has been reported to transcriptionally repress TrkB synthesis (Pombo et al., 2000). In addition, the concentration of insulin in our medium was decreased before neuronal studies to avoid baseline occupancy of IGF-1 receptors. However, it should be pointed out that findings from both studies were similar at time points greater than 15 min, where IGF-1-stimulated activity was found to be somewhat higher than that of BDNF. This was the result of differences in kinetics, with BDNF-induced activation of Akt being maximal at 15 min and then slowly decreasing. In contrast, IGF-1-activation was not maximal until 15 min of treatment and remained at maximal levels until at least 1 h of treatment.

Our finding of cumulative activation of Akt by BDNF and IGF-1 was somewhat surprising. BDNF stimulated a large activation of Akt at low concentrations (5 ng/ml being maximal), suggesting the possibility that the pathways for Akt activation in neurons might be maximally stimulated by BDNF. In fact, with a K_D for BDNF at the TrkB receptor of 1.8 nM (equivalent to 48 ng/ml; Soppet et al., 1991), it seems that there is a significant TrkB receptor reserve for coupling to Akt and ERK. However, neither maximal BDNF- nor maximal IGF-1-stimulated Akt activity was found to be attenuated by simultaneous treatment with the other growth factor. Apparently, upstream cellular components, including PI 3-kinase and the inositol 1,4,5-trisphosphate-dependent kinases that phosphorylate Akt, were not maximally activated in hippocampal neurons by BDNF. In contrast, Yamada et al., (2001) reported an additive activation of PI

3-kinase in cortical neurons but not an additive activation of Akt.

In studies using submaximal concentrations of peptides, we found no evidence for synergistic interactions between BDNF and IGF-1. Nor did we find any evidence for pretreatment with one peptide causing an inhibition or synergistic activation of the other peptide. Interestingly, simultaneous cotreatment with submaximal concentrations of IGF-1 (20 ng/ml) and BDNF (1 ng/ml) did not result in cumulative Akt activation. Instead, the magnitude of activation was similar to that induced by either compound alone, suggesting a possible small negative interaction.

These findings suggest that TrkB receptor- and IGF-1 receptor- mediated signaling pathways are primarily but not completely segregated. This segregation might be achieved by lipid rafts, in which microenvironments are created containing specific receptors and the relevant signaling molecules. Such colocalization enhances required interactions, while preventing interaction with proteins outside the rafts (Simons and Toomre, 2000). In fact, roles for lipid rafts in BDNF- and IGF-1- stimulated events have been reported. In 3T3-L1 cells, the IGF-1 receptor has been reported to be located in lipid rafts and disruption of the lipid rafts by depletion of cellular cholesterol inhibits IGF-1 receptor-mediated signaling (Huo et al., 2003; Hong et al., 2004). Although TrkB receptors on cortical neurons have been reported to be located primarily in nonraft regions, BDNF has been found to rapidly stimulate receptor translocation to lipid rafts (Suzuki et al., 2004). Significantly, the translocation of TrkB receptors to lipid rafts was reported to be selective, with no translocation of transferrin receptor or epidermal growth factor receptors observed.

Consistent with our findings of IGF-1 and BDNF activating Akt and inhibiting GSK-3, we found that both peptides enhanced neuronal survival. It should be noted that our model system was designed to demonstrate neurotrophic actions rather than neuroprotection. Cells were cultured in media containing minimal requirements, including insulin. Although the concentration of insulin was reduced, it was reduced only to 100 ng/ml, a concentration more than sufficient to fully occupy insulin receptors (Whittaker and Whittaker, 2005). These culture conditions are in contrast to a study in which IGF-1 and BDNF were tested for their efficacy as neuroprotective agents after the withdrawal of B27 supplement from the media of 7-day-old hippocampal cultures (Zheng and Quirion, 2004). IGF-1 was found to be more effective than BDNF in protecting against the complete withdrawal of insulin and other required culture reagents. This may have reflected the closer similarity of insulin to IGF-1 than to BDNF.

Our survival study was also different, in that immature cultures were used such that neurons had not established extensive processes. This may be relevant in that we found that BDNF was more effective than IGF-1 in enhancing process formation. Establishment of synapses can be hypothesized to confer protection by facilitating release of neuronally synthesized growth factors in close proximity to postsynaptic neurons. We are currently exploring how differences in modulation of signaling pathways could account for difference in IGF-1- and BDNF-stimulated process formation. One possibility is the more effective activation of ERK by BDNF. In fact, it has been reported that ERK can mediate the formation of dendritic spines in cultured hippocampal neurons (Goldin and Segal, 2003).

Our findings that BDNF and IGF-1 cumulatively activated Akt and enhanced survival may also be indicative of similar interactions between agonists for other receptors that exhibit overlap in coupling to cellular signaling pathways. Cumulative activation provides a context for understanding the significance of hippocampal neurons expressing receptors for multiple growth factors. It can be hypothesized that some cellular responses require a magnitude of signal activation that cannot be achieved through treatment with a single growth factor. With respect to Akt, possibilities include the various proteins negatively regulated by Akt: GSK-3, Bad, Caspase 9, and the Forkhead family transcription factor FKHRL1 (Cross et al., 1995; Brunet et al., 1999; Datta et al., 1999). In fact, we found that cotreatment with BDNF plus IGF-1 induced greater inhibition (phosphorylation) of proapoptotic GSK-3 than did either individual compound. Such a combinatory model may be useful in understanding some of the numerous complex actions elicited by growth factors in the hippocampus and other brain regions.

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