Inhibition of glycogen synthase kinase-3β (GSK3β) is one of the mechanisms by which phosphatidylinositol 3-kinase (PI3K) activation protects neurons from apoptosis. Here, we report that inhibition of ERK1/2 increased the basal activity of GSK3β in cortical neurons and that both ERK1/2 and PI3K were required for brain-derived neurotrophic factor (BDNF) suppression of GSK3β activity. Moreover, cortical neuron apoptosis induced by expression of recombinant GSK3β was inhibited by coexpression of constitutively active MKK1 or PI3K. Activation of both endogenous ERK1/2 and PI3K signaling pathways was required for BDNF to block apoptosis induced by expression of recombinant GSK3β. Furthermore, cortical neuron apoptosis induced by LY294002-mediated activation of endogenous GSK3β was blocked by expression of constitutively active MKK1 or by BDNF via stimulation of the endogenous ERK1/2 pathway. Although both PI3K and ERK1/2 inhibited GSK3β activity, neither had an effect on GSK3β phosphorylation at Tyr-216. Interestingly, PI3K (but not ERK1/2) induced the inhibitory phosphorylation of GSK3β at Ser-9. Significantly, coexpression of constitutively active MKK1 (but not PI3K) still suppressed neuronal apoptosis induced by expression of the GSK3β(S9A) mutant. These data suggest that activation of the ERK1/2 signaling pathway protects neurons from GSK3β-induced apoptosis and that inhibition of GSK3β may be a common target by which ERK1/2 and PI3K protect neurons from apoptosis. Furthermore, ERK1/2 inhibits GSK3β activity via a novel mechanism that is independent of Ser-9 phosphorylation and likely does not involve Tyr-216 phosphorylation.

It has become increasingly evident that there is a complex balance between survival and apoptotic signaling pathways in neurons that determines whether they survive or die. For example, BDNF1 activates the ERK1/2 and PI3K/Akt pathways in neurons and protects them from several forms of apoptosis, including those induced by DNA damage, microtubule damage, and trophic deprivation (1–6). The relative contribution of the ERK1/2 and PI3K/Akt pathways to neuronal survival depends on the specific type of cellular injury (7). In contrast, activation of the stress-activated protein kinases, including the c-Jun NH2-terminal protein kinase and the p38 MAPK, contributes to apoptosis in cortical neurons (6, 8, 9) and other types of neurons (for a review, see Ref. 10).

Recently, GSK3β was discovered as another apoptosis-inducing kinase in the nervous system (5, 11). Expression of recombinant wild-type GSK3β is sufficient to induce apoptosis in PC12 cells (11), primary cortical neurons (5), and sympathetic neurons (12). The basal activity of GSK3β in PC12 cells and cortical neurons is relatively high, but can be further activated by inhibition of PI3K/Akt signaling (5, 11). Blocking GSK3β suppresses apoptosis induced by PI3K inhibition in PC12 cells, cortical neurons, cerebellar granule cells, and sympathetic neurons (5, 11–13). Although inhibition of GSK3β is not sufficient to inhibit sympathetic neuron apoptosis triggered by nerve growth factor withdrawal (12), it protects against trophic deprivation in PC12 cells, cortical neurons, and cerebellar granule cells (5, 11, 13, 14). These data suggest that inhibition of GSK3β is one of the mechanisms by which PI3K activation protects neurons from apoptosis.

The activity of GSK3β is negatively regulated by phosphorylation at Ser-9 (15) and may be positively regulated by phosphorylation at Tyr-216 (14). Studies in non-neuronal cells suggest that activation of several signaling pathways can phosphorylate Ser-9 in GSK3β and inhibit its activity. These include the PI3K/Akt (15, 16), protein kinase A (17), protein kinase C (18–20), and ERK1/2-activated p90 MAPK (21, 22) pathways, all of which are implicated in promoting neuronal survival. This suggests that GSK3β-induced apoptosis may be inhibited by these signaling pathways via GSK3β phosphorylation at Ser-9. Indeed, cAMP protects cerebellar granule cells from trophic withdrawal-induced apoptosis via protein kinase A-dependent Ser-9 phosphorylation and inhibition of GSK3β (13). Similarly, PI3K-promoted sympathetic neuron survival correlates with GSK3β phosphorylation at Ser-9 (12).

In this report, we present data supporting the hypothesis that ERK1/2 protects neurons from GSK3β-induced apoptosis.

1 The abbreviations used are: BDNF, brain-derived neurotrophic factor; ERK, extracellular signal-regulated kinase; PI3K, phosphatidylinositol 3-kinase; MAPK, mitogen-activated protein kinase; MKK, MAPK kinase; GSK3β, glycogen synthase kinase-3β; GBP, GSK3β-binding protein; DIV, day in vitro; ANOVA, analysis of variance; CREB, cAMP-responsive element-binding protein.
and that inhibition of GSKβ may be a common mechanism by which ERK1/2 and PI3K protect neurons from apoptosis. However, ERK1/2 inhibits GSKβ activity in cortical neurons via a novel mechanism independent of Ser-9 phosphorylation.

**EXPERIMENTAL PROCEDURES**

**Materials**—The following plasmids have been previously described: pON260 (23); rat GSKβ (both wild-type GSKβ and kinase-dead dominant-negative mutant GSKβ) (24); expression constructs for Xenopus GBP (25); hemagglutinin-tagged expression vectors for MKK1 (wild-type MKK1, kinase-dead dominant-negative MKK1(K97M), and constitutively active MKK1(ΔN3-S218E/S222D)) (26) and constitutively active p110 (p110<sup>ΔN</sup>) and dominant-negative p110 (p110<sup>ΔN/KIN</sup>) (27). The anti-GSKβ polyclonal antibody used for immunoprecipitation was from Santa Cruz Biotechnology (sc8257; Santa Cruz, CA). The anti-Akt and anti-phospho-Ser-473 Akt antibodies were from New England BioLabs (Beverly, MA). The anti-ERK2 antibody and the mouse anti-GSKβ/α monoclonal antibody were from Upstate Biotechnology, Inc. The polyclonal antibody to β-galactosidase was from Sigma. The anti-hemagglutinin monoclonal antibody (12CA5) was from Roche Molecular Biochemicals. The rabbit anti-phospho-Ser-9 GSKβ and anti-phospho-Tyr-279 GSKβ/p-hydroxy-Tyr-216 GSKβ polyclonal antibodies were from BIOSOURCE, In Vivo Technologies (Camarillo, CA). PD98059 and LY294002 were purchased from Calbiochem. BDNF was purchased from Alomone Labs (Jerusalem, Israel). SL327 was a gift from DuPont.

**Cell Culture and Transfection**—Cortical neurons were prepared from newborn Sprague-Dawley rats and transiently transfected at day 3 or 4 in vitro (DIV3/4) after seeding using a calcium phosphate coprecipitation protocol (2). In Fig. 7, cortical neurons were transfected with LipofectAMINE 2000 (Invitrogen). Briefly, cells were seeded at 500,000/well in 24-well plates. At DIV3–4, the conditioned medium were removed and saved. Cells were placed in serum-free basal Eagle’s medium (Sigma) containing 0.8 μg of DNA mixed with 1.5 μl of LipofectAMINE 2000/Well. After a 2-h incubation at 5% CO<sub>2</sub> and 37°C, the transfection medium were replaced with conditioned medium. Cells were fixed and immunostained 2 days after transfection.

**Drug Treatment**—PD98059, LY294002, and SL327 were dissolved in Me<sub>2</sub>SO, and Me<sub>2</sub>SO was used as a vehicle control for these drugs. The final concentration of Me<sub>2</sub>SO was 0.2%. When cultures were cotreated with PD98059 and LY294002 or with SL327 and LY294002, the final concentration of Me<sub>2</sub>SO was 0.2%. When cultures were cotreated with PD98059, LY294002, and SL327, they were washed twice with serum-free basal Eagle’s medium supplemented with 10 μM cytosine arabinoside, and the final concentration of Me<sub>2</sub>SO was 0.4%. BDNF was diluted in phosphate-buffered saline containing 0.1% bovine serum albumin before addition to the cells.

**Trophic Deprivation**—When cells were stimulated with BDNF in serum-free and MK801 (dizocilpine maleate)-containing medium (see Fig. 2), they were washed twice with serum-free basal Eagle’s medium and incubated in serum-free basal Eagle’s medium supplemented with 35 mM glucose, 1 mM l-glutamine, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 2.5 μg cysteine arabinoside, and the N-methyl-D-aspartate receptor antagonist MK801 (10 μM). BDNF stimulation of the two caused a significant increase in GSKβ activity induced by trophic deprivation was blocked by N-methyl-D-aspartate receptor antagonist MK801 (5). LY294002 completely blocked BDNF stimulation of Akt phosphorylation. PD98059 significantly inhibited BDNF stimulation of Akt phosphorylation (Fig. 2). The combination of LY294002 and PD98059 was more effective than either drug alone and caused a 63% increase in GSKβ activity (Fig. 1B). This supports the notion that ERK1/2, like PI3K, negatively regulates GSKβ activity in central nervous system neurons.

**Activation of GSKβ** induced by trophic deprivation was counteracted by BDNF treatment (Fig. 2) (5). Because BDNF activates both ERK1/2 and PI3K in cortical neurons, we tested whether BDNF-driven ERK1/2 activation contributes to BDNF inhibition of GSKβ. Cortical neurons were incubated with BDNF under trophic deprivation in the presence of various inhibitors (Fig. 2). Trophic deprivation was achieved by serum withdrawal in the presence of the N-methyl-D-aspartate receptor antagonist MK801 (5). LY294002 completely blocked BDNF stimulation of Akt phosphorylation. PD98059 significantly inhibited BDNF stimulation of ERK1/2 without affecting Akt phosphorylation (Fig. 1A). It also caused a 40% increase in GSKβ activity (Fig. 1B). The combination of LY294002 and PD98059 was more effective than either drug alone and caused a 63% increase in GSKβ activity (Fig. 1B). This supports the notion that ERK1/2, like PI3K, negatively regulates GSKβ activity in central nervous system neurons.

**RESULTS**

**GSKβ Activity Is Negatively Regulated by ERK1/2 in Cortical Neurons**—To test the hypothesis that ERK1/2 antagonizes GSKβ-induced apoptosis in cortical neurons, we first determined whether GSKβ activity is negatively regulated by ERK1/2 in these cells. We previously showed that LY294002 treatment, which inhibits PI3K activity, activates GSKβ (5). Therefore, we used LY294002 treatment as a positive control for the assessment of GSKβ activity. To inhibit the ERK1/2 pathway, we applied PD98059, an inhibitor of MKK1 and MKK2 (30), which are upstream kinases that phosphorylate and activate ERK1/2. The effect of these inhibitors on ERK1/2 or PI3K activity was indirectly measured by Western analysis using antibodies that specifically recognize phosphorylated and activated ERK1/2 or Akt, respectively. The effect of these inhibitors on GSKβ activity was directly measured by an immune complex kinase assay.

Cortical neurons maintained in the presence of 10% serum under normal culture conditions had high basal ERK1/2 and PI3K/Akt activities (Fig. 1A). LY294002 inhibited the basal phosphorylation of Akt, but not ERK1/2 (Fig. 1A), and activated GSKβ (Fig. 1B), consistent with previous observations (5). PD98059 specifically inhibited basal ERK1/2 phosphorylation without affecting Akt phosphorylation (Fig. 1A). It also caused a 40% increase in GSKβ activity (Fig. 1B). The combination of LY294002 and PD98059 was more effective than either drug alone and caused a 63% increase in GSKβ activity (Fig. 1B). This supports the notion that ERK1/2, like PI3K, negatively regulates GSKβ activity in central nervous system neurons.

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**Cortical Neuron Apoptosis Induced by Expression of Recombinant GSKβ Is Inhibited by Activation of Both ERK1/2 and PI3K Signaling Pathways**—Expression of wild-type GSKβ is sufficient to induce apoptosis in cortical neurons (5). To elucidate the functional significance of ERK1/2 inhibition of GSKβ, we determined whether apoptosis induced by expression of wild-type GSKβ is blocked by coexpression of constitutively active MKK1 (MKK1CA), which selectively activates ERK1/2 (Fig. 3A). Cortical neurons were cotransfected with a wild-type expression vector for GSKβ<sub>3β</sub> and MKK1CA. The empty
cloning vectors and kinase-dead dominant-negative MKK1 (MK1KIN) were used as controls. Basal cell death in cells transfected with vectors only was 16%. Expression of GSK3β increased apoptosis to 34%. Coexpression of constitutively active MKK1 (but not dominant-negative MKK1) reduced GSK3β-induced apoptosis to 23% (p < 0.01). Similarly, GSK3β-induced apoptosis was partially suppressed when the PI3K pathway was selectively activated by transient expression of an active form of the catalytic subunit of PI3K (p110α), but not a kinase-dead mutant of p110 (p110*KIN) (Fig. 3B).

To determine whether stimulation of endogenous ERK1/2 is sufficient to suppress cortical neuron apoptosis due to expression of recombinant GSK3β, cortical neurons were treated with BDNF to activate endogenous ERK1/2 and PI3K (Fig. 4). BDNF completely suppressed apoptosis induced by expression of recombinant GSK3β.

Cortical Neuron Apoptosis Induced by Expression of Recombinant GSK3β Is More Potently Inhibited by Activation of Both ERK1/2 and PI3K Signaling Pathways than by Activation of Either Pathway Alone—Because both ERK1/2 and PI3K can negatively regulate GSK3β activity and inhibit GSK3β-induced apoptosis, it was important to determine whether these pathways are redundant neuroprotective pathways. We transfected cortical neurons with a wild-type expression vector for GSK3β to induce apoptosis and treated these cultures with BDNF to activate both endogenous ERK1/2 and PI3K (Fig. 5). A combination of LY294002 and PD98059 or SL327 was used to block both ERK1/2 and PI3K signaling. As reported in Figs. 3 and 4, expression of wild-type GSK3β by itself was sufficient to increase basal cortical neuron apoptosis from 20 to 40–50% under normal culture conditions (Fig. 5). Cotreatment with LY294002 and PD98059 or SL327 greatly exacerbated GSK3β expression-induced apoptosis, which was now seen in 80% of the GSK3β-expressing cells (Fig. 5). This suggests that under normal culture conditions, apoptosis induced by expression of wild-type GSK3β is attenuated by the endogenous basal ERK1/2 and PI3K activities. As shown earlier, addition of BDNF completely suppressed apoptosis induced by expression of GSK3β. Neuroprotection provided by BDNF was totally reversed by cotreatment with PD98059 and LY294002 (Fig. 5A) or with SL327 and LY294002 (Fig. 5B). These data suggest that
coactivation of ERK1/2 and PI3K provides combinatorial neuroprotection against GSK3β.

Apoptosis Induced by Activation of Endogenous GSK3β Is Blocked by ERK1/2—We previously reported that addition of LY294002 to cortical neuron cultures maintained in the presence of serum inhibits the endogenous PI3K/Akt pathway, activates endogenous GSK3β, and induces apoptosis in cortical neurons (5). Cortical neuron apoptosis induced by LY294002 was significantly blocked by expression of a dominant-negative mutant form of rat GSK3β or inhibitory GBP (p < 0.001) (Fig. 6A). This confirms that endogenous GSK3β plays a significant role in apoptosis induced by LY294002. To determine whether apoptosis induced by activation of endogenous GSK3β is blocked by ERK1/2, cortical neurons were treated with LY294002 in the presence of SL327, BDNF, or SL327 plus BDNF (Fig. 6B). LY294002-induced apoptosis was prevented by cotreatment with BDNF (p < 0.001). Because LY294002 directly inhibited PI3K, and BDNF did not activate PI3K in the presence of LY294002 (Fig. 2A), BDNF protection against LY294002 is very likely mediated by ERK1/2 signaling. Moreover, BDNF protection against LY294002 was reversed by cotreatment with SL327 (p < 0.001) (Fig. 6B), further supporting a role for endogenous ERK1/2 in neuroprotection against activation of GSK3β.

Cortical neurons were also transfected with MKK1CA or its vector control and treated with LY294002. Expression of MKK1CA was sufficient to inhibit cortical neuron apoptosis induced by LY294002 treatment (p < 0.01) (Fig. 6C). Together, these data suggest that apoptosis induced by activation of endogenous GSK3β is blocked by ERK1/2 signaling.

GSK3β Phosphorylation at Ser-9 or Tyr-216 Is Not Affected by Inhibition of ERK1/2 in Cortical Neurons—GSK3β activity is negatively regulated by phosphorylation at Ser-9. To elucidate mechanisms by which PI3K and ERK1/2 inhibit GSK3β activity and GSK3β-induced apoptosis in cortical neurons, we carried out experiments to determine whether these signaling pathways regulate GSK3β phosphorylation at Ser-9. This was accomplished by Western analysis using a phosphopeptide-
specific antibody that recognizes GSK3β phosphorylated at Ser-9 (Figs. 1 and 2). Under normal culture conditions (i.e. in the presence of serum), LY294002 treatment inhibited Ser-9 phosphorylation by 70% (p < 0.01) (Fig. 1, D and E). In contrast, PD98059 treatment caused a slight reduction in Ser-9 phosphorylation that was statistically insignificant (p > 0.05). Furthermore, cotreatment with PD98059 and LY294002 did not inhibit Ser-9 phosphorylation more than LY294002 treatment alone. Because both PD98059 and LY294002 treatment caused an increase in GSK3β activity under the same conditions (Fig. 1B), these data suggest that serum-activated PI3K (but not ERK1/2) inhibits GSK3β activity in primary cortical neurons via Ser-9 phosphorylation.

Trophic deprivation stimulated GSK3β activity, which was reversed by BDNF (Fig. 2B) (5). Accordingly, basal GSK3β phosphorylation at Ser-9 was suppressed upon trophic deprivation and restored upon BDNF addition (Fig. 2D). Treatment with LY294002 (but not SL327 or PD98059) inhibited BDNF-induced GSK3β phosphorylation at Ser-9 by 85% (Fig. 2, E and F). This suggests that BDNF-activated PI3K (but not ERK1/2) induces GSK3β phosphorylation at Ser-9 in primary cortical neurons.

FIG. 3. Cortical neuron apoptosis induced by expression of recombinant GSK3β is partially inhibited by coexpression of constitutively active MKK1 or PI3K, which selectively activates the ERK1/2 or PI3K signaling pathway, respectively. Cortical neurons (DIV3) were transfected with a rat GSK3β cDNA (1 μg of plasmid DNA/2 × 10^6 cells/35-mm plate) to induce apoptosis in transfected cells. Its cloning vector (pEF1α) was used as a control. Cells were also cotransfected with 2 μg of plasmid DNA encoding β-galactosidase as a marker for transfection. A, cells were cotransfected with constitutively active MKK1 (MKK1CA), kinase-dead MKK1 (MKK1KIN), or a vector control (4 μg of plasmid DNA/plate). B, cells were cotransfected with constitutively active PI3K (p110CA), dominant-negative PI3K (p110kin), or a vector control (4 μg of plasmid DNA/plate). Two days after transfection, cells were fixed and immunostained with anti-β-galactosidase antibody to identify transfected neurons. Apoptosis in the transfected cell population (β-galactosidase-positive) was scored. Data are from four independent experiments of duplicate determinations. Error bars are S.E., **, p < 0.01 (ANOVA).

A GSK3β mutant was then generated in which Ser-9 was replaced with a non-phosphorylatable Ala residue (GSK3β(S9A)). Like wild-type GSK3β, expression of GSK3β(S9A) was sufficient to induce apoptosis in cortical neurons (Fig. 7). Significantly, GSK3β(S9A)-induced apoptosis was suppressed by coexpression of constitutively active MKK1, but not PI3K. Together, the data from Figs. 1, 2, and 7 indicate that inhibition of GSK3β by the ERK1/2 signaling pathway in neurons is not mediated by inhibitory phosphorylation at Ser-9.

Another possible mechanism for ERK1/2 inhibition of GSK3β in neurons is through inhibition of GSK3β phosphorylation at Tyr-216, a phosphorylation that may activate GSK3β (14). However, there was no significant change in Tyr-216 phosphorylation following BDNF treatment, inhibition of ERK1/2, or inhibition of PI3K in cortical neurons (Figs. 1D and 2F). Thus, inhibition of GSK3β phosphorylation at Tyr-216 is an unlikely mechanism for ERK1/2 inhibition of GSK3β in neurons.

DISCUSSION

The objective of this study was to test the hypothesis that neuronal apoptosis induced by activation of GSK3β may be suppressed by stimulation of the ERK1/2 signaling pathway. We report that ERK1/2 negatively regulated GSK3β activity in cortical neurons and that this regulation did not involve the inhibitory phosphorylation of GSK3β at Ser-9. In addition, this regulation probably does not require the activating phosphorylation of GSK3β at Tyr-216. Furthermore, expression of a constitutively active MKK1, which directly activated ERK1/2, suppressed cortical neuron apoptosis induced by expression of
PI3K inhibitors on phosphorylation of GSK3

ERK1/2 pathway. This is in contrast to the profound effect of

vation at Ser-9 was not significantly affected by inhibitors of the

a functional consequence of ERK1/2 inhibition of GSK3

protection against LY294002 or GSK3

phosphorylation site. Studies in non-neuronal cells suggest

GSK3

knows and novel mechanism, which most likely does not in-

recombinant wild-type GSK3β or the GSK3β(S9A) mutant. MKK1 also suppressed cortical neuron apoptosis induced by activation of endogenous GSK3β caused by LY294002 inhibition of PI3K. Moreover, ERK1/2 contributed to BDNF neuroprotection against LY294002 or GSK3β expression. Maximal BDNF neuroprotection against GSK3β expression-induced apoptosis was dependent upon activation of both ERK1/2 and PI3K signaling pathways. Our data provide the first example of a functional consequence of ERK1/2 inhibition of GSK3β.

Mechanisms for ERK1/2 inhibition of GSK3β in neurons are still unclear. Both Akt and protein kinase A have been shown to directly phosphorylate GSK3β at Ser-9 (13, 15), an inhibitory phosphorylation site. Studies in non-neuronal cells suggest ERK1/2 activation of p90S6K, which also phosphorylates GSK3β at Ser-9 (21, 22). However, our data indicate that this mechanism is not operative in primary neurons. This conclusion is based on the observation that the level of GSK3β phosphorylation at Ser-9 was not significantly affected by inhibitors of the ERK1/2 pathway. This is in contrast to the profound effect of PI3K inhibitors on phosphorylation of GSK3β at Ser-9. In addition, MKK1 (but not PI3K) could still suppress GSK3β(S9A)-induced apoptosis.

Our data do not support the hypothesis that ERK1/2 inhibition of GSK3β in neurons is mediated by inhibition of the activating GSK3β phosphorylation at Tyr-216. Alternatively, ERK1/2 may increase the expression or improve the function of the GSK3β inhibitory protein GBP. Regardless, it seems that in cortical neurons, ERK1/2 inhibits GSK3β activity by an unknown and novel mechanism, which most likely does not in-

FIG. 5. Inhibition of both ERK1/2 and PI3K signaling completely abrogates BDNF protection against cortical neuron apoptosis induced by expression of recombinant GSK3β.

Cortical neurons (DIV3) were transfected with a rat GSK3β cDNA or its cloning vector as described in the legend to Fig. 3. One day after transfection, cells were treated for 24 h with BDNF (10 ng/ml), 40 μM PD98059 (PD), 50 μM SL327 (SL), 30 μM LY294002 (LY), or a vehicle control (C) as indicated. Cells were then fixed and immunostained with anti-β-galactosidase antibody to identify transfected neurons. Apoptosis in the transfected cell population (β-galactosidase-positive) was scored. As shown in Fig. 4, BDNF completely protected against GSK3β-induced apoptosis. A, BDNF neuroprotection was completely reversed by cotreatment with PD98059 and LY294002. B, BDNF neuroprotection was completely reversed by cotreatment with SL327 and LY294002. Data are from three independent experiments of duplicate determinations. Error bars are S.E.

FIG. 6. Cortical neuron apoptosis induced by activation of endogenous GSK3β can be blocked by stimulating the ERK1/2 signaling pathway. A, cortical neuron apoptosis induced by LY294002 treatment is mediated by endogenous GSK3β. Cortical neurons (DIV3) were transfected with plasmid DNA encoding wild-type GBP, a domi-

nant-negative mutant form of rat GSK3β (GSK3dn), or a vector control (4 μg of DNA/2 × 10⁶ neurons/35-mm plate). Cells were also cotransfected with 2 μg of plasmid DNA encoding β-galactosidase as a marker for transfection. Two days after transfection, cells were treated with 30 μM LY294002 for 0 or 24 h, and apoptosis in the transfected cells was scored. Data are from two independent experiments of duplicate determinations. ***, p < 0.001 (ANOVA). B, cortical neuron apoptosis induced by LY294002 can be inhibited by activation of the endogenous ERK1/2 signaling pathway. Cortical neurons (DIV5) were treated with 0 or 30 μM LY294002 to induce apoptosis. In addition, cells were cotreated with 50 μM SL327, 10 ng/ml BDNF, or a vehicle control as indicated. Apoptosis was scored 24 h after drug treatment. Data are from two independent experiments of triplicate determinations. ***, p < 0.001 (ANOVA). C, activation of ERK1/2 signaling by expression of constitutively active MKK1 offers protection against LY294002-induced apoptosis. Cortical neurons (DIV3) were transfected with a plasmid DNA encoding constitutively active MKK1 (MKK1CA) or its vector control (4 μg of DNA/2 × 10⁶ neurons/35-mm plate). Cells were also cotransfected with 2 μg of plasmid DNA encoding β-galactosidase as a marker for transfection. Two days after transfection, cells were treated with 30 μM LY294002 for 0 or 24 h, and apoptosis in the transfected cells was scored. Data are from two independent experiments of duplicate determinations. Error bars are S.E. ***, p < 0.01 (ANOVA).
bition of GSKβ may explain why cotreatment of neurons with inhibitors of both signaling pathways activates GSKβ more effectively than a single inhibitor alone.

GSKβ was first discovered as a pro-apoptotic signaling molecule in PC12 cells (11) and subsequently in primary cortical neurons (5) as well as in sympathetic neurons (12). GSKβ-induced apoptosis is suppressed by activation of the PI3K/Akt and cAMP/protein kinase A signaling pathways (5, 11). Induction of the apoptotic insult. For example, camptothecin, a DNA-damaging agent, induces cortical neuron apoptosis, which is blocked by ERK1/2 (2). However, expression of either GBP or expression of PI3K activity is inhibited by LY294002, but not against serum deprivation plus MK801. In addition, ERK1/2 activation does not play a major role in BDNF protection against serum deprivation (2). It seems likely that other pathways, in addition to PI3K and ERK1/2, may also contribute to the neuronal survival provided by serum or BDNF. These may include protein kinase C (54), ERK5 (55, 56), and isoforms of p38 MAPKs (57). Although ERK1/2 alone does not promote survival against serum deprivation, which presumably down-regulates all these pathways, it is sufficient to antagonize LY294002-induced apoptosis, which specifically targets PI3K inhibition.

Mechanisms downstream of ERK1/2 activation that lead to neuronal survival are not clearly defined. Activation of p90 ribosomal S6 kinase and the subsequent phosphorylation of CREB as well as increased CREB-mediated gene expression are potential mechanisms (3, 58). Alternatively, ERK1/2 may activate p90 ribosomal S6 kinase and inactivates BAD (3). Our discovery that activation of the ERK1/2 signaling pathway protects against GSKβ-induced apoptosis suggests another novel downstream target for the ERK1/2 survival pathway.

Why are there multiple downstream effectors mediating the anti-apoptotic activity of ERK1/2? Activation of CREB-mediated transcription and inhibition of BAD or GSKβ may function as parallel downstream targets of the anti-apoptotic ERK1/2 pathway. Alternatively, ERK1/2 neuroprotection may employ different downstream targets depending on the nature of the apoptotic insult. For example, camptothecin, a DNA-damaging agent, induces cortical neuron apoptosis, which is blocked by ERK1/2 (2). However, expression of either GBP or dominant-negative GSKβ did not protect cortical neurons against camptothecin, excluding GSKβ inhibition as a mechanism for ERK1/2 neuroprotection against camptothecin. Finally, there may be cross-talk between the various downstream targets of ERK1/2. For instance, inhibition of GSKβ may contribute to ERK1/2 activation of CREB (59).

In summary, our data suggest that ERK1/2 negatively regulates GSKβ activity in neurons via a novel mechanism that is independent of phosphorylation of GSKβ at Ser-9 and likely does not involve phosphorylation of GSKβ at Thr-216. Furthermore, activation of ERK1/2 suppresses GSKβ-induced neuronal apoptosis. Our data suggest a new mechanism for the anti-apoptotic action of the ERK1/2 pathway in neurons and implicate GSKβ inhibition as a convergence point for several pro-survival signal transduction systems in neurons.

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