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Glutamatergic Regulation of the p70S6 Kinase in Primary Mouse Neurons^{*[5]}

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Brief glutamatergic stimulation of neurons from fetal mice, cultured *in vitro* for 6 days, activates the mTOR-S6 kinase, ERK1/2 and Akt pathways, to an extent approaching that elicited by brain-derived neurotrophic factor. In contrast, sustained glutamatergic stimulation inhibits ERK, Akt, and S6K. Glutamatergic activation of S6K is calcium/calmodulin-dependent and is prevented by inhibitors of calcium/calmodulin-dependent protein kinase 2, phosphatidylinositol 3-OH-kinase and by rapamycin. 2-Amino-5-phosphonovaleric acid, an inhibitor of *N*-methyl-D-aspartate receptors, abolishes glutamatergic activation of ERK1/2 but not the activation of mTOR-S6K; the latter is completely abolished by inhibitors of voltage-dependent calcium channels. Added singly, dopamine gives slight, and norepinephrine a more significant, activation of ERK and S6K; both catecholamines, however, enhance glutamatergic activation of S6K but not ERK. After 12 days in culture, the response to direct glutamatergic activation is attenuated but can be uncovered by suppression of γ -aminobutyric acid interneurons with bicuculline in the presence of the weak K⁺ channel blocker 4-aminopyridine (4-AP). This selective synaptic activation of mTOR-S6K is also resistant to APV and inhibited by Ca²⁺ channel blockers and higher concentrations of glutamate. Elongation factor 2 (EF2) is phosphorylated and inhibited by the eEF2 kinase (CaM kinase III); the latter is inhibited by the S6K or Rsk. Bicuculline/4-AP or KCl-induced depolarization reduces, whereas higher concentrations of glutamate increases, EF2 phosphorylation. Thus the mTOR-S6K pathway in neurons, a critical component of the late phase of LTP, is activated by glutamatergic stimulation in a calcium/calmodulin-dependent fashion through a calcium pool controlled by postsynaptic voltage-dependent calcium channels, whereas sustained stimulation of extrasynaptic glutamate receptors is inhibitory.

The storage of information for behavioral purposes requires that changes in synaptic strength be maintained for periods that greatly exceed the half-life of activation or even the lifetime of the proteins that participate in this process. Several positive feedback mechanisms have been proposed to account for this long lasting effect, involving mainly kinases, phosphatases, and the protein synthesis machinery (1, 2).

Protein synthesis in dendrites has emerged as an important process for the induction of the late phase of long term potentiation (LTP)² inasmuch as it

provides a mechanism by which a single synapse can undergo rapid and specific changes in protein composition, and therefore in physiological activity, without significantly affecting most other synapses in the same neuron. The regulation of dendritic protein synthesis seems to be largely independent of the cell soma, inasmuch as isolated synaptoneurosomes respond to short pulses of a mixture of glutamate and NMDA with an increase in general protein synthesis, which is accompanied by an increase in the levels of the CaMKII protein (3). Furthermore, BDNF as well as a D1/D5 dopaminergic agonist induce the expression in dendrites of a green fluorescent protein reporter whose coding sequence is flanked by the sequences encoding the 5'- and 3'-untranslated region of CaMKII mRNA, sequences known to control CaMKII mRNA translation (4, 5).

Mammalian target of rapamycin (mTOR) plays an important role in the late phase of LTP; thus rapamycin, a specific inhibitor of TOR complex 1, prevents late phase LTP when applied during the induction phase (6). BDNF increases protein synthesis in neurons through activation of the mTOR/S6K pathway (7, 8) and potentiates LTP (9), responses that are inhibited by rapamycin (6, 10, 11). TOR complex 1 controls the component of mRNA translation necessary for cell growth, presumably through the phosphorylation of its substrates, which include eukaryotic initiation factor 4E-binding protein and the p70S6 kinase (S6K). Thus, interference or deletion of S6K results in a marked decrease in cell size during development and in response to mitogen stimulation in culture (reviewed in Ref. 12). mTOR can regulate both translational initiation and elongation, the latter by promoting the inactivation of elongation factor 2 kinase (EF2 kinase, also known as CaMKIII). EF2 kinase phosphorylates EF2 at Thr⁵² in the GTP-binding domain and inhibits EF2-catalyzed mRNA transit through the ribosome (13). Whereas intracellular Ca²⁺ activates EF2 kinase (14), activation of the mTOR/S6K as well as ERK/Rsk pathways phosphorylate and inhibit EF2 kinase, thereby promoting the elongation activity of EF2 (15).

Glutamatergic receptors are central to several activity-dependent alterations in synaptic strength that play a role in memory and learning. These receptors are also involved in the neuronal death that occurs in many chronic neurodegenerative disorders (16). The factors that promote the physiological and pathological functions of the NMDA receptors are incompletely defined, but the intensity and duration of stimulation (17) together with the synaptic *versus* extrasynaptic localization of the receptor (18) are suggested as factors that can direct the response to one or the other outcome. Herein we characterize the effect of glutamatergic activation on the mTOR/S6K pathway in cultured mouse neurons and demonstrate that physiologic stimulation promotes mTOR activation independently of the NMDA receptor through a calcium/calmodulin signal controlled by voltage-dependent Ca²⁺ channels; by contrast, excitotoxic glutamatergic stimulation inhibits mTOR signaling.

MATERIALS AND METHODS

The "Materials and Methods" are provided in the supplemental material.

RESULTS

A Short Glutamatergic Stimulus Induces the Activation of the S6K Pathway, whereas a Sustained Stimulus Is Inhibitory—Mimicking the physiological glutamatergic stimulus that occurs at a synapse is not a simple task, inasmuch as neurons in culture are very sensitive to supra-physiologic concentrations of, and/or inappropriately prolonged exposure to, glutamatergic agonists. For example, NMDA at 25 μ M activates, whereas 100 μ M inhibits, ERK signaling (19), and continuous exposure to glutamate for periods needed to study protein synthesis regulation, *i.e.* 30 min or more, leads to massive cell death (20). In an effort to achieve a glutamatergic stimulation that mimics, as closely as possible, a physiologic stimulus, we employed a very short glutamatergic treatment protocol; this was accomplished by adding a mixture of glutamate (10 μ M) and NMDA (50 μ M) followed 5 s later by the addition of the NMDA receptor antagonist APV (120 μ M). This treatment protocol is hereafter referred to as GNA.

TORC1, rapamycin-sensitive TOR complex 1; S6K, p70S6 kinase; ERK, extracellular regulated protein kinase; PI3K, phosphatidylinositol 3-OH-kinase; CaMKII, calcium/calmodulin-dependent protein kinase 2; EF2, elongation factor 2; EF2K, EF2 kinase; GABA, γ -aminobutyric acid; BDNF, brain-derived neurotrophic factor; NE, norepinephrine.

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^[5] The on-line version of this article (available at <http://www.jbc.org>) contains the Materials and Methods and supplemental Discussion and Refs. S1–S8.

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² The abbreviations used are: LTP, long term potentiation; APV, 2-amino-5-phosphonovaleric acid; NMDA, *N*-methyl-D-aspartate; GNA, glutamate + NMDA + APV; 4-AP, 4-aminopyridine; BAP, bicuculline + 4-AP; VDCC, voltage-dependent calcium channel(s); GSK3, glycogen synthase kinase 3; mTOR, mammalian target of rapamycin;

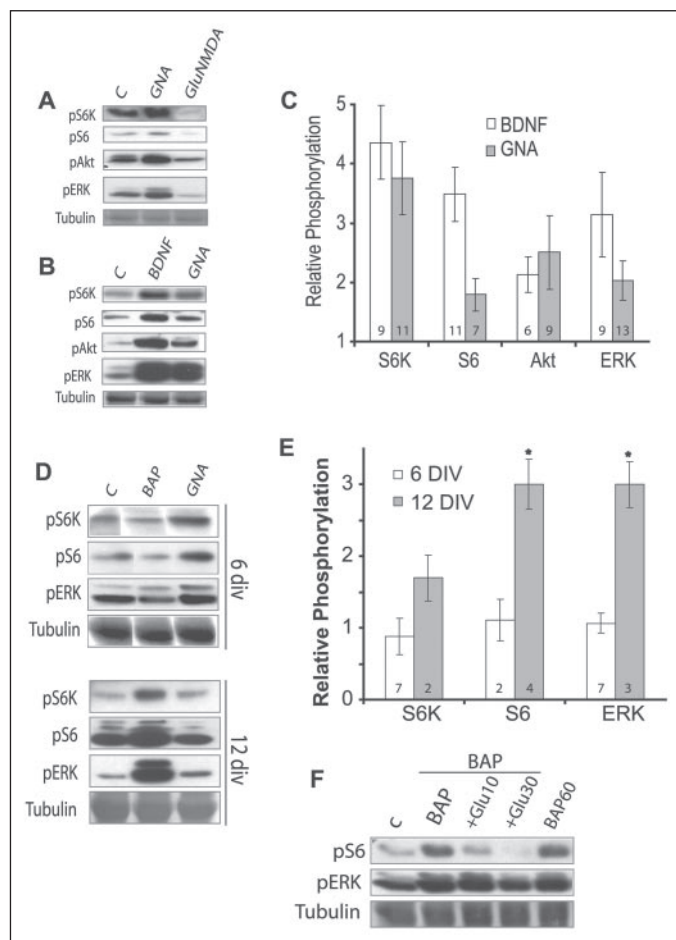


FIGURE 1. Brief glutamatergic stimulation and synaptic stimulation induce activation of the S6K pathway. A, neuronal cultures were harvested 30 min after treatment with GNA (glutamate, 10 μ M plus NMDA, 50 μ M, followed 5 s later by APV, 120 μ M) or with continuous stimulation with glutamate (10 μ M) plus NMDA (50 μ M) (GluNMDA). B, treatment with GNA as described for A or BDNF (50 ng/ml). Immunoblots were performed against the phosphorylated forms of S6K (pS6K), ribosomal S6 protein (pS6), Akt, and ERK. C, BDNF and GNA induce an increase in phosphorylation of S6K, ribosomal S6 protein, Akt, and ERK; $p < 0.05$ compared with control; the number of experiments is indicated within each bar. D, 6-day (upper) or 12-day (lower) old cultures were treated with GNA or 50 μ M bicuculline, supplemented with 2.5 mM 4-AP. E, BAP-stimulated S6 and ERK phosphorylation is significantly higher in twelve day cultures, when compared with 6-day cultures; $*p < 0.05$. F, 12-day cultures were pretreated for 30 min with BAP followed by the addition of glutamate (100 μ M) or carrier; cultures were harvested 10 or 30 min thereafter. All blots shown except for tubulin are phosphospecific; blots of kinase polypeptides (data not shown) indicated equal loading. In A, B, D, and F, lane C = control.

Whereas the GNA protocol resulted in an increase in the phosphorylation of S6K, S6, Akt, and ERK in cortical neurons when analyzed 30 min after application of the stimulus, the same concentrations of glutamate and NMDA applied without the addition of APV 5 s later led to a reduction in the phosphorylation of these proteins below the initial levels (Fig. 1A). The magnitude of the increase in the phosphorylation of S6K, Akt, and ERK induced by GNA (estimated by immunoblot) is comparable but slightly less than that elicited by BDNF (Fig. 1, B and C); however, the GNA-induced increase in S6 phosphorylation is only about half that elicited by BDNF (Fig. 1C). The very brief nature of the GNA stimulus biases toward underestimation and makes a quantitative comparison to BDNF unreliable.

Stimulation of Synaptic Activity in Culture Activates the S6K Pathway—Neuronal cultures contain inhibitory interneurons that control basal neuronal activity through the activation of the GABA_A receptor. Therefore, the inhibition of this receptor with bicuculline, a GABA_A receptor antagonist, together with 4-AP, a weak potassium channel blocker, is able to produce a selective increase in basal synaptic activity, *i.e.* activating receptors localized at synapses, without activating extrasynaptic receptors (18).

The elaboration of these inhibitory GABAergic interneurons requires considerable maturation of primary neuronal cultures (21). Thus whereas a vigor-

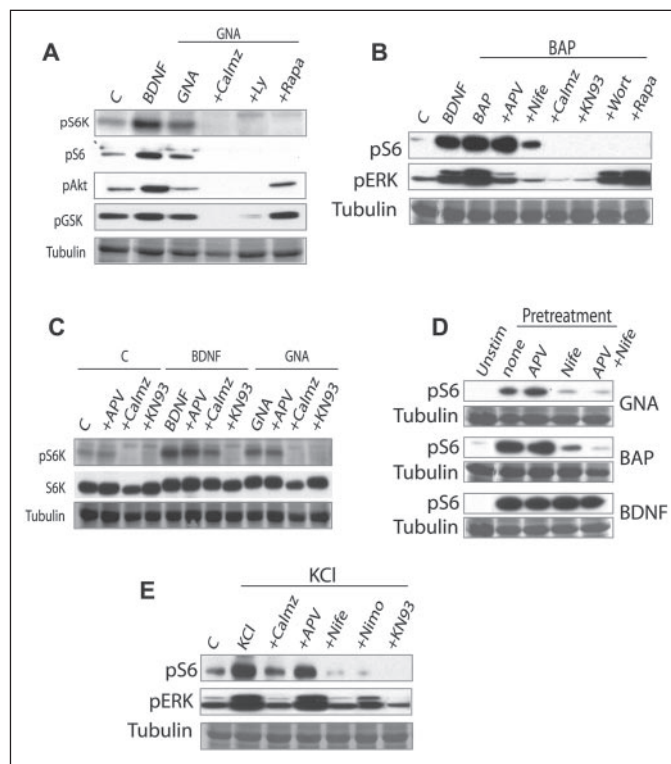


FIGURE 2. Activation of S6K by GNA and BAP is dependent on calcium signaling. Neuronal cultures were pretreated for 30 min with the indicated inhibitors at the concentrations described in the methods and then stimulated with BDNF (A–D) or GNA (A, C, D) or BAP (B, D) or KCl (E). Thirty min thereafter, cells were harvested, and extracts were subjected to immunoblot as indicated. In A, B, C, and E, lane C = control.

ous response to GNA is evident in cultures stimulated 6 days after plating/attachment, the response to GNA is fully attenuated by 12 days in culture. In contrast, bicuculline (50 μ M) plus 4-aminopyridine (2.5 μ M) (hereafter called BAP), which elicits little or no response in 6-day cultures, produces a robust activation of p70S6 kinase and ERK when applied to neurons after twelve days in culture. These results reflect the maturation of inhibitory GABAergic interneurons and/or glutamatergic synapses (22) and indicate that synaptic stimulation is a very strong activator of signaling pathways leading to the S6K and ERK (Fig. 1, D and E).

In contrast to BAP, which selectively disinhibits receptors localized at synapses, bath application of glutamate activates both synaptic and extrasynaptic receptors. Activation of extrasynaptic glutamatergic receptors was shown previously to block the induction of cAMP-response element-binding protein (CREB) phosphorylation by BAP (18). Herein we show that when neurons, 12 days in culture, are treated with glutamate (100 μ M) after a 30-min pretreatment with BAP, the BAP-induced phosphorylation of S6 is reduced by 10 min and abolished after 30 min. Thus activation of extrasynaptic glutamate receptors, in a dominant fashion, blocks the activation of the mTOR/S6K pathway induced by synaptic activation (Fig. 1F).

Glutamatergic Stimulation of the mTOR Pathway Requires Calcium Entry through L-type Voltage-dependent Calcium Channels—We sought to characterize the pathway responsible for glutamatergic activation of mTOR signaling. Activation of p70S6 kinase by receptor tyrosine kinases (*e.g.* BDNF/TrkB) requires the activation of Type 1 PI3K and is ultimately mediated by the sequential phosphorylation of S6 kinase by the rapamycin-sensitive TOR complex 1 (TORC1) and the kinase PDK1; thus BDNF activation of the mTOR pathway to p70S6K is inhibited by the PI3K inhibitors, Ly294002 or wortmannin, and by the TORC1 inhibitor, rapamycin (7, 8). Similarly, GNA- and BAP-stimulated phosphorylation of p70S6K and S6 is strongly inhibited by Ly294002 or wortmannin and by rapamycin (Fig. 2, A and B). The PI3K inhibitor also prevents the GNA phosphorylation of Akt (Thr⁴⁷³) and of the Akt substrate, GSK3 β (Ser⁹) (Fig. 2A); the latter are, however, resistant to rapamycin. Neither Ly294002 nor rapamycin has any significant effect on BAP (Fig. 2A) or GNA (data not shown) activation of ERK (Fig. 2B). Thus, as with BDNF, PI3K is necessary for GNA/BAP activation of Akt and p70S6 kinase, and the

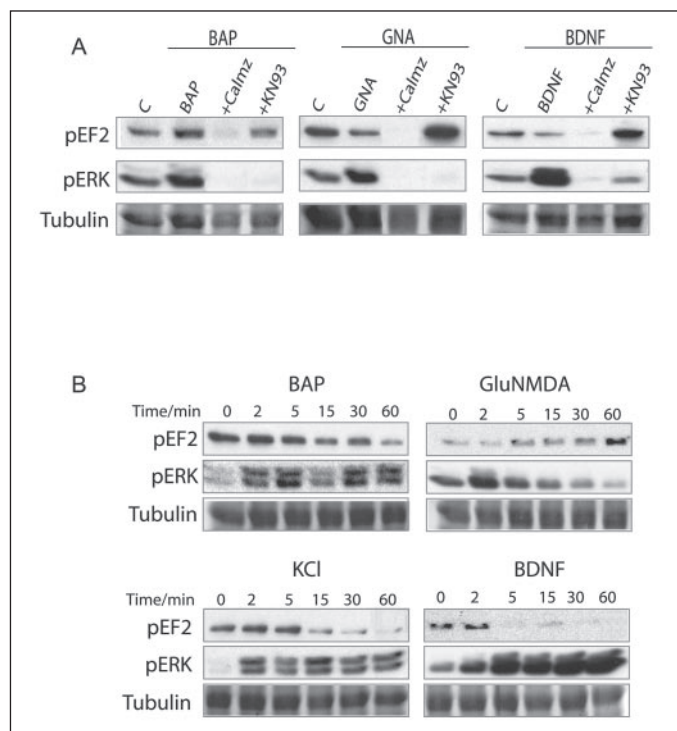


FIGURE 3. Regulation of EF2 phosphorylation by various stimuli. A, the effect of BAP (left) GNA (middle) or BDNF (right) on EF2 phosphorylation in neuronal cultures, directly or pretreated with calmidazolium (Calmiz) or KN93. Lane C = control. B, the time course of EF2 and ERK phosphorylation in response to BDNF, synaptic stimulation (BAP), KCl depolarization, or sustained (GluNMDA) glutamatergic stimulation.

latter also requires the TORC1, whereas activation of ERK is presumably driven by Ras-GTP through a PI3K-independent mechanism.

As regards the GNA/BAP responsive elements further upstream, APV, a potent and selective inhibitor of the ionotropic NMDA receptor, strongly suppresses BAP (Fig. 2B) and GNA (data not shown) activation of ERK. Surprisingly, however, APV does not alter GNA or BAP activation of the mTOR pathway (Fig. 2, B–D). Glutamatergic stimulation of the mTOR pathway does require Ca^{2+} /calmodulin, inasmuch as blockade of calmodulin with calmidazolium (Fig. 2, A–C) or inhibition of CaMKII with KN93 (Fig. 2, B and C) each completely inhibits the activation of S6K and the phosphorylation of S6. BDNF-stimulated phosphorylation of S6K is also inhibited by both KN93 and by calmidazolium (Fig. 2C). Nifedipine, an inhibitor of L-type voltage-dependent calcium channels (L-VDCC), strongly inhibits both S6 and ERK phosphorylation induced by GNA or BAP (Fig. 2, B and D) but not by BDNF (Fig. 2D), which can mobilize intracellular calcium stores by activation of phospholipase C. Direct activation of voltage-dependent Ca^{2+} channels, achieved by depolarization of neurons with KCl (60 mM for 30 min), induces a strong increase in the phosphorylation of S6, in a mechanism blocked by calmidazolium, the L-type voltage-dependent Ca^{2+} channel blockers nifedipine and nimodipine as well as by KN93 (Fig. 2E). These results indicate that the GNA and BAP glutamatergic stimulation protocols promote activation of the mTOR/S6K pathway by an NMDA receptor-independent activation of L-VDCC; in contrast, BDNF signaling to CaMKII is completely independent of both NMDA receptor as well as L-VDCC.

Elongation Factor 2 Phosphorylation Is Inhibited by Depolarization or Synaptic Stimulation but Increased by Extrasynaptic Glutamatergic Stimulation—EF2 facilitates peptide chain elongation and can be negatively regulated by EF2 kinase (EF2K). The latter is activated by Ca^{2+} /calmodulin and inhibited by both the ERK and the mTOR pathways, through direct phosphorylation of EF2K in its calmodulin-binding domain by either Rsk or p70S6 kinase, respectively. Treatment of neurons with calmidazolium completely abolishes phosphorylation of EF2 (Fig. 3A), presumably by direct inhibition of EF2K, whereas the relatively selective CaMKII inhibitor KN93 increases the phosphorylation of EF2 in the presence of GNA and BDNF (Fig. 3A), presumably reflecting the ability of KN93 to abolish activation of ERK and mTOR signaling (Fig. 2, B, C, and E); inhibition of mTOR with rapamycin also increases the phosphorylation of EF2 (data not shown).

Neurotrophic and physiological glutamatergic stimulation leads to the increase in intracellular Ca^{2+} but also results in an increase in protein synthesis; this suggests that the activation of ERK and S6K accompanying such stimuli is dominant in the regulation of EF2. Consistent with this view, BDNF gives a very robust activation of ERK paralleled by a profound inhibition of EF2 phosphorylation (Fig. 3B). BAP- and KCl-induced depolarization each cause a progressive decrease in the phosphorylation of EF2, which, compared with BDNF, is relatively slow in onset, perhaps due to the less robust activation of ERK. In contrast to BAP and KCl, the continuous presence of glutamate plus NMDA without the addition of APV (*GluNMDA*), which suppresses ERK and mTOR signaling, results in a progressive increase in EF2 phosphorylation (Fig. 3B). Thus, despite the ability of all these stimuli to increase intracellular Ca^{2+} , the Ca^{2+} -dependent phosphorylation of EF2 is inhibited if there is concomitant activation of ERK/Rsk and/or mTOR/p70S6 kinase.

Catecholamines Enhance GNA-stimulated S6 Phosphorylation—Activation of D1/D5 dopaminergic receptors was recently shown to increase protein synthesis in dendrites of hippocampal neurons (5). Dopaminergic and NMDA receptors interact directly and dopaminergic receptor activation protects neurons from NMDA-induced excitotoxicity; this protection is prevented by a PI3K inhibitor (23). To assess whether there is an interaction between the glutamatergic and dopaminergic activated pathways in the regulation of the mTOR/S6K pathway, neurons were treated with dopamine, resulting in a small, statistically insignificant increase in the phosphorylation of S6. In contrast to the borderline stimulation by dopamine alone, the concomitant presence of dopamine and GNA produces a further increase in the phosphorylation of S6 when compared with that elicited by GNA alone (Fig. 4, A and B). An augmentation of GNA-stimulated S6 phosphorylation is even more clear-cut with another catecholamine, norepinephrine (NE) (Fig. 4, A and B), which significantly stimulates S6 phosphorylation by itself and when present with GNA promotes a further increase in S6 phosphorylation that is significantly greater than that elicited by GNA alone. The response of ERK to dopamine and NE alone is similar to that seen with S6 phosphorylation; however, ERK activation in response to GNA is not further increased by the presence of NE or dopamine (Fig. 4C).

DISCUSSION

The mTOR/S6K pathway controls the component of protein synthesis that is particularly important for cell growth as well as for neuronal plasticity, inasmuch as the late phase of LTP is inhibited comparably by inhibitors of overall protein synthesis and by rapamycin. Because the glutamatergic system is a fundamental player in synaptic plasticity, we investigated the regulation of the mTOR/S6K pathway using stimulation protocols designed to mimic the plasticity-inducing stimulation that occurs in the intact brain.

Previous studies using hippocampal slices (24) showed that ERK is activated by LTP-inducing stimulation, whereas excessive NMDA receptor stimulation inactivates this kinase (19). Similarly, we find that short and long exposures to glutamatergic stimulation produce, respectively, stimulatory and inhibitory effects on S6K, S6, Akt, and ERK phosphorylation, reinforcing the importance of a short pulse of glutamatergic stimulation for the activation of these pathways. Thus, despite the short duration between agonist and antagonist addition, GNA appears to provide a *bona fide* physiologic glutamatergic stimulation. The slow development in culture of inhibitory postsynaptic GABAergic interneurons enables the selective stimulation of endogenous synapses, through inhibition of GABA_A receptor; such a maneuver (*i.e.* BAP) also activates the S6K and ERK pathways (Fig. 1D). As with GNA, sustained and generalized glutamatergic stimulation generates a dominant inhibition over BAP stimulation. A third illustration of this difference between physiologic and excitotoxic stimulation is seen in the phosphorylation of EF2; as shown previously (14), supraphysiologic glutamatergic stimulation, which suppresses ERK and S6K activity, increases EF2 phosphorylation. In contrast, physiologic glutamatergic stimulation by BAP and GNA results in a decrease in phosphorylation of EF2. Thus although all require a Ca^{2+} signal, the mTOR/S6K and/or ERK/Rsk pathways are dominant to the EF2K pathway.

The dependence of glutamatergic S6K activation on PI3K and the rapamycin-sensitive TORC1, as seen with BDNF stimulation of neurons (7), is not surprising. More unexpected is the finding that activation of the S6K pathway by GNA and BAP is strongly inhibited by L-VDCC blockers but is unaffected by the NMDA receptor antagonist APV (Fig. 2, B–D) or by the PLC inhibitor U73122 (data not shown). Thus the L-VDCC are indispensable for activation of the mTOR/S6K pathway in neurons by glutamatergic stimulation, by endogenous synaptic stimulation as well as by KCl-induced depolarization.

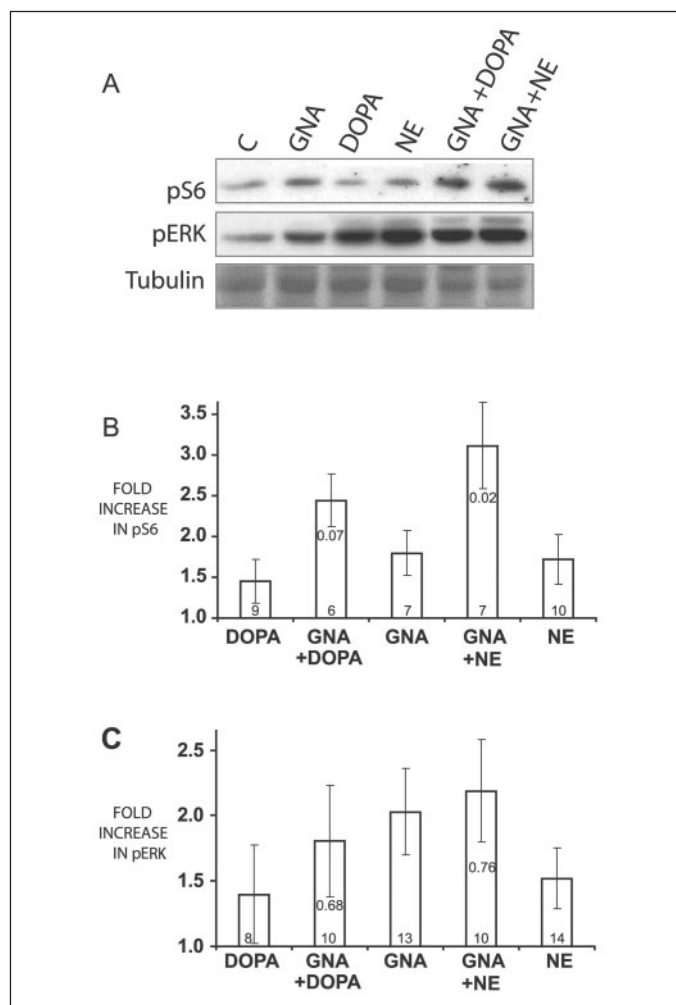


FIGURE 4. Dopamine or norepinephrine enhance selectively the glutamatergic stimulation of S6 phosphorylation. A, cortical neuronal cultures were incubated with GNA, NE, or dopamine (DOPA) singly or in the combinations shown and harvested 30 min later. A representative immunoblot of phospho-S6 and pERK is shown. Lane C = control. B, the densitometric intensity of the pS6 band in extracts from stimulated cells was divided by the intensity of the pS6 band in unstimulated cells and expressed as fold stimulation. The number of independent experiments is indicated in each bar. The effect of dopamine is not significant ($p = 0.13$), whereas the response to GNA ($p = 0.03$), GNA + dopa ($p = 0.01$), NE ($p = 0.04$) and GNA + NE ($p = 0.01$) is significant; the p value of the comparison between GNA alone and GNA + dopamine (0.07) or GNA + NE (0.02) is indicated in the latter bars. C, as described for B, for the fold stimulation of pERK.

Glutamatergic or synaptic stimulation can cause depolarization through activation of AMPA receptors and thereby activate the L-VDCC (25). Synaptic NMDA receptors, although critical for BAP-induced ERK activation, do not participate in BAP activation of the mTOR/S6K pathway. These results illustrate that Ca^{2+} entry through postsynaptic NMDA receptors and L-type VDCC contributes to different postsynaptic pools of Ca^{2+} , in that the signal transduction pathways engaged by these two Ca^{2+} entry pathways differ, at least in part. Although both components are engaged during most protocols productive of LTP, earlier work has indicated that the two pathways can be recruited differentially by different patterns of electrical stimulation (26). Moreover, the selective activation of Ca^{2+} entry by one or the other of these two pathways can each generate LTP responses *in vitro* that are distinct in their sensitivity to various protein kinase inhibitors (27), and when engaged selectively *in vivo*, these two pathways have distinguishable effects in a test of spatial memory, with inhibition of L-VDCC impairing the retention but not the acquisition of a learned task (28).

Catecholaminergic neurons are concentrated in specific brain areas, such as substantia nigra in the case of dopaminergic neurons and locus coeruleus for norepinephrine neurons. Axons sprouting from these structures irrigate vir-

tually all brain areas modulating the activity in these regions (29). It is believed that catecholamines are key regulators of mood and thus are responsible for the emotional background of memories (30). The ability of concomitant glutamatergic and catecholaminergic stimulation to enhance glutamatergic S6 phosphorylation is consistent with a variety of previous reports describing mutual reinforcement between these two synaptic inputs. Dopamine and NE affect synaptic potentiation, with dopamine alone or agonists of D1/D5 dopamine receptors inducing a slow, protein synthesis-dependent, synaptic potentiation in hippocampal slice that requires glutamatergic activation of NMDA receptors (31). Norepinephrine is also known to modulate LTP, restoring the LTP-inducing potency of high frequency stimulation in older animals (32). Activation of D1/D5 receptors lead to an increase in protein synthesis, in an mTOR-dependent way. Moreover, NMDA receptor activity is required for the dopamine agonist-induced increase in surface GluR and miniature excitatory postsynaptic currents (5), indicating the importance of the signaling of the glutamatergic and dopaminergic receptors toward protein synthesis. Thus, the additive effect of GNA with dopamine/norepinephrine on activation of the mTOR/S6K pathway suggests that this phenomenon may account in part for the synergistic regulation of protein-synthesis dependent synaptic plasticity.

In conclusion, glutamatergic or synaptic stimulations designed to mimic physiological stimuli increase the activation of S6K and the phosphorylation of S6 in a VDCC-, Ca^{2+} -calmodulin-, CaMKII-, and PI3K-dependent mechanism, and this increase is further augmented by co-stimulation with catecholamines. In contrast, excitotoxic glutamatergic stimulation dominantly inhibits the mTOR/S6K pathway.

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