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Functional Role of RNA Polymerase II and P70 S6 Kinase in KCl Withdrawal-induced Cerebellar Granule Neuron Apoptosis*

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Background: Neurons undergoing apoptosis show increased expression of cell cycle regulatory proteins.
Results: The expression of *CDK7* and *CDK9* and phosphorylation and activation of RNA polymerase II and P70S6K are increased in apoptotic neurons, and their inhibition prevents apoptosis.
Conclusion: The P70S6K-dependent translation of specific mRNAs may contribute to neuronal vulnerability.
Significance: Inhibitors of P70S6K could be beneficial in preventing neurodegeneration and neuronal apoptosis.

KCl withdrawal-induced apoptosis in cerebellar granule neurons is associated with aberrant cell cycle activation, and treatment with cyclin-dependent kinase (Cdk) inhibitors protects cells from undergoing apoptosis. Because the Cdk inhibitor flavopiridol is known to inhibit RNA polymerase II (Pol II)-dependent transcription elongation by inhibiting the positive transcription elongation factor b (P-TEFb, a complex of CDK9 and cyclin T), we examined whether inhibition of RNA Pol II protects neurons from apoptosis. Treatment of neurons with 5, 6-dichloro-1-β-D-ribobenzimidazole (DRB), an RNA Pol II-dependent transcription elongation inhibitor, and flavopiridol inhibited phosphorylation and activation of Pol II and protected neurons from undergoing apoptosis. In addition to Pol II, neurons subjected to KCl withdrawal showed increased phosphorylation and activation of p70 S6 kinase, which was inhibited by both DRB and flavopiridol. Immunostaining analysis of the neurons deprived of KCl showed increased nuclear levels of phospho-p70 S6 kinase, and neurons protected with DRB and flavopiridol showed accumulation of the kinase into large spliceosome assembly factor-positive speckle domains within the nuclei. The formation of these foci corresponded with cell survival, and removal of the inhibitors resulted in dispersal of the speckles into smaller foci with subsequent apoptosis induction. Because p70 S6 kinase is known to induce translation of mRNAs containing a 5′-terminal oligopyrimidine tract, our data suggest that transcription and translation of this subset of mRNAs may contribute to KCl withdrawal-induced apoptosis in neurons.

Although programmed cell death is essential for the sculpting of the developing nervous system (1–4), its occurrence in mature neurons leads to neurodegeneration and pathological transformations in the brain. *In vitro* studies have shown that growth factor deprivation, oxidative stress, activity withdrawal, and treatment with various insults can induce apoptosis in primary neurons (5–13). This is partly brought about by aberrant expression of cell cycle regulatory proteins, indicating a role for deregulation of the cell cycle in this phenomenon (14–21). Inhibition of cyclin-dependent kinases (Cdk)[2] or overexpression of retinoblastoma protein and $p16^{INK4}$ protected the neurons, thereby establishing a role for cell cycle deregulation in neuronal apoptosis (16, 18, 19, 22–24).

It has been shown that apoptosis in neurons is associated with enhanced transcription and translation (25, 26). RNA polymerase II (Pol II) is the main enzyme that initiates transcription and induces generation of mRNAs. Pol II, in association with other general transcription factors, enhances transcription initiation and elongation (27, 28). The C-terminal domain of Pol II, which is important in linking transcription and mRNA processing, is phosphorylated and activated by the positive transcription elongation factor P-TEFb, a complex of CDK9 and cyclin T (28–32). In addition to CDK9, CDK7, in association with cyclin H, also plays a role in transcription activation by complexing with transcription factor IIH (TFIIH) (32–34). The Cdk inhibitors flavopiridol and roscovitine are known to inhibit CDK9 and CDK7 and hinder transcription (35–37). We have shown previously that these inhibitors protect neuronal apoptosis by inhibiting CDK4/CyclinD1 and CDK2/CyclinE enzyme activation and retinoblastoma protein phosphorylation (16, 19). On the basis of our previous data and the known inhibitory effect of these kinase inhibitors on CDK9 and CDK7, we hypothesize that neuronal apoptosis is brought about not only by aberrant activation of the cell cycle but also by

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[2] The abbreviations used are: Cdk, cyclin-dependent kinase; Pol II, RNA polymerase II; DRB, 5,6-dichloro-1-β-D-ribobenzimidazole; mTOR, mammalian target of rapamycin; CGN, cerebellar granule neuron; P-TEFb, positive transcription elongation factor b.

activation of CDK9/CDK7-dependent phosphorylation of Pol II and activation of transcription. Here we used a more specific inhibitor of Pol II-dependent transcription elongation, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) (28, 38), to determine whether KCl withdrawal-induced apoptosis in cerebellar granule neurons (CGNs) is associated with activation of Pol II.

In addition to Pol II, DRB has been shown to inhibit ribosomal p70 S6 kinase 1 (P70S6K), a serine/threonine protein kinase involved in enhanced protein synthesis at ribosomes (39–41). It is one of the two ribosomal S6 kinases that is known to phosphorylate the 40 S ribosomal protein S6, thereby enhancing the translational capacity at the ribosomes. P70S6K is specifically known to induce translation of a selected set of mRNAs containing a polypyrimidine tract at their 5′ transcriptional start site (5′-terminal oligopyrimidine) (42–44). P70S6K activity is regulated by phosphorylation at specific sites by cyclin-dependent and -independent kinases (45–48). It is known that phosphorylation of P70S6K at Ser-411, Ser-418, Thr-421, and Ser-424, within the autoinhibitory C-terminal domain of the kinase, is important for the conformational change prior to further phosphorylation at Thr-229 and Thr-389 and activation of the kinase. Phosphorylation at Thr-389 is mediated by an mTOR-dependent pathway (49). It has been shown that the phosphorylation and activation of P70S6K are important for the transition of cells through G_1 phase of the cell cycle, the period during which cell size and protein expression are increased (50). Additionally, studies have shown that mitotic Cdks phosphorylate P70S6K at the C terminus (45). Because neurons undergoing apoptosis show activation of G_1 cyclins and Cdks, indicative of G_1 progression, we postulate that P70S6K phosphorylation and activation occur during early phases of the cell cycle and that they play a role in apoptosis induction.

Here we examined the status of Pol II and P70S6K in cerebellar granule neurons undergoing KCl withdrawal-induced apoptosis. The results presented here show that treatment of neurons with DRB or flavopiridol inhibits the phosphorylation and activation of Pol II and P70S6K and induces accumulation of the kinase in nuclear speckle domains. Studies in transformed cells have shown that nuclei under active transcription contain several small interconnected chromatin granules (51) and that, upon transcription inhibition, these granules aggregate to form large transcriptionally inactive foci that are positive for the spliceosome assembly factor SC35 (52). Here we show evidence for SC35- as well as P70S6K-positive focus formation in neurons treated with DRB or flavopiridol and dissolution of the foci upon inhibitor withdrawal. These are novel and significant findings, and these data imply that apoptosis in neurons is associated with transcriptional activation and translation of a selected set of mRNAs, the identification of which will enable the development of targeted drugs to effectively prevent neuronal apoptosis.

MATERIALS AND METHODS

Materials—Cell culture reagents were purchased from Invitrogen. Poly-D-lysine, Percoll, and all other chemicals, unless mentioned otherwise, were purchased from Sigma. Antibodies to Ser-411 P70S6K, total P70S6K, and RNA polymerase II were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Thr-421/Ser-424 P-P70S6K antibodies were from New England Biolabs (Beverly, MA) or Cell Signaling Technology (Danvers. MA). MAP-2 antibody was purchased from Abcam (Cambridge, MA), and P-histone H3 was from Cell Signaling Technology. SC35 clones were purchased from the ATCC, and concentrated tissue culture supernatant from the cultured cells was used as the antibody source. Alexa Fluor 488 and Alexa Fluor 594 secondary antibodies were purchased from Invitrogen/Molecular Probes.

Cell Culture—Neurons were prepared according to published protocols (16). Briefly, cerebella from 8-day-old Sprague-Dawley rat pups were dissected out, meninges were removed, and tissue was trypsinized and treated with DNase as reported previously (53). The resulting cell suspension was layered on top of a step gradient that consisted of 35 and 60% Percoll and centrifuged at 3000 rpm (800 × *g*) for 10 min. The cerebellar granule cells were collected from interphase (54) and washed with cold PBS. The cells were resuspended in basal medium eagle (BME) containing 10% fetal bovine serum and 25 mM KCl. Neurons were plated into 24-well or 60-mm tissue culture dishes coated with 200 μg/ml poly-D-lysine at a cell density of ~400,000 cells/well or $8–10 \times 10^6$ cells/60-mm dish. Eighteen hours after cell plating, cytosine arabinoside was added to a final concentration of 10 μM to prevent proliferation of non-neuronal cells. Experiments were done in cultures that were 5–6 days old. At the time of the experiments, >95% of the cell population consisted of granule neurons.

Cell Survival Assay—After 5–6 days in culture, the CGNs were washed with serum-free medium and maintained in BME with 25 mM KCl for 24 h before lowering the KCl to 5 mM. The cells were treated with or without inhibitors of apoptosis in BME containing 5 mM KCl for different time periods, as indicated under "Results." The Cdk inhibitor flavopiridol was used at 1 μM, and DRB was used at 300 μM concentrations. Survival was assessed at various times after KCl deprivation by cell lysis and nucleus counting as described previously (55). The number of intact nuclei under each condition was counted, and cell survival was expressed as a percentage of the cells present in the control cultures containing 25 mM KCl.

Lysate Preparation and Western Blot Analysis—CGNs were cultured in 60-mm tissue culture dishes at a density of $8–10 \times 10^6$ cells/dish and subjected to different treatments in BME with low KCl. At the end of the treatment, cells were washed with cold PBS, and cell lysates were made in either 1× Laemmli sample buffer or lysis buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 0.1% Tween 20, 10% glycerol, 1 mM DTT, 0.1 mM PMSF, 10 mM β-glycerophosphate, 0.1 mM orthovanadate, 1 mM NaF, 10 μg/ml leupeptin, and 1 μg/ml aprotinin (Hepes lysis buffer). The lysate was centrifuged at 15,000 rpm for 15 min at 4 °C, and the soluble protein was collected into a fresh tube. For Western blot analysis, equal amounts of proteins (20–30 μg) were resolved by PAGE and transferred to nitrocellulose membrane. Equal protein loading on the blot was confirmed by Ponceau S staining. The membranes were incubated with 5% nonfat dry milk in PBS for 2 h at room temperature to inhibit nonspecific binding by the antibodies. Primary antibody dilutions were done in 3% BSA/PBS,

and the blots were incubated overnight at 4 °C with gentle rocking. Blots were washed four times for 5 min each and incubated with goat anti-rabbit or goat anti-mouse peroxidase-conjugated secondary antibodies (Amersham Biosciences, Arlington Heights, IL) at 1:1000 dilution for 2 h at room temperature and developed after further washes using Pierce Super Signal ECL reagent (Thermo Fisher Scientific, Pittsburgh, PA).

Immunocytochemistry—Cells were plated in 8-chamber slides coated with poly-D-lysine for immunocytochemical analysis. After 1 week in culture, neurons were deprived of KCl in the presence or absence of DRB or flavopiridol for different time periods and fixed with ice-cold methanol for 5 min at −20 °C. The slides were washed with PBS, and nonspecific binding was blocked by incubation for 1 h with 10% normal goat serum diluted in PBS containing 0.1% Triton X-100 (blocking buffer). This was followed by incubation with primary antibodies diluted in blocking buffer for 2 h at room temperature. Cells were washed thoroughly and incubated for 1 h at room temperature in the dark with anti-mouse or anti-rabbit Alexa Fluor 488 or 568 secondary antibodies (1:1000 and 1: 4000, respectively) diluted in blocking buffer. Cells were incubated with 1 μg/ml Hoechst diluted in PBS for 5 min at room temperature for visualization of nuclei. Subsequently, cells were washed, and the wells and gasket were removed from the chamber slide. The slides were mounted using Gel-Mount (Fisher Scientific) and stored at 4 °C, protected from light. After 24 h, the slides were analyzed using a Zeiss fluorescent Axio Imager using Axio Vision Rel 4.8 software.

^{3}H and ^{35}S Incorporation Assay—Neurons under different treatment conditions were incubated with 10 μCi/ml [^{3}H]uridine for the indicated time periods and washed with PBS to remove excess uridine. Cellular proteins were precipitated with 10% TCA, washed with 5% TCA twice, and solubilized in NaOH, and then incorporation of ^{3}H was examined using a LKB liquid scintillation counter. Samples were done in triplicate, experiments were repeated at least three times, and average incorporation was taken to plot the graphs.

For the ^{35}S incorporation assay, granule neurons were incubated for 1 h with methionine- and cysteine-free DMEM containing 25 mM KCl. 100 μCi/ml [^{35}S]methionine/cysteine (New England Nuclear, Boston, MA) was added to the medium with or without depolarizing concentrations of KCl, and the cells were incubated in the presence and absence of DRB or flavopiridol for different time periods. At the end of the treatment, cells were washed, and TCA precipitable counts were determined using a scintillation counter.

BrdU Incorporation Analysis—Neurons cultured in 8-chamber slides were incubated with 10 μM BrdU for 6 h. At the end of the incubation period, cells were fixed with 70% ethanol, washed, and hydrolyzed using 2 N HCl by incubation at 37 °C for 30 min. HCl was removed, and cells were incubated with sodium borate buffer (pH 8.0) for 5 min, washed, and incubated with 1% BSA solution in TBS to block nonspecific staining. Cells were incubated with rat monoclonal anti-BrdU (1:300 dilution, Abcam) and mouse monoclonal anti NeuN (1:500 dilution, Millipore) antibodies for 2 h at room temperature and visualized after staining with Alexa Fluor 594 and 488 secondary antibodies (1:1000 dilution) on a Zeiss fluorescent Axio Imager using Axio Vision Rel 4.8 software.

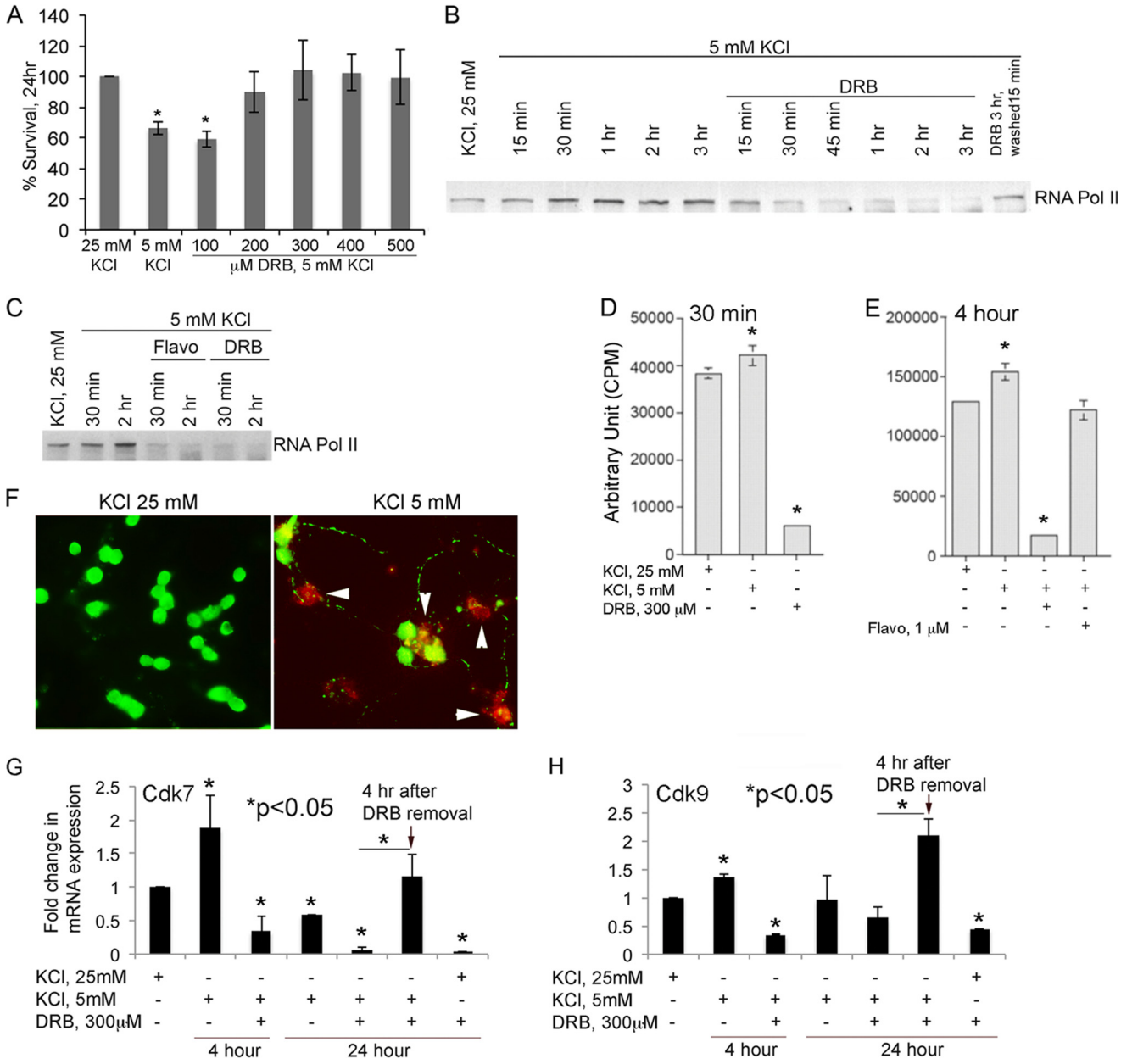
P70S6 Kinase Activity Assay—The S6 kinase activity assay kit was purchased from Enzo Life Sciences (Farmingdale, NY), and the assay was performed following the protocol of the manufacturer using a substrate-coated ELISA plate. Briefly, neurons were subjected to different treatments, and lysates were prepared in Hepes lysis buffer (buffer A). Approximately 100 μg of protein was used for the activity assay. The kit contents were warmed up to room temperature, and the ELISA plate was incubated for 10 min with the kinase assay buffer provided in the kit. The samples were diluted in kinase assay buffer and added to the ELISA plate, followed by addition of 10 μl of ATP to initiate the reaction. The plate was incubated at 30 °C for 1 h on a rocker. The contents of the plate were removed, and the plate was incubated with phospho-specific substrate antibody for another hour at room temperature. The wells were washed and incubated with HRP-conjugated anti-rabbit IgG for 30 min at room temperature. At the end of the incubation period, the wells were washed again and incubated with TMB substrate until the desired color was reached. The reaction was stopped by addition of acid stop solution, absorbance was read at 450 nm, and the relative kinase activity was calculated after subtracting the absorbance of the blank from the samples.

Real-time PCR Analysis for CDK7 and CDK9 mRNA Expression—Cerebellar granule neurons cultured in BME containing 25 mM KCl were left untreated or changed to medium with low KCl (5 mM KCl) with or without DRB (300 μM) for 2 h, 4 h, or overnight. The cells treated with DRB overnight were left undisturbed or washed to remove DRB and replenished with medium containing 5 mM KCl to determine the changes in RNA expression upon withdrawal of DRB. At the end of the treatment, neurons were washed with PBS, and RNA was extracted using the Qiagen RNeasy kit (Qiagen, Inc. Valencia, CA). Purified RNA was quantified on a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific) and stored at −80 °C until ready to be analyzed. To determine the RNA expression levels, a two-step, real-time PCR analysis was performed. First, RNA (0.5–1 μg) was used to make cDNA using the iScript cDNA synthesis kit (Bio-Rad). Next, real-time PCR with SYBR Green was performed using the ABI 7500 Fast System (Applied Biosystems, Grand Island, NY). We examined the expression of *CDK7* and *CDK9* using the following primers specific for the rat sequences: *CDK7*, 5′-AGG-ATG-TAC-GGT-GTG-GGA-GT-3′ (forward) and 5′-TGC-CAG-GGA-AAC-TCT-TGA-AC-3′ (reverse); *CDK9*, 5′-AGC-TTC-ACC-CTA-TAA-CCG-CTG-CAA-3′ (forward) and 5′-ACT-AAG-ACA-TTG-CTC-AGC-AGC-CCA-3′ (reverse). *GAPDH* was used as housekeeping gene for normalization of the results. The following primers were used for amplification of *GAPDH*: 5′-TGA-CTC-TAC-CCA-CGG-CAA-GTT-CAA-3′ (forward) and 5′-TGG-TGA-TGG-GIT-TCC-CGT-TGA-TGA-3′ (reverse). Relative amounts of mRNA levels were calculated using the $2\text{-}\Delta\Delta C_T$ formula.

RESULTS

Inhibition of RNA Pol II Protects CGN from KCl Withdrawal-induced Cell Death—Our earlier studies have shown that KCl withdrawal-induced apoptosis in CGNs could be inhibited by treatment with the Cdk inhibitor flavopiridol (16). Because fla-

vopiridol has been shown to inhibit Pol II through its interaction with P-TEFb, we examined whether inhibition of Pol II protects neurons against apoptosis. CGNs were deprived of KCl in the presence or absence of the Pol II inhibitor DRB, cells were lysed, and nuclei were counted (55). Neurons treated with DRB at 300 μM and above showed a significant protection against KCl withdrawal-induced apoptosis at 24 h (Fig. 1*A*). At 100 μM, we did not observe any inhibition on apoptosis, and, at 200 μM, the results were inconsistent. A single dose of DRB gave significant protection for up to 48 h, suggesting that Pol II-dependent transcription is involved in activity withdrawal-induced neuronal apoptosis. Analysis of cell lysates prepared from CGNs treated with or without DRB showed that KCl withdrawal is associated with an increase in the level of Pol II phosphorylation within 30 min, which was inhibited upon treatment with DRB or flavopiridol (Fig. 1, *B* and *C*). [^{3}H]Uridine incorporation analysis confirmed that neurons deprived of KCl show an ~10–20% increase in transcription compared with the control untreated neurons (Fig. 1, *D* and *E*), and treatment with DRB inhibited [^{3}H]uridine incorporation significantly (control = 100%, DRB = 15%). Neurons treated with flavopiridol maintained [^{3}H]uridine incorporation at the basal level, which was equivalent to that observed in CGNs cultured in 25 mM KCl (Fig. 1*E*). The reason for the differential effect elicited by DRB and flavopiridol on the level of transcription inhibition is unclear. Although flavopiridol is known to inhibit CDK2, CDK4, CDK7, and CDK9, DRB is mainly considered a P-TEFb (CDK9-Cyclin T complex) inhibitor, and cells treated with DRB have been shown to accumulate short transcripts. Because both flavopiridol and DRB protected the neurons from undergoing apoptosis, we predict that transcription of



a subset of genes that are inhibited by these drugs is important for KCl withdrawal-induced cell death in CGNs.

To confirm that new DNA synthesis occurs upon induction of apoptosis, we performed a BrdU incorporation analysis and found that neurons deprived of KCl show an increase in BrdU staining, indicative of new DNA synthesis (Fig. 1*F*). The neurons that stained positively for BrdU showed a decrease in NeuN staining. The reason for this is unclear. NeuN is a marker for mature differentiated neurons, and it is possible that the neurons undergoing apoptosis may undergo dedifferentiation.

Neurons Undergoing Apoptosis Show Increased Expression of CDK7 and CDK9—Because the elongation by RNA Pol II is regulated by CDK9 and CDK7 (28, 32, 33, 56), we examined whether the neurons undergoing apoptosis show any change in expression of these kinases. Our results show that expression of *CDK7* and *CDK9* was increased within 4 h after KCl withdrawal, which were inhibited by DRB (Fig. 1, *G* and *H*). At 24 h, the levels of *CDK7* and *CDK9* were lower than that in the control, which was decreased further upon DRB treatment. Analysis of neurons that were treated with DRB for 24 h, washed off, and replenished with low KCl medium showed a significant increase in *CDK7* and *CDK9* expression, indicating that apoptosis in neurons is associated with activation of CDK7 and CDK9.

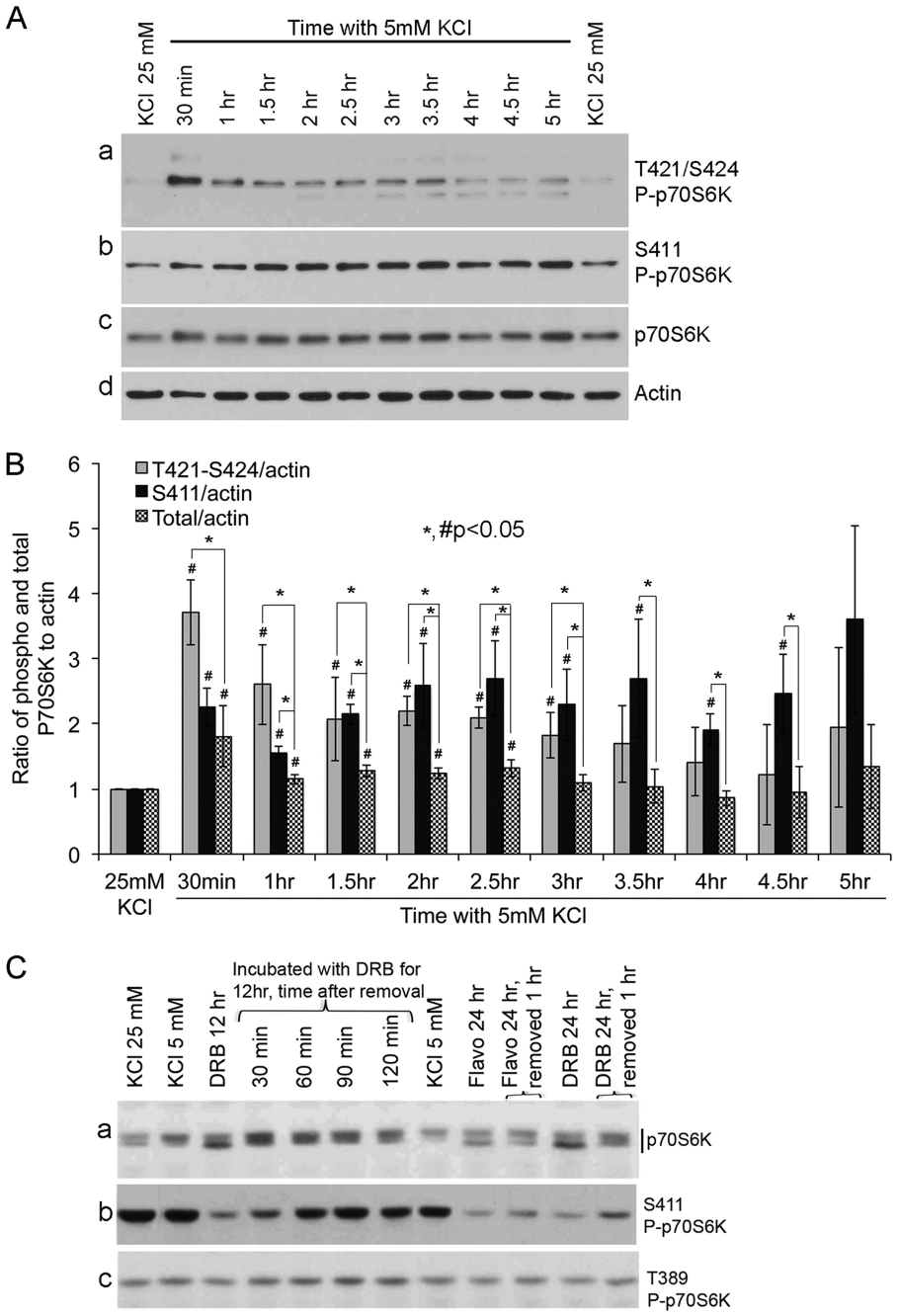
Neurons Undergoing Apoptosis Show Increased Phosphorylation of P70S6K—In addition to Pol-II, DRB has also been shown to inhibit nuclear and cytosolic P70S6K, which is involved in the phosphorylation of the S6 subunit of the 40 S ribosome (39). Because the activity of this kinase is associated with enhanced protein synthesis as well as cell cycle activation, we examined whether KCl depletion in CGNs has any effect on P70S6K. Neurons were deprived of KCl for different time periods, and cell lysates were prepared and analyzed by Western blot using phospho-specific (Ser(P)-411 and Thr(P)421/Ser(P)-424) and total P70S6K antibodies. KCl withdrawal resulted in a time-dependent increase in phosphorylation of P70S6K at Ser-411 and Thr-421/Ser-424 (Fig. 2*A*, *a* and *b*). These sites are known to be phosphorylated by Cdks and suggest that P70S6K could be a downstream target of these kinases during KCl withdrawal-induced apoptosis. Levels of total P70S6K were also elevated, and showed very similar pattern as Thr-421/Ser-424 P-P70S6K but to a lesser extent. P70S6K showed a slight mobility shift after 30 min of KCl withdrawal (Fig. 2*A*, *c*). The results with total P70S6K antibody suggest that, in addition to phosphorylation, its expression may also be increased in cells undergoing apoptosis. We speculate that the mobility shift in P70S6K is brought about by the increased phosphorylation, as reported by others (48). The blots were reprobed with actin antibody for normalization for protein loading (Fig. 2*A*, *d*). For quantification, the percentage of change in the levels of phospho- or total P70S6K was calculated on the basis of the corresponding levels in untreated neurons cultured with 25 mM KCl and normalized to actin levels prior to plotting the graph (Fig. 2*B*). The data show that both phosphorylation and total levels of P70S6K are elevated within 30 min after KCl deprivation compared with that in the untreated sample (Fig. 2*B*, $p < 0.05$). Furthermore, the data also show a significant increase in Thr-421/Ser-424 and Ser-411 phosphorylation of P70S6K compared with its total level (Fig. 2*B*, $p < 0.05$). The increase in phosphorylation was transient and decreased to non-significant levels over the time course.

To examine the effect of DRB and flavopiridol on P70S6K phosphorylation, neurons were deprived of KCl, treated with the drugs for 12 or 24 h, and examined for changes in total P70S6K and Ser-411 P-P0S6K. Analysis of neurons deprived of KCl for 12 and 24 h using total P70S6K antibody showed an increase in the slower migrating band, indicating phosphorylation of the kinase upon cell death induction (Fig. 2*C*, *a*, *lanes 2* and *8* compared with lane *1*). Treatment with DRB and flavopiridol inhibited the mobility shift, indicating an inhibition of P70S6K phosphorylation (Fig. 2*C*, *a*, *lanes 3*, *9*, and *11* compared with lanes *2* and *8*). Removal of the drugs led to a time-dependent reversal of this inhibition and reduced migration of P70S6K, indicative of its increased phosphorylation (Fig. 2*C*, *a*,

FIGURE 1. *A*, DRB protects neurons from activity withdrawal-induced apoptosis. Granule neurons cultured in the presence of 25 mM KCl were switched to medium with 5 mM KCl in the presence and absence of DRB at a concentration ranging from 100–500 μM. Cell death was measured after 24 h by lysing the cells and counting the nuclei. DRB concentrations above 200 μm protected KCl withdrawal-induced apoptosis in the neurons with 300 μM, consistently showing protection. Data are represented as mean ± S.E. from three independent experiments done in triplicates. *, $p < 0.05$. *B*, DRB inhibits hyperphosphorylation of Pol II in CGNs undergoing apoptosis. Neurons deprived of KCl were treated with or without 300 μM DRB for 15 min to 3 h and analyzed by Western blot using an antibody specific to the large subunit of Pol II. DRB inhibited the KCl withdrawal-induced increase in Pol II phosphorylation and brought it down to below basal levels. Upon withdrawal of DRB, the inhibition of phosphorylation was reversed (*last lane*, 15 min after withdrawal of DRB). *C*, flavopiridol (*Flavo*) showed a similar inhibition of Pol II phosphorylation as DRB. Neurons were deprived of KCl in the presence and absence of DRB (300 μM) or flavopiridol (1 μM) for 30 min and 2 h, and cell lysates were Western-blotted using a polyclonal antibody to the Pol II large subunit. Both DRB and flavopiridol showed a similar inhibition of Pol II phosphorylation. *D* and *E*, increased [^{3}H]uridine incorporation in cerebellar granule neurons deprived of KCl. Cerebellar granule neurons left untreated or deprived of KCl were incubated with [^{3}H]uridine in the presence or absence of DRB (300 μM) or flavopiridol (1 μM) for the indicated times, and cell lysates were analyzed for incorporated radioactivity. *D*, the level of incorporation at 30 min after KCl withdrawal. We observed an ~5–10% increase at this time point, and treatment with DRB inhibited the transcription significantly, as evident by the very low level of incorporation (10–15% of control value). *E*, incorporation after 4 h, where neurons cultured in 5 mM KCl showed an ~15–20% increase in transcription, whereas the presence of DRB kept the level significantly below the basal level (15% of the untreated control). Flavopiridol treatment showed similar levels of incorporation, as seen with 25 mM KCl, suggesting that the basal level of transcription is maintained by this drug. Data are represented as mean ± S.E. from three independent experiments. *, $p < 0.05$. *F*, KCl deprivation leads to new DNA synthesis, as evident by BrdU incorporation in neurons. Neurons cultured in 8-chamber slides were deprived of KCl and incubated with 10 μM BrdU for 6 h. At the end of the incubation, cells were fixed and stained using rat anti-BrdU and mouse anti-NeuN antibodies and Alexa Fluor 594 anti-rat and 488 anti-mouse secondary antibodies. Neurons subjected to KCl deprivation showed an increase in BrdU incorporation, indicating new DNA and RNA synthesis upon apoptosis induction. The neurons that were positive for BrdU showed reduced NeuN staining. Magnification, ×63. *G* and *H*, KCl deprivation enhances expression of *CDK7* and *CDK9* in neurons. Neurons were deprived of KCl in the presence or absence of DRB (300 μM) for 4 or 24 h, and, from one set of DRB treated samples, the drug was washed off, and cells were replenished with medium containing 5 mM KCl for 4 h. Real-time PCR analysis was performed with RNA prepared from these samples using rat-specific *CDK7* and *CDK9* primers. The data show that removal of KCl leads to increased expression of *CDK7* (*G*) and *CDK9* (*H*) at 4 h, which is inhibited by DRB. At 24 h, the cells generally showed low levels of RNA expression, possibly because of cell death. DRB protected the cells and showed very low levels of *CDK7* and *CDK9* mRNA expression. Removal of DRB reversed the inhibition, and the neurons showed a significant increase in the expression of *CDK7* and *CDK9*. The data shows mean ± S.E. from three independent experiments. *, $p < 0.05$.

lanes 4–7, *10*, and *12* compared with lanes *3*, *9*, and *11*, respectively). Reprobing of the blot using Ser-411 P-P70S6K antibody showed inhibition of phosphorylation with DRB and flavopiridol and reversal of the inhibition upon drug withdrawal (Fig. 2*C*, *b*). One of the Cdks that can induce Ser-411-specific phosphorylation is CDC2/cyclin B (45). Studies in CGNs have shown that apoptosis is associated with activation of cdc2 (14). Therefore, it is possible that CDC2-dependent phosphorylation of P70S6K at Ser-411 plays an important role in activity withdrawal-induced apoptosis. Because phosphorylation of P70S6K at Thr-389 by mTOR has been shown to induce kinase activation (49), we examined whether Thr-389 phosphorylation is induced upon KCl withdrawal or is affected by the inhibitor treatment. We did not observe any significant change in Thr-389 phosphorylation on P70S6K (Fig. 2*C*, *c*). Furthermore, rapamycin, an inhibitor of mTOR (57), did not protect the neurons against apoptosis (data not shown).

KCl Withdrawal Is Associated with Increased P70S6K Enzyme Activity—Because the apoptosis in CGNs is associated with an increase in phosphorylation of P70S6K, we examined whether this correlates with the enzyme activity. CGNs deprived of KCl were treated with DRB or flavopiridol for 15 min, 30 min, or 1 h, and cell lysates were analyzed for kinase activity. KCl withdrawal resulted in a transient increase in P70S6K activity that peaked at 30 min and diminished after 1 h (Fig. 3*A*). Treatment of neurons with DRB or flavopiridol inhibited enzyme activity significantly at 30 min and 1 h. To determine whether the increased P70S6K activity correlates with an



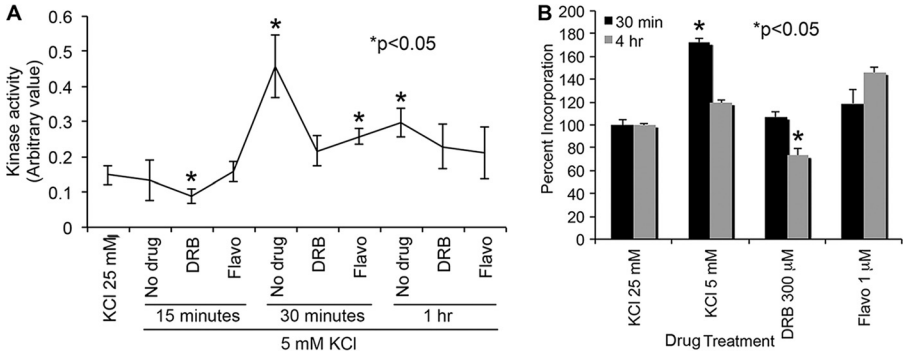


FIGURE 3. **KCl deprivation induces P70S6K activity and protein synthesis in cerebellar granule neurons.** *A*, neurons were deprived of depolarizing concentrations of KCl, and cell lysates were made in Hepes lysis buffer at 15 min, 30 min, or 1 h in the presence or absence of DRB or flavopiridol (*Flavo*). The kinase assay was performed using the assay kit from Enzo Life Sciences. The data show that KCl withdrawal induces kinase activity significantly at 30 min and 1 h, with maximum activity visible at 30 min. DRB inhibited the kinase activity at all times tested. Treatment with flavopiridol showed significant inhibition at all time points compared with cells deprived of KCl, but, at 30 min, the levels were higher that the basal level. Data are represented as mean ± S.E. from three independent experiments. *, $p < 0.05$. *B*, apoptosis is associated with enhanced protein synthesis in neurons. Neurons were incubated with [^{35}S]methionine/cysteine in methionine-free medium with low KCl in the presence or absence of the inhibitors, samples were collected after 30 min and 4 h, and incorporated radioactivity was measured using a scintillation counter. Results showed that KCl withdrawal is associated with a significant increase in ^{35}S incorporation at 30 min, indicating an increase in translation. By 4 h, the samples showed lower ^{35}S incorporation compared with that at 30 min, but it was still above basal level. DRB and flavopiridol inhibited the incorporation at 30 min after KCl withdrawal. Although DRB continued to inhibit the incorporation even at 4 h, the effect with flavopiridol was weak. Data are represented as mean ± S.E. from three independent experiments. *, $p < 0.05$.

increase in protein synthesis, we examined[^{35}S]methionine incorporation in the presence or absence of DRB or flavopiridol. There was a significant increase (60–70%) in the levels of [^{35}S]methionine incorporation within 30 min after KCl withdrawal (Fig. 3*C*). The incorporation was reduced at 4 h, which correlated very well with the time course of P70S6K activation. DRB treatment inhibited [^{35}S]methionine incorporation at 30 min, and sustained the lower levels even after 4 h of treatment. Flavopiridol treatment showed a transient but significant inhibition of ^{35}S incorporation at 30 min, and, by 4 h, the incorporation was close to or slightly higher than that observed with cells cultured in low KCl medium. These data suggest that cell death is intimately associated with translation of a subset of mRNAs, which could be responsible for KCl withdrawal-induced apoptosis in CGNs.

Nuclear Accumulation of P70S6K in CGNs Undergoing Apoptosis—Studies have suggested that P70S6K translocates into the nucleus upon activation (39), and we examined whether cellular distribution of this kinase is altered in CGNs subjected to KCl withdrawal-induced apoptosis. Neurons were deprived of KCl for 14 h in the presence or absence of DRB, and cells were examined by immunostaining analysis using monoclonal Ser(P)-411-specific P70S6K and polyclonal Pol II antibodies. We observed a significant increase in the levels of Ser(P)-411 P70S6K in the nuclei of cells deprived of KCl compared with untreated cells (Fig. 4*A*, *second row*, *arrows*). The neurons that already underwent apoptosis, as evident by DNA condensation, showed loss of P-P70S6K (Fig. 4*A*, *second row*, *arrowheads*). P-P70S6K appeared to colocalize with Pol II in the neuronal nuclei upon induction of apoptosis. The cells treated with DRB for 14 h showed redistribution of P-P70S6K into large speckles in the nucleus (Fig. 4*A*, *third row*). Because the effect of the inhibitors on neurons was reversible, we examined whether the speckles are affected upon withdrawal of DRB. Neurons containing DRB were washed to remove the drug, replenished with low KCl medium without DRB for 3 h, and examined for

FIGURE 2. *A*, KCl deprivation induces P70S6K phosphorylation. Neurons were deprived of KCl, and cell lysates were prepared at the indicated times. Equal amounts of proteins were separated on a 12% tris-glycine gel, and Western blot analysis was performed using phospho- and total P70S6K antibodies. Analysis using Thr(P)-421/Ser(P)-424 P70S6K (*a*) antibody showed that KCl withdrawal induces phosphorylation at these sites, with maximum phosphorylation visible at 30 min. The levels remained high up to 3–4 h after KCl withdrawal. Analysis using the Ser(P)-411 P70S6K (*b*) antibody also showed an increase within 30 min after KCl withdrawal, and the levels remained high up to 4 h, at which point they started showing a decline. Analysis using total P70S6K antibody also showed an increase in the total level of the kinase upon KCl withdrawal, and this was associated with a mobility shift in the band (*c*). Blots were reprobed with actin antibody (*d*) for normalizing protein loading on gels. The figure represents one of three independent experiments. *B*, percentage of change in the levels of Thr(P)-421/Ser(P)-424, Ser(P)-411, and total P70S6K were calculated in comparison with the corresponding levels in untreated neurons cultured in 25 mM KCl and normalized to the percent of actin in the sample. Significance for phospho- and total P70S6K was calculated in relation to untreated samples (#) or between phospho and total P70S6K (*). The *y* axis shows the ratio of phospho- or total P70S6K to actin in each sample. The bar graph shows mean ± S.E. from three independent experiments. * and #, $p < 0.05$). *C*, DRB and flavopiridol inhibits phosphorylation of P70S6K in a reversible manner. Neurons were incubated with or without DRB (300 μM) or flavopiridol (1 μM) in low KCl medium for 12 (DRB) or 24 h (DRB or flavopiridol) or were depleted of drugs, and the incubation continued in low KCl medium for 30 min, 1 h, 1.5 h, and 2 h (in the case of DRB) or for 1 h (in the case of cells treated with DRB or flavopiridol for 24 h) (*lanes 9* and *10* in the case of flavopiridol and lanes *11* and *12* in the case of DRB). Cell lysates were analyzed using total P70S6K (*a*), Ser(P)-411 P70S6K (*b*), or Thr-389 (*c*) P-P70S6K antibodies. *a*, shows an increase in the slower migrating band upon KCl withdrawal, indicating increased phosphorylation of P70S6K at 12 and 24 h (*lanes 2* and *8* compared with *lane 1*). Treatment with DRB inhibited this increase and showed an increase in the level of the faster migrating band (*lanes 3* and *11* compared with *lanes 2* and *8*). Withdrawal of DRB after 12 h resulted in a time-dependent mobility shift and increase in the slower migrating hyperphosphorylated P70S6K (*a*, *lanes 4—7* compared with *lane 3*), suggesting that inhibition by DRB is reversible. Withdrawal of the inhibitors after 24 h of treatment also showed a similar effect. The inhibition of P70S6K brought about by flavopiridol and DRB treatment was reversed upon removal of the drugs, which is evident by the appearance of the slower migrating P70S6K bands (*a*, *lane 10* compared with *lane 9* with flavopiridol and *lane 12* compared with *lane 11* with DRB). *b*, reprobing of the blot with Ser(P)-411 P70S6K antibody, which showed almost similar levels of phosphorylation as untreated cells after 12 h of KCl withdrawal, which was inhibited by DRB and flavopiridol. Removal of the drugs reversed the inhibition, and the levels of Ser(P)-411 P70S6K increased in a time-dependent manner. Reprobing of the blot with Thr(P)-389 P70S6K, the rapamycin-sensitive, site-specific antibody, did not show any significant change in phosphorylation of P70S6K at this site.

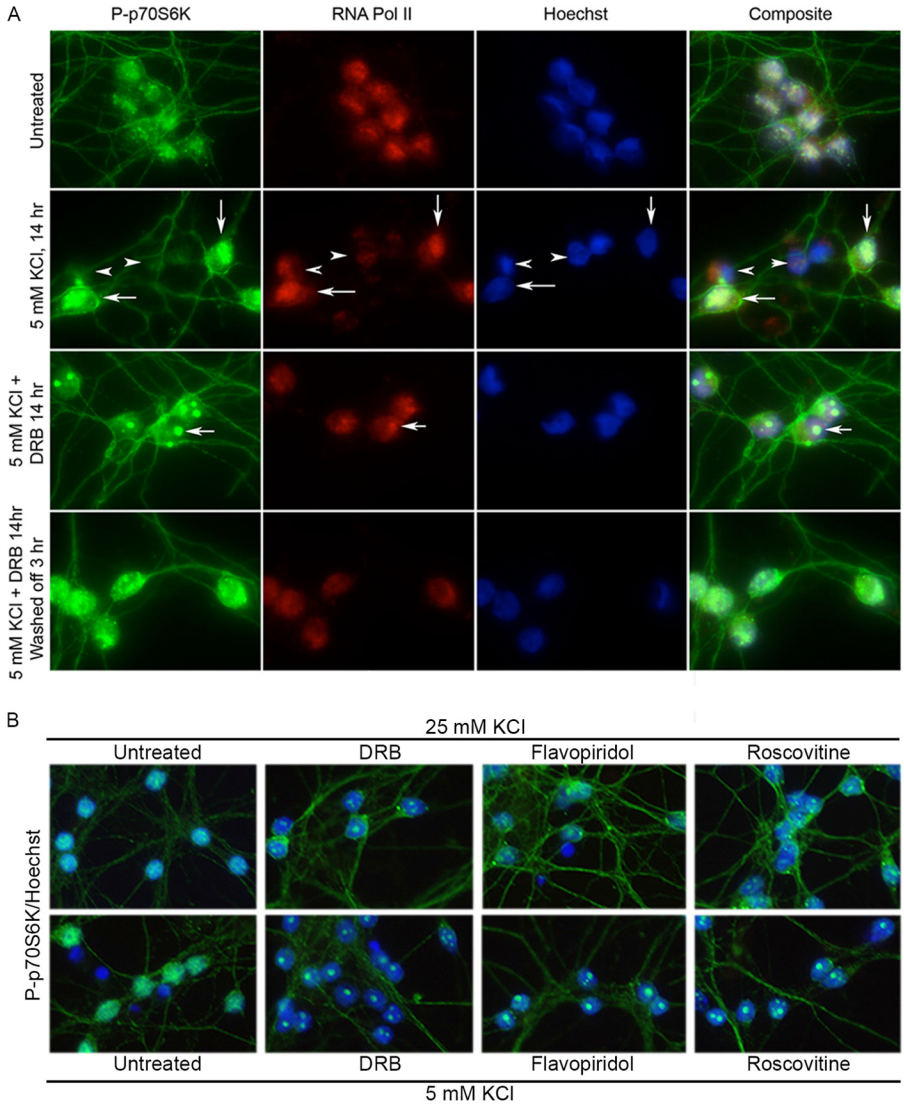


FIGURE 4. **Nuclear accumulation of P70S6K in neurons undergoing apoptosis.** *A*, neurons were treated with or without DRB in low KCl medium, analyzed after 14 h using Ser(P)-411 P70S6K mouse monoclonal and Pol II rabbit polyclonal antibodies, and staining was visualized using Alexa Fluor 488 and 594 secondary antibodies, respectively. Results show that KCl withdrawal is associated with an increase in localization of Ser(P)-411 P70S6K to the nucleus (*arrows*), which colocalizes with Pol II (*second row* compared with *first row*). The neurons that showed DNA condensation showed a decrease in P-P70S6K levels (*arrowheads*). In neurons treated with DRB, staining appeared to localize to one or two large speckles in the nucleus (*third row*, *arrows*). *Fourth row*, cells where DRB was withdrawn after 14 h and cells were replenished with medium containing 5 mM KCl without DRB for an additional 3 h. Removal of DRB resulted in dispersal of large speckles into smaller ones and an increase in the nuclear distribution of Ser(P)-411 P70S6K. Magnification, ×63. *B*, P-P70S6K-positive speckle formation correlates with inhibitor treatment. Neurons cultured with 25 mM and 5 mM KCl were treated with or without DRB (300 μM), flavopiridol (1 μM), or roscovitine (20 μM) and stained using P-P70S6K (Ser-411) antibodies. The results showed the formation of large P-P70S6-positive speckles in cells treated with the inhibitors under both apoptotic (KCl 5 mM) and non-apoptotic conditions. These speckles appeared to be more pronounced in the cells deprived of KCl. Nuclei were visualized using Hoechst. Magnification, ×40.

cellular distribution of P-P70S6K. Results showed that the larger bright spots formed after inhibitor treatment dispersed into many smaller ones upon removal of the drug (Fig. 4*A*, *fourth row*), indicative of a direct link between speckle size increase and transcription inhibition. To determine whether the speckles are formed under non-apoptotic conditions or in the presence of other cell cycle inhibitors, we examined neurons cultured in the presence of 25 or 5 mM KCl after treatment with DRB, flavopiridol, or roscovitine. It was found that inhibitor treatment induced the formation of large speckles under apoptotic and non-apoptotic conditions, indicating that this is not solely formed by cells undergoing apoptosis and is brought about by the effect of inhibitors on the neurons (Fig. 4*B*).

The Speckle Domains Are Immunopositive for the Spliceosome Assembly Factor SC35—To determine whether the bright spots we observed in the neuronal nuclei represent speckle domains associated with transcription, we performed an immunostaining analysis of CGNs with antibodies specific to the splicing factor compartment-associated protein SC35 (58). CGNs were deprived of KCl in the presence or absence of DRB or flavopiridol for 14 h and stained with monoclonal SC35 and polyclonal Pol II antibodies. The SC35 antibody showed dispersed staining in untreated and KCl-deprived neurons, whereas the cells treated with the drugs showed accumulation of SC35 into large speckles (Fig. 5*A*), which stained positive for Pol II as well. As in the case of P-P70S6K, formation of large

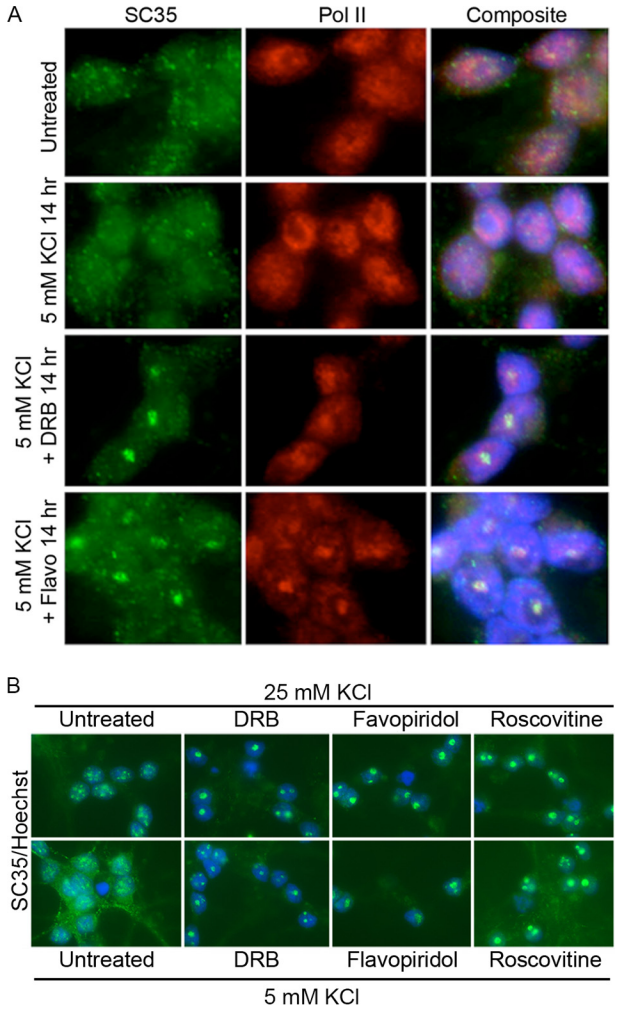


FIGURE 5. **Nuclear speckle domains in granule neurons are SC35-positive.** *A*, neurons were deprived of KCl in the presence and absence of DRB or flavopiridol (*Flavo*) and stained with SC35 and Pol II antibodies. Cells were fixed and stained using a monoclonal SC35 and polyclonal Pol II antibodies, and staining was visualized using Alexa Fluor 488 and 594 secondary antibodies, respectively. Treatment of the cells with flavopiridol and DRB resulted in fewer and larger SC35 positive speckle domains (*third* and *fourth rows*). Pol II also showed a similar localization pattern with more dispersed nuclear speckles under low KCl conditions and accumulation of these speckles into larger ones in cells treated with DRB or flavopiridol. This indicates that apoptosis induction and inhibition affect the organization of nuclear speckle domains in neurons. Magnification, ×100. *B*, SC35-positive large speckles are formed in neurons treated with DRB, flavopiridol, or roscovitine under apoptotic and non-apoptotic conditions. Neurons cultured in 25 mM or 5 mM KCl were treated with 300 μM DRB, 1 μM flavopiridol, or 20 μM roscovitine for 24 h and stained using SC35 antibodies. Nuclei were visualized using Hoechst. Magnification, ×40.

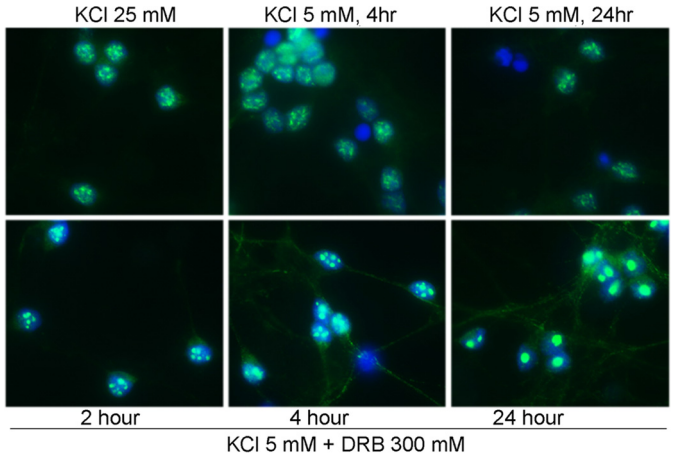


FIGURE 6. **Neurons show a time-dependent increase in nuclear speckle size upon DRB treatment.** CGNs were treated with or without DRB in low-KCl medium for 2, 4, and 24 h, and immunostaining analysis was performed using SC35 antibody. Hoechst was used to visualize the nuclei. Magnification, ×63.

SC35-positive speckles occurred under apoptotic and non-apoptotic conditions and was induced upon treatment with DRB, flavopiridol, and roscovitine (Fig. 5*B*). Additionally, a time course analysis of the nuclear speckles showed that treatment with DRB leads to a time-dependent increase in speckle size (Fig. 6, compare *bottom row* with *top row*). After 24 h with the drugs, the cells mainly contained one or two large speckles within the nucleus, which may be indicative of global transcription inhibition.

CGNs Undergoing Apoptosis Show Increased Levels of P-histone H3—Because mitotic stimulation of mammalian cells is associated with induction of histone H3 phosphorylation (59, 60), we examined the neurons undergoing apoptosis for P-histone H3 levels. Staining of neurons using Ser(P)-10 histone H3 and MAP2 antibodies showed that KCl withdrawal led to an increase in P-histone H3 levels in neurons, which was diminished in cells treated with DRB (Fig. 7*A*, compare *center row* with *top* and *bottom rows*). We examined whether the P-histone H3-positive neurons showed an increase in P-P70S6K levels. Immunostaining analysis of neurons deprived of KCl using P-histone H3 and Ser(P)-411-P70S6K antibodies did not show any colocalization of these two phosphoproteins (Fig. 7*B*). Similar to the results shown in Fig. 4, the neurons deprived of KCl showed an increase in nuclear localization of Ser(P)-411-P70S6K, but these neurons did not show increased P-histone H3 (Fig. 7*B*, *second row*, *arrowheads* and *arrows*). P-histone H3-positive neurons showed nuclear condensation and DNA fragmentation, indicative of apoptosis induction. Removal of DRB resulted in reversal of inhibition of histone H3 phosphorylation, implying that mitotic progression is linked to apoptosis induction in KCl-deprived CGNs (Fig. 7*B*, *fourth row*).

DISCUSSION

The results presented here show that KCl withdrawal-induced apoptosis in CGNs is associated with an increase in transcriptional and translational activation, as evident by increased DNA, mRNA, and protein synthesis. The increased transcription appears to be brought about by increased phosphorylation and activation of RNA polymerase II, and the increased translation appears to correlate with phosphorylation and activation of P70S6K. The neurons undergoing apoptosis showed enhanced phosphorylation of histone H3, indicative of transcriptional activation or mitotic progression in neurons undergoing apoptosis.

Inhibitors of transcription, such as DRB and flavopiridol, prevented transcription and reentry into mitosis and protected neurons from undergoing apoptosis. DRB is a specific reversible inhibitor of Pol II, and it preferentially inhibits mRNA elongation by the positive elongation factor P-TEFb (38). P-TEFb is a complex of CDK9 and cyclin T, and our analysis showed that neurons deprived of KCl show enhanced expression of not only

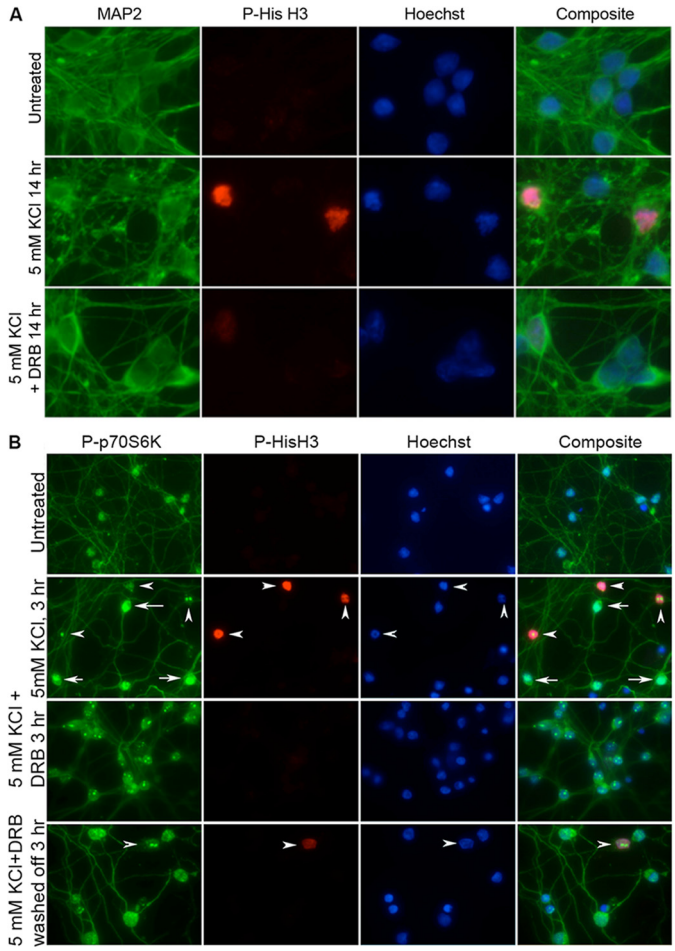


FIGURE 7. *A*, increased phosphorylation of histone H3 in neurons undergoing apoptosis. Neurons treated with or without DRB for 14 h were fixed and stained using Ser(P)-10-histone H3 polyclonal and MAP2 monoclonal antibodies. The staining was visualized using Alexa Fluor 594 and 488 secondary antibodies. The results show an increase in P-histone H3-positive neurons after KCl withdrawal (*center row*), and DRB inhibited this increase (*bottom row*). Magnification, ×63. *B*, coimmunostaining analysis did not show any colocalization of P-histone H3 and P-P70S6K. To determine whether the neurons that showed increased P-histone H3 show increased P-P70S6K, we performed coimmunostaining using the corresponding antibodies. Neurons were treated with or without DRB for 3 h, and, in a few wells, DRB was washed off, and neurons were replenished with medium deprived of KCl and cultured for an additional 3 h. The results showed an increase in P-histone H3- and P-P70S6K-positive neurons after KCl withdrawal (*arrowheads* and *arrows* in the *second row*, respectively). We did not observe any colocalization of these two proteins in the neurons. Treatment with DRB inhibited the increase (*third row*), and removal of DRB led to an increase in the nuclear dispersion of P-P70S6K and appearance of P-histone H3-positive neurons. Magnification, ×40.

CDK9 but also its activator *CDK7*, both of which were inhibited upon treatment with DRB. Removal of DRB resulted in reversal of this inhibition and led to an increase in expression of *CDK7* and *CDK9*, subsequently enhancing Pol II phosphorylation and apoptosis induction. These data indicate that apoptosis in neurons is associated with Pol II-dependent transcriptional activation and that neuronal protection can be achieved by treating neurons with inhibitors of transcription.

In addition to increased Pol II activation, neurons showed phosphorylation and activation of P70S6K, which was inhibited by DRB and flavopiridol. This is intriguing and may suggest that P70S6K may be regulated by CDK7 or CDK9. P70S6K is known for its role in modulation of cell cycle progression, cell survival, differentiation, and motility (40, 41, 61). It is activated in response to mitogen stimulation, and it up-regulates ribosomal biosynthesis and translational capacity of the cell. Because it is known to regulate mRNAs with 5′-terminal oligopyrimidine tracts, which generally encode ribosomal proteins and elongation factors (62), it is possible that the proteins generated from translation of 5′-terminal oligopyrimidine mRNAs might play a role in cell death induction. PI3K has been shown to induce P70S6K phosphorylation at serine/threonine residues (63). Similarly, mTOR, MAP kinases, and Cdks (45, 64) have been shown to induce phosphorylation and activation of P70S6K. Our analysis showed that, although the mTOR inhibitor rapamycin or a MAP kinase inhibitor, U0126, had no effect on KCl withdrawal-induced apoptosis in CGNs (data not shown), transcriptional inhibitors inhibited P70S6K phosphorylation and protected neurons from apoptosis. This specifies a role for aberrant transcriptional activation and cell cycle deregulation in KCl withdrawal-induced neuronal apoptosis, which is independent of mTOR or ERK signaling cascades. It has been shown that the proline-directed phosphorylations are sensitive to inhibitors of cell cycle progression but not to inhibitors of mTOR. Therefore, it is possible that the increased phosphorylation of P70S6K at Ser-411, Thr-421, and Ser-424, but not at the rapamycin-sensitive Thr-389 site, in neurons undergoing cell death is brought about by activation of Cdks. Activation of P70S6K has been implicated in increased translation of *Tau* mRNA and Aβ-dependent Tau hyperphosphorylation and tangle formation in Alzheimer disease (65–67). Deletion of P70S6K has been shown to enhance learning and behavior and to increase life span and resistance to age-related pathologies in mice (68, 69). Furthermore, studies in *Caenorhabditis elegans* have shown that P70S6K inhibits axon regeneration and that loss of its kinase function leads to accelerated growth cone formation and elongation after injury (70). Together, these data suggest that activation or aberrant expression of P70S6K may negatively affect neuronal function and that targeting this kinase is beneficial for neuronal survival and function.

The neurons treated with DRB and flavopiridol showed sequestration of Pol II and P70S6K into large speckle domains in the nucleus. Nuclear speckles or spots have been reported in cells other than neurons, where they are associated with transcriptional inhibition. For example, treatment of HeLa cells with DRB has been shown to induce formation of SC35-positive nuclear speckles with increasing size (58, 71). Speckle domains have been shown to function as storage units for different transcription factors. In cells actively undergoing proliferation and division, these speckle domains are smaller and interconnected and are known as interchromatin granule clusters. A nucleus under active transcription has many interchromatin granule clusters. On inhibition of transcription, the interconnections are lost, and the granules form enlarged speckles. One of the proteins associated with the active interchromatin granule clusters is Pol II. It has been shown that the large subunit of Pol II associates with the interchromatin granule clusters when there is active transcription (58). During transcriptional inhibition, Pol II is stored in the large speckle domains. Our analysis of CGNs with the SC35 antibody showed that neurons contain

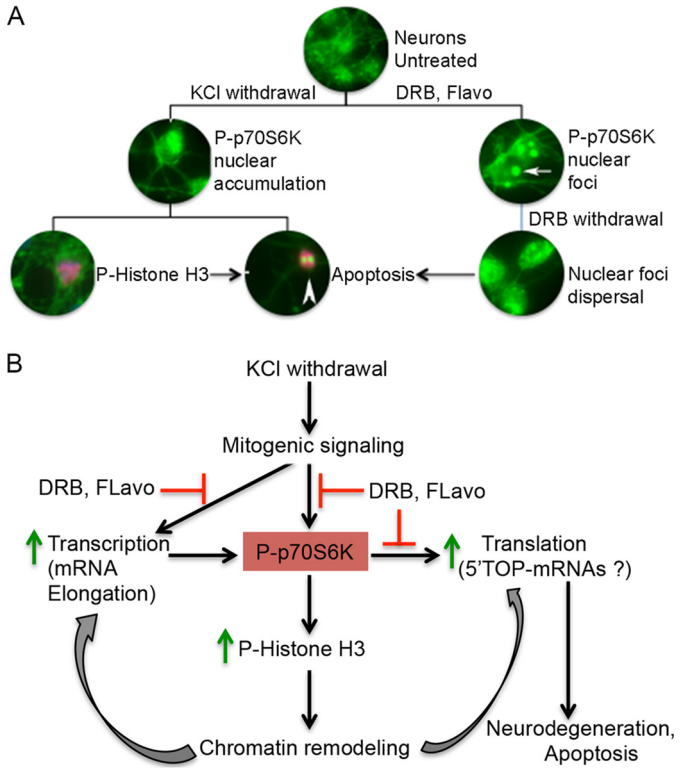


FIGURE 8. *A*, the sequence of events in neurons undergoing apoptosis or inhibited with DRB or flavopiridol (*Flavo*). *B*, proposed signaling cascade involved in mitosis-specific induction of neuronal apoptosis. Upon removal of KCl, transcriptional mechanisms are activated that induce P70S6K activation and mRNA translation. DRB and flavopiridol inhibit Pol II-dependent elongation of the newly synthesized RNA, prevent P70S6K-specific mRNA translation, and protect neurons against apoptosis.

several nuclear speckles. Although KCl withdrawal did not show any significant change in the appearance of the speckles, treatment of the neurons with DRB and flavopiridol showed a time-dependent increase in size with a concomitant decrease in the number of speckles in neuronal nuclei. This may imply that DRB and flavopiridol protect neurons by aggregating the active transcription foci into large inactive foci. Isolation and analysis of these large speckle domains may enable us to determine whether any of the associated components are activated upon apoptosis induction and whether inhibition of the specific genes/proteins associated with these foci can prevent neuronal apoptosis.

In summary, as shown in the schematic provided in Fig. 8, our studies demonstrate that RNA Pol II-dependent transcriptional activation and P70S6K-dependent translation play a crucial role in KCl withdrawal-induced apoptosis in neurons. Additionally, cells undergoing apoptosis show an increase in expression of *CDK7* and *CDK9*, activators of Pol II. Therefore, it is possible that targeted inhibition of CDK9, CDK7, P70S6K, or the specific mRNAs regulated by P70S6K may prevent neuronal loss. On the basis of our data and the finding that P70S6K activation induces Tau hyperphosphorylation and tangle formation (65, 72), we hypothesize that inhibition of P70S6K may not only prevent apoptosis but also protect neurons from microtubule destabilization and neurodegeneration. An identification of the mRNAs regulated by P70S6K may provide us with more insights into their role in apoptosis, and these mRNAs could be targeted to prevent neurodegeneration and neuronal loss associated with various neurodegenerative diseases.

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