

The Transcription Factor NFAT3 Mediates Neuronal Survival*

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Neuronal apoptosis is critical for normal development of the mammalian nervous system and also contributes to the pathogenesis of ischemic and degenerative diseases of the brain. Apoptosis of neurons is tightly regulated by extrinsic signals including growth factors and neuronal activity, but the intracellular mechanisms by which these signals promote neuronal survival are incompletely understood. We report that the transcription factor NFAT3 plays a critical role in mediating survival of granule neurons of the developing cerebellum. NFAT3 accumulated in the nucleus of primary granule neurons under survival conditions of serum growth factors and neuronal activity that was elicited by depolarization with high K^+ . In contrast, deprivation of serum and K^+ , which leads to neuronal apoptosis, triggered NFAT3 nuclear export. Treatment of granule neurons with Li^+ , an inhibitor of the NFAT export kinase GSK3, prevented the nuclear export of NFAT3 and increased granule cell survival even under pro-apoptotic conditions. Thus, the nuclear localization of NFAT3 correlated tightly with granule neuron survival. Consistent with a pro-survival function for NFAT3, genetic knockdown of NFAT3 by RNA interference in primary granule neurons led to increased apoptosis even in neurons cultured under survival conditions. Conversely, expression of a constitutively active form of NFAT protected neurons against apoptosis induced by serum withdrawal and low K^+ . Taken together, these results reveal an essential function for NFAT3-mediated transcription in neuronal survival that may play important roles in the developing and mature brain.

The study of neuronal survival and apoptosis is a central focus in neurobiology and neurology. Neurons are generated in excess during the development of the nervous system, and therefore their numbers need to be controlled by programmed

cell death or apoptosis to ensure the proper wiring of the nervous system (1). In the developing mammalian brain, a number of extrinsic factors tightly control apoptosis. Polypeptide growth factors and neuronal activity are all thought to play critical roles in suppressing apoptosis and promoting neuronal survival (1, 2). In the adult brain, apoptosis of neurons occurs under pathological conditions in response to a variety of insults including ischemia and neurodegenerative processes (3–5). Because of the importance of neuronal survival and apoptosis in brain development and disease, there is tremendous interest in characterizing the intracellular mechanisms by which extrinsic factors control neuronal apoptosis.

A model system that has been widely used in studies of neuronal survival is the primary cerebellar granule neuron culture system (6, 7). In the developing rodent cerebellum, granule neurons undergo developmentally regulated apoptosis peaking at the end of the first week of postnatal life (8). Granule neurons cultured from rats or mice around this time of development undergo cell death in culture unless they are provided with extrinsic survival factors. Maximal survival is provided by a combination of growth factors typically provided by serum together with neuronal activity and mimicked by high extracellular concentrations of potassium chloride that depolarize the membrane and induce activation of voltage-sensitive calcium channels (9–12).

The signaling mechanisms by which growth factors and neuronal activity promote the survival of cerebellar granule neurons are beginning to be characterized. Protein kinase cascades figure prominently in the control of neuronal survival. The ERK1/2-Rsk, phosphatidylinositol 3-kinase-Akt, and ERK5 protein kinase signaling pathways play critical roles in mediating the survival of granule neurons upon exposure to the neurotrophin brain-derived neurotrophic factor (13, 14). The phosphatidylinositol 3-kinase-Akt signaling pathway plays a central role in mediating insulin-like growth factor 1-mediated neuronal survival (15). The influx of calcium via voltage-sensitive calcium channels promotes neuronal survival through activation of the protein kinases p38MAPK and calcium-calmodulin-dependent protein kinase IV (CaMKIV) (16, 17). Removal of survival factors promotes neuronal apoptosis in part because of inactivity of pro-survival protein kinases. However, deprivation of survival factors also leads to stimulation of other protein kinases that impart an apoptotic signal in neurons. These protein kinases include c-Jun NH₂-terminal kinase, Cdc2, and GSK3 (18–25).

Although the mechanisms by which pro-survival and pro-apoptotic kinases regulate neuronal survival remain to be elucidated, a common theme that has emerged from studies of neuronal apoptosis and survival is that many of these kinases act via transcription factors. For instance, the ERK1/2-Rsk and

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CaMKIV protein kinase pathways activate CREB¹ to promote survival (13, 17), whereas the p38MAPK and ERK5 signaling pathways activate the transcription factor MEF2 (14, 16, 26). Akt, which is activated via the phosphatidylinositol 3-kinase pathway, promotes neuronal survival in part by phosphorylating and thereby inhibiting FOXO3-mediated transcription (15); it may also act by inactivating GSK3 and therefore activating the transcription factor NFAT as discussed later. Among the apoptotic kinases, c-Jun NH₂-terminal kinase induces transcription of apoptotic genes via the transcription factor c-Jun (18–21), whereas for GSK3 the most likely transcription factor target is NFAT.

NFAT is a family of highly phosphorylated transcription factors that undergo shuttling between the cytoplasm and nucleus (27–29). The nuclear import of NFAT proteins requires dephosphorylation by the Ca²⁺/calmodulin-dependent phosphatase calcineurin, whereas their nuclear export requires rephosphorylation, which can be mediated by a variety of kinases including GSK3 (30–32). Overexpression of GSK3 promotes NFAT nuclear export (33, 34); conversely, treatment of cells with the GSK3 inhibitor LiCl slows the rate of NFAT nuclear export, thus prolonging the nuclear residence time of NFAT (35). GSK3 is a downstream target of Akt, which promotes an inhibitory phosphorylation of GSK3 on a serine residue near the amino terminus (36). Thus conditions that activate Akt concurrently inhibit GSK3, prolonging the nuclear residence of NFAT (37). In neurons, GSK3 has been demonstrated to inhibit the transcriptional activity of the NFAT family member NFAT3 (34). NFAT3 was shown to be regulated by neuronal activity in hippocampal neurons, and NFAT proteins including NFAT3 have been implicated in regulating axonal growth (34, 38). However, the role of endogenous NFAT3 in mediating cell survival responses has not yet been characterized.

In this study, we report that NFAT3 plays a critical role in mediating growth factor and activity-dependent survival of cerebellar granule neurons. Both endogenous NFAT3 and exogenously expressed NFAT3 in granule neurons were found in the nucleus under standard culture conditions in which the combination of serum and high K⁺ promotes neuronal survival. By contrast, serum withdrawal and low K⁺ conditions triggered the nuclear export of both endogenous and exogenous NFAT3 to the cytoplasm, and this process was tightly correlated with subsequent neuronal cell death. Both NFAT3 nuclear export and granule cell apoptosis were blocked by the GSK3 inhibitor LiCl. Furthermore, genetic knockdown of NFAT3 by RNA interference triggered apoptosis of granule neurons even under survival conditions of serum growth factors and neuronal activity, whereas expression of a constitutively active form of NFAT protected granule neurons against apoptosis induced by serum/K⁺ withdrawal. Together, these findings suggest that NFAT3-dependent transcription plays a critical role in promoting the survival of neurons and that GSK3 may promote neuronal apoptosis by inhibiting NFAT3-mediated neuronal survival in the developing brain. Because GSK3 is thought to contribute to neuronal loss in the adult brain in response to neurodegenerative insults (39), NFAT3-promoted neuronal survival may also play important roles in the mature brain.

EXPERIMENTAL PROCEDURES

Antibodies—For immunocytochemistry to detect endogenous NFAT3, we used commercial anti-NFAT3 (Affinity Bioreagents). For Western blot experiments to detect exogenous NFAT3, we used anti-

NFAT3 polyclonal antibody from an immunization of New Zealand rabbits against a carboxyl-terminal peptide of mouse NFAT3. Unfortunately none of these antibodies were effective in detecting endogenous NFAT3 in Western blotting of protein lysates from cerebellar granule neurons.

Plasmids—FLAG-tagged NFAT3 and Rep4-NFAT3 constructs were kindly provided by Dr. Chi-Wing Chow (Albert Einstein College of Medicine). The RNAi constructs targeting NFAT3 were designed following the method of Gaudillière *et al.* (40). The NFAT3-C RNAi targets the mouse/human sequence 564 gggagcgtctctctagatt 584, and the NFAT3-N RNAi targets the rat sequence 2305 ggccggaggagcagctccc 2326. The RNAi construct for cdk2 was a gift from Dr. Yang Shi (Harvard Medical School).

Culture and Treatment of Cerebellar Granule Neurons—Cerebellar granule neurons were isolated from cerebellum of 6-day-old Long Evans rat pups and cultured as described by (14). After 3 days, 35 mM glucose was added to the culture medium. After 2–5 days *in vitro*, cultures were washed three times in basal medium (basal medium eagle (Sigma) containing penicillin-streptomycin-glutamine and 17.5 mM glucose) followed by incubation either in basal medium or in depolarizing/survival medium (basal medium eagle supplemented with 10% calf serum + 25 mM KCl). The incubation time was 6 h for the NFAT3 localization assay or 24 h for apoptosis assays. Where indicated, 10 mM LiCl (or the same volume of 10 mM NaCl as control) was added 1 h prior to the washes with basal medium eagle and then included in the subsequent culture with basal medium or KCl/serum medium.

Cerebellar granule neurons were transfected with CsCl gradient-purified plasmid DNA using a modified calcium phosphate method (23). Briefly, granule neurons cultured for 4 days *in vitro* were transfected with 1 μg of the test plasmid per well of a 24-well plate. For the RNAi experiments, granule neurons cultured for 2 days *in vitro* were transfected with 2 μg of the RNAi construct or control plasmids as modified by Konishi *et al.* (23) together with 0.25 μg of expression plasmids encoding β-galactosidase or green fluorescent protein (GFP) to identify the transfected cells.

Immunocytochemistry—Cells were fixed in 24-well plates with 4% paraformaldehyde in PBS, pH 7.4, containing 4% sucrose. After 20 min at room temperature, the fixed cells were washed three times with 0.5 ml of PBS. Then they were permeabilized with 0.4% Triton X-100 in PBS for 20 min and washed three times with 0.5 ml of PBS per well. Incubation with blocking solution (10% dry milk, 1–2% goat serum (Sigma), 0.02% Tween in Tris-buffered saline (TBS)) was performed for 1 h. The coverslips were transferred to a bed of paraffin and incubated overnight at 4 °C in 50 μl of primary antibody diluted in 3% bovine serum albumin, 0.02% Tween in TBS. The primary antibodies used were NFAT3 polyclonal antibody (Affinity Bioreagents), Tuj1 monoclonal antibody (Covance) or M2 FLAG monoclonal antibody (Sigma) in 1% goat serum. After washes, 1:500 of Cy3- or Cy2-conjugated goat secondary antibody (Amersham Biosciences) in 3% bovine serum albumin, 0.02% Tween in TBS was added. After incubation for 1 h and three washes of 10 min each, the cell nuclei were stained as described below (see “Apoptosis Assay”). The cells were imaged using an Eclipse TE2000-U Nikon microscope using a ×60 oil immersion lens.

For the peptide blocking experiment, the commercial rabbit NFAT3 antibody (Affinity Bioreagents) was preincubated with control peptide or buffer and then centrifuged in an Eppendorf microcentrifuge at 4 °C. The supernatant was carefully transferred to a fresh tube and diluted for the immunocytochemistry procedure.

Apoptosis Assay—For this assay, cells were fixed and permeabilized as described above, and their nuclei were stained by adding 50 μl of 0.2 μg/ml DNA dye bisbenzimidazole (Hoechst 33258) in 0.1% Triton X-100 in PBS. After 5 min, the cells were washed three times with PBS, and the coverslips were mounted on slides containing 6 μl of 5% *n*-propyl gallate in 1:1 (v/v) glycerol:10× PBS. The cells were imaged as described above (see “Immunocytochemistry”). ~200 cells or ~100 transfected β-galactosidase or GFP-positive cells were counted per condition. They were classified according to the appearance of their nuclei and the integrity of their neurites. Cells showing a condensed nucleus and disintegrated neurites were classified as apoptotic.

Quantifying Nuclear-Cytoplasmic Distribution of NFAT3—Images were acquired with a fully motorized wide field epifluorescence microscope (Axiovert 200M, Carl Zeiss, Inc., Thornwood, NY) under control of SlideBook (Intelligent Imaging Innovations, Denver, CO). The microscope was equipped with phase contrast optics, motorized filter turret, and lens holder, and a ×60 oil immersion lens (Pan Apochromat, NA 1.3; Carl Zeiss, Inc.). Samples were illuminated with a 175-watt xenon lamp source (Sutter Instruments Co., Novato, CA) optically coupled to the microscope with a liquid guide. Three-dimensional image stacks

¹ The abbreviations used are: CREB, cAMP-response element-binding protein; NFAT, nuclear factor of activated T cells; RNAi, RNA interference; GFP, green fluorescent protein; PBS, phosphate-buffered saline; TBS, Tris-buffered saline.

were recorded by sequential acquisition of optical sections recorded along the z axis every $0.25\ \mu\text{m}$ (step size) by varying the position of the lens holder. Then images were restored in three dimensions by constrained iterative deconvolution (41) with Slidebook using experimentally determined point-spread functions corresponding to $0.17\ \mu\text{m}$ beads labeled with fluorescent dyes (Molecular Probes, Eugene, OR) compatible with the 4',6-diamidino-2-phenylindole and Cy3 filter sets. Three-dimensional surface rendering of the restored images was obtained with Velocity (Improvision, Inc., Lexington, MA). ~150 cells were analyzed blinded for NFAT3 localization.

Transfection of HEK 293T Cells—293T cells were maintained in 60-mm plates (Corning) in 10% fetal bovine serum (Invitrogen) and penicillin-streptomycin-glutamine (Invitrogen) in Dulbecco's modified Eagle's medium (Cellgro). Confluent cultures were dissociated by incubation with 0.3 ml of trypsin-EDTA (Invitrogen) for 3 min before passaging. Cells were transfected 1 day after passaging using the calcium phosphate method described in Ref. 23. For each well of a 6-well plate, 0.18 ml of CaCl_2 /Hanks' buffered salt solution precipitate was added to the cells. The cells were co-transfected with either 0.5 μg of cdk2 RNAi, NFAT3-N RNAi, or NFAT3-C RNAi constructs together with 0.5 μg of FLAG-tagged NFAT3 or Rep4-NFAT3. For an internal control of transfection efficiency, 0.25 μg of FLAG-tagged 14.3.3 protein was used. Negative controls were performed with 0.5 μg of empty pU6, pcDNA3, or Rep4 vectors.

Western Blot Analysis—Whole cell lysates of 293T cells were prepared after 4 or 5 days of transfection as indicated. Protein amounts were measured by the Bradford method (Bio-Rad), and 10 μg of each lysate was analyzed by electrophoresis on 7% SDS-polyacrylamide gels. The proteins were transferred to a nitrocellulose membrane (Bio-Rad) at 250 mA for 1 h. The membrane was blocked in 5% dry milk in TBS for 2 h, washed with 0.05% Tween 20 in TBS, and incubated for 2 h with the primary antibody diluted in 5% milk, 0.05% Tween 20 in TBS and then washed again and incubated with the secondary antibody. The dilutions utilized were 1:7500 for the NFAT3 antibody produced in a New Zealand rabbit, 1:500 for M2 FLAG antibody (Sigma), and 1:20,000 for anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibodies (Sigma).

RESULTS

LiCl Inhibits Apoptosis Induced by Serum and K^+ Deprivation—Cerebellar granule neurons were obtained from 6-day-old rats and cultured in 10% calf serum and 30 mM KCl (depolarizing conditions) for 5 days *in vitro*. These concentrations of KCl lead to depolarization of the neuronal membrane and mimic neuronal activity, an extrinsic cue that promotes survival of neurons in the developing brain (2). Membrane depolarization induces activation of voltage-sensitive calcium channels and consequent entry of calcium into the neurons (12). Serum is provided as a source of pro-survival growth factors.

We tested whether LiCl was protective against apoptosis under our culture conditions (Fig. 1). When granule neurons that had been cultured for 4 days *in vitro* were incubated with basal medium for 24 h, 88% of the neurons showed apoptosis as judged by Hoechst staining for apoptotic nuclei, whereas neurons that were maintained in full survival medium were viable (<10% apoptosis) (compare *first* and *fourth* bars). However if the neurons were pretreated with 10 mM LiCl for 1 h before transfer into basal medium, fewer than 30% were apoptotic at the end of 24 h (compare *first* and *third* bars). Control treatment with 10 mM NaCl was much less effective in protecting against apoptosis (70% apoptosis; compare *first* and *second* bars). Neither NaCl nor LiCl had a significant effect on survival of the neurons in medium containing KCl and serum (*fifth* and *sixth* bars). These results confirmed previous studies showing that LiCl promotes survival of mature cerebellar granule neurons (25). Because LiCl is a known inhibitor of GSK3 (42), an NFAT kinase that deactivates NFAT and promotes its export from the nucleus, we asked whether NFAT3 had a role in the survival of cerebellar granule neurons.

NFAT3 Is Expressed in Cerebellar Granule Neurons—First we asked which of the four members of the NFAT family were expressed in cerebellar granule neurons. Although NFAT1 and

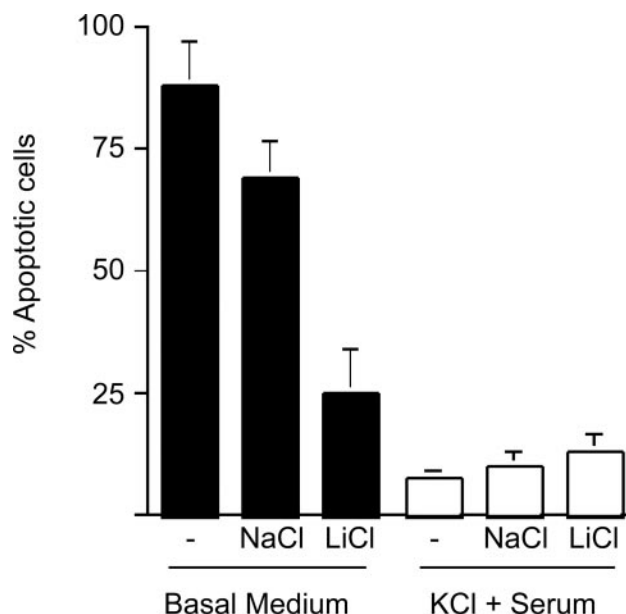


FIG. 1. LiCl inhibits apoptosis induced by KCl and serum deprivation. Cerebellar granule neurons that had been cultured for 4 days *in vitro* were pretreated with 10 mM LiCl or NaCl and incubated for 24 h in basal medium or medium containing calf serum and KCl. After 24 h, the neurons were fixed and their nuclei were stained with Hoechst dye. Neurons showing features of apoptosis were counted, and the percentage of apoptotic over total cells was plotted.

NFAT2 were present by immunocytochemistry and Western blot analysis, respectively, NFAT2 was expressed at low levels, and NFAT1 was cytoplasmic in complete medium containing KCl and serum (data not shown), leading us to focus on NFAT3. We demonstrated the presence of NFAT3 in granule neurons by immunocytochemistry with a polyclonal antiserum against a peptide of NFAT3 (Fig. 2). Cerebellar granule neurons maintained in survival medium with serum and high K^+ were co-stained with anti-NFAT3 and a monoclonal antibody for the neurofilament marker Tuj1, a marker for postmitotic neuronal cells (Fig. 2A). Under these conditions, endogenous NFAT3 can be detected in Tuj1-positive neurons where it is localized to the nucleus. The specificity of the NFAT3 antibody was confirmed by peptide competition experiments; the immunofluorescence signal in neurons stained with the antibody/peptide complex was greatly reduced compared with the staining observed using antibody incubated with buffer alone (Fig. 2B).

Serum and K^+ Deprivation Promotes the Nuclear Export of NFAT3—As shown above (Fig. 2), endogenous NFAT3 was nuclear in most cerebellar granule neurons under depolarizing conditions. However in basal media lacking KCl and serum, NFAT3 appeared largely cytoplasmic (Fig. 3). To quantify this effect, we performed immunocytochemistry for NFAT3, concurrently staining the nuclei with Hoechst dye. Then we acquired images in diverse planes of a random field and analyzed NFAT3 localization in a stack of images that were overlaid. This procedure allowed us to determine the specific nuclear signal. Fig. 3A shows examples of the three-dimensional reconstruction in two neurons showing nuclear and cytoplasmic localization of NFAT3, respectively. In several such experiments, 66 + 5% of cerebellar granule neurons maintained in full survival medium showed nuclear NFAT3 consistent with the fact that KCl treatment activates an increase in intracellular calcium levels, thus promoting the nuclear localization of NFAT (Fig. 3B). In contrast after 6 h of serum deprivation and KCl deprivation, just 11 + 6% of neurons showed nuclear NFAT3 (Fig. 3B). Thus, KCl and serum withdrawal triggers the nuclear export of NFAT3.

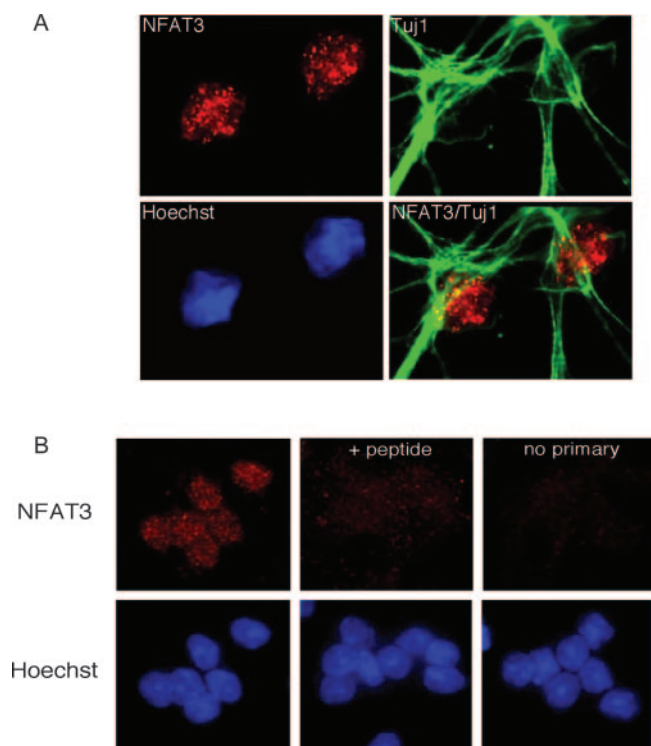


FIG. 2. **NFAT3 is expressed in cerebellar granule neurons.** A, cerebellar granule neurons that had been cultured for 5 days *in vitro* in KCl/serum medium were fixed and co-stained for NFAT3 and the neuronal marker Tuj-1. Nuclei were stained with Hoechst dye. B, the specificity of NFAT3 antibody was demonstrated by competition assay with the control peptide and by staining with secondary antibody alone. $\times 60$ magnification; red, NFAT3; green, Tuj1; blue, Hoechst.

To confirm the differential localization of NFAT3 in full survival media compared with basal media, FLAG-tagged NFAT3 was expressed in cerebellar granule neurons for 24 h. Immunocytochemistry against the FLAG epitope showed nuclear localization of exogenous NFAT3 in neurons incubated with KCl + serum medium, whereas in contrast neurons starved of both serum and KCl for 6 h showed cytoplasmic NFAT3 (Fig 3C). These data suggest that the subcellular localization of exogenous NFAT3 like that of endogenous NFAT3 is regulated in granule neurons by neuronal activity and pro-survival growth factors.

Next we determined the functional consequences of differential NFAT3 localization on NFAT3-mediated transcription. First, we tested in transient transfections of granule neurons the ability of FLAG-NFAT3 to induce the expression of a luciferase reporter gene that is controlled by four NFAT binding sites (*k3-luciferase*) (43). We found that FLAG-NFAT3 as compared with control vector induced the expression of the *k3-luciferase* reporter in granule neurons that were maintained in full medium (Fig. 3D). In other experiments, we found that FLAG-NFAT3-mediated transcription was significantly reduced in granule neurons upon KCl and serum withdrawal (Fig. 3D). Because KCl and serum withdrawal induce the nuclear export of NFAT3 in granule neurons (Fig. 3, B and C), these results indicate that the nuclear localization of NFAT3 correlates tightly with the ability of NFAT3 to induce transcription. Taken together, these results suggest that the pro-survival stimulus of growth factors and depolarization promotes the nuclear localization of NFAT3 leading to NFAT3-induced transcription.

LiCl Promotes the Nuclear Localization of NFAT3—As shown above (Fig. 1), LiCl inhibits apoptosis of cerebellar granule neurons induced by K^+ deprivation. Because LiCl is a

widely used inhibitor of GSK3 (42), an NFAT kinase that deactivates NFAT and promotes its export from the nucleus (33), we asked whether LiCl also influenced the localization of NFAT3. Cerebellar granule neurons were treated with 10 mM LiCl or 10 mM NaCl as control, prior to treatment with depolarizing or basal media, and NFAT3 localization was assessed by immunocytochemistry (Fig. 3B). $\sim 67\%$ of neurons incubated in complete medium (KCl + serum) showed nuclear NFAT3 compared with only 14% of neurons incubated in basal medium for 6 h (compare the *first* and *second* bars). In neurons that had been pretreated with NaCl for 1 h before transfer into basal medium for 6 h, 30% showed nuclear NFAT3 (*fourth* bar); in contrast, $\sim 90\%$ of neurons showed NFAT3 nuclear localization when pretreated with LiCl for 1 h and deprived of KCl and serum for 6 h. These data demonstrate that LiCl potently promotes the nuclear localization of NFAT3 consistent with its ability to inhibit the NFAT export kinase GSK3.

Because LiCl treatment promoted both NFAT nuclear localization and cell survival in cerebellar granule neuron cultures, we examined the extent to which these two effects correlated (Fig. 4). We plotted the results of every experiment in which nuclear NFAT3 localization and cell survival were assessed in parallel. Localization was assessed at 6 h and apoptosis at 24 h to avoid artifacts resulting from leakage of NFAT3 out of frankly apoptotic cells. Clearly, neuronal viability was high in cultures in which more than 65% of neurons were scored as showing predominantly nuclear NFAT3 (Fig. 4).

Knockdown of NFAT3 Levels by RNAi Induces Apoptosis—Our results thus far revealed that when cerebellar granule neurons are cultured under survival conditions (*i.e.* in media containing growth factors and KCl), the transcription factor NFAT3 is nuclear. In contrast, under conditions of growth factor and KCl deprivation, which leads to apoptosis, the cells relocate NFAT3 from the nucleus to the cytoplasm. This led us to test whether nuclear NFAT3 actively promotes survival of cerebellar granule neurons.

For this purpose, we generated RNAi plasmids designed to knockdown protein levels of endogenous NFAT3. Two different constructs were made, targeting either the amino terminus (*NFAT3 NRNAi*) or the carboxyl terminus (*NFAT3 CRNAi*) of NFAT3 (Fig. 5A). Blast searches did not show any match of greater than three nucleotides with proteins other than NFAT3, suggesting that both constructs were specific for NFAT3. To assess the effectiveness of the RNAi plasmids at knocking down NFAT3, HEK 293T cells were co-transfected with two different expression vectors for NFAT3 (FLAG-NFAT3 or Rep4-NFAT3) together with NFAT3-N or NFAT3-C RNAi plasmids. A plasmid encoding FLAG-tagged 14-3-3 protein was co-transfected as a loading control. 4–5 days later, Western blotting of total cell lysates demonstrated a reduced level of NFAT3 in cells co-transfected with the RNAi plasmids compared with cells co-transfected with empty U6 vector (Fig. 5B, *first* through *third* lanes and *sixth* through *eighth* lanes). In addition, RNAi for *cdk2* did not alter the expression of NFAT3 protein (Fig. 5B, *ninth* lane).

We used the RNAi plasmids to address the role of NFAT3 in survival of cerebellar granule neurons. The plasmids were transfected into neurons that had been cultured *in vitro* for 2 days together with a β -galactosidase expression vector to identify transfected neurons. After 5 additional days of culture in complete KCl + serum medium, the neurons were fixed and β -galactosidase-positive neurons were evaluated for apoptosis by Hoechst staining. RNAi for NFAT3 had a robust effect with more than 60% of the neurons becoming apoptotic after 5 days of transfection with either NFAT3-C or NFAT3-N RNAi plasmids compared with only $\sim 30\%$ of neurons transfected with

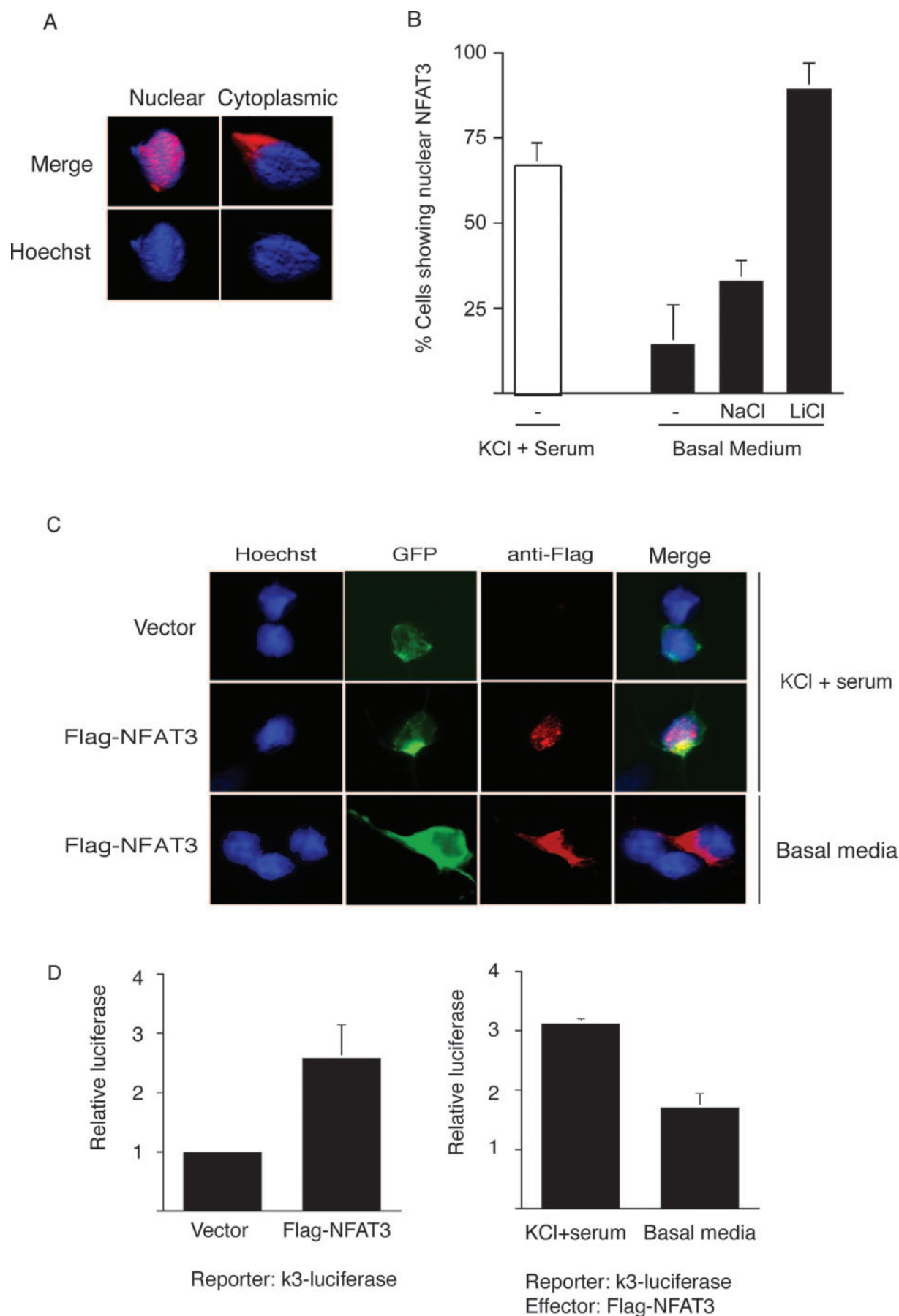


FIG. 3. KCl and serum deprivation promotes the nuclear export of NFAT3. *A*, the nuclear-cytoplasmic localization of endogenous NFAT3 was determined by immunocytochemistry, analysis of images in diverse planes, and three-dimensional reconstruction as described under "Experimental Procedures." Two different neurons are shown to exemplify nuclear and cytoplasmic localization of NFAT3, respectively. *B*, a

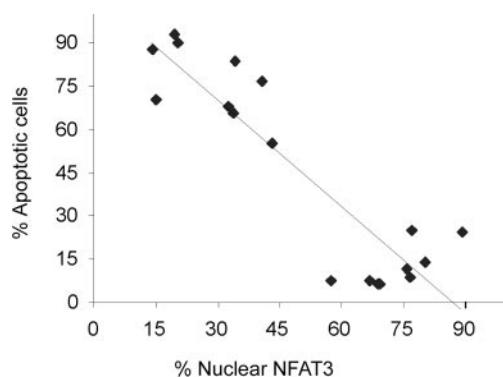


FIG. 4. **Cell survival correlates with nuclear NFAT3.** We plotted the results of three independent experiments in which cultures incubated under various conditions after serum and KCl withdrawal were assessed in parallel for apoptosis and NFAT3 localization (plotted on the y and x axes, respectively). The linear regression shows a good correlation between localization of NFAT3 in the nucleus (assessed at 6 h after serum and KCl withdrawal) and survival of cerebellar granule neurons (assessed at 24 h).

empty U6 vector or with RNAi for *cdk2* (Fig. 5C). We attribute the higher background level of apoptosis in these cultures (30% compared with ~10% in Fig. 1) to the toxicity of the transfection and the fact that the cells were cultured for a longer time, 7 days instead of only 5. These results show that apoptosis of cerebellar granule neurons is triggered even under depolarizing conditions when NFAT3 levels are decreased by RNAi, suggesting strongly that NFAT3 is required for survival of these cells.

Expression of Constitutively Active NFAT Inhibits Apoptosis Induced by Serum and K^+ Deprivation—The previous experiment suggested that a decrease in NFAT3 activity led to apoptosis of cerebellar granule neurons. Therefore we asked if expression of constitutively active NFAT would have the opposite effect, promoting neuronal survival (Fig. 6). Because the DNA-binding domains of all four NFAT proteins recognize the same consensus binding sites (44), we used a constitutively active NFAT1 (CA-NFAT1) that was previously validated in T cells (45). For a negative control we used a plasmid encoding GFP. The constructs were co-transfected into cerebellar granule neurons together with the β -galactosidase expression plasmid; 16 h later, the neurons were kept for 24 h in basal medium lacking KCl and serum. Over 60% of the β -galactosidase-positive neurons that were co-transfected with GFP were apoptotic under these conditions, contrasting with only 15% apoptosis in neurons co-transfected with constitutively active NFAT1 (Fig. 6B). In complete medium with KCl and serum, only 10 and 18% of the neurons were apoptotic, respectively (Fig. 6). The CA-NFAT1 protein was constitutively present in the nucleus of

transfected cerebellar granule neurons and was highly active at driving transcription, as shown by immunocytochemistry and reporter assays, respectively (data not shown). Thus, these data show that constitutively active NFAT1 inhibits apoptosis induced by deprivation of growth factors and KCl, suggesting that NFAT controls the expression of pro-survival genes in cerebellar granule neurons.

DISCUSSION

In this report, we have characterized the mode of regulation and function of the transcription factor NFAT3 in cerebellar granule neurons. We found that membrane depolarization of granule neurons promotes the nuclear accumulation of NFAT3, whereas serum and KCl deprivation triggers the nuclear export of NFAT3. In survival assays, we found that genetic knock-down of NFAT3 by RNAi significantly reduced survival of granule neurons even under conditions of membrane depolarization, whereas expression of a constitutively active form of NFAT protected neurons against apoptosis induced by serum and KCl deprivation. We also found that LiCl counteracted apoptosis, most likely by inhibiting the NFAT export kinase GSK3 and therefore promoting nuclear localization of NFAT3. Our data point to a tight correlation between NFAT3 nuclear localization and granule neuron survival and suggest a direct role for NFAT3 in regulating expression of pro-survival genes in these cells.

The NFAT family of transcription factors has important roles in the development and homeostasis of multicellular organisms. NFAT proteins are expressed in many cell types and contribute to diverse cellular functions, including gene expression and cell differentiation in cells of the immune system as well as cardiomyocytes, endothelial cells, smooth muscle cells, neurons, osteoclasts, and skeletal muscle (32, 46). NFAT proteins appear to have redundant functions in most cell types because knockouts of individual NFAT proteins tend to have mild effects, and multiple knockouts are generally required to observe a striking phenotype (reviewed in Refs. 46 and 47). Interestingly, however, NFAT3 is selectively expressed in the brain and heart, suggesting specific roles for NFAT3 in excitable tissues.

Our data suggest that NFAT3 is an important mediator of neuronal survival in the developing mammalian brain. In a recent study, neurons in which the NFAT3 gene was disrupted either alone or in combination with disruption of the NFAT1 and NFAT2 genes did not show increased apoptosis in trigeminal ganglion sensory and spinal cord commissural neurons (38). However, the triple NFAT knock-out mice die prior to the development of cerebellar granule neurons, and the absence of a neuronal survival phenotype in the NFAT3^{-/-} mice may reflect potential compensation by other NFAT proteins in these

striking difference in localization of endogenous NFAT3 in cerebellar granule neurons cultured for 5 days *in vitro* and then incubated in depolarizing (KCl + serum, first bar) or basal medium (second through fourth bars) for 6 h. KCl + serum deprivation decreases the number of neurons showing nuclear NFAT3 (compare first and second bars), and this decrease is prevented by treatment of neurons with 10 mM LiCl (fourth bar) compared with 10 mM NaCl (third bar) used as the negative control. NFAT3 localization was assessed as in A, and the percentage of neurons showing nuclear NFAT3 was plotted. C, exogenous NFAT3 shows the same behavior as the endogenous protein. Cerebellar granule neurons cultured for 4 days *in vitro* were co-transfected with FLAG-NFAT3 and GFP expression plasmids and then incubated for 24 h in depolarizing (KCl + serum) or basal media. Immunocytochemistry and GFP fluorescence show that FLAG-NFAT3 is nuclear in neurons incubated under depolarizing conditions (second row) but cytoplasmic under conditions of KCl/serum deprivation (third row). The first row shows neurons expressing the empty pcDNA3 vector that was used as a control. $\times 60$ magnification; blue, Hoechst dye; green, GFP; red, NFAT3. D, growth factors and neuronal activity stimulate NFAT3-dependent transcription in granule neurons. Left panel, cerebellar granule neurons were transfected with an expression vector encoding FLAG-NFAT3 or the control plasmid together with a firefly luciferase gene that is controlled by four NFAT binding sites (k3-luc) (43). A *Renilla* luciferase reporter gene was co-transfected to control for transfection efficiency. After transfection, neurons were left in full medium. The expression of FLAG-NFAT3 relative to the control plasmid induced reporter gene activity measured as firefly luciferase activity that was normalized against *Renilla* luciferase activity (Student's *t* test; $p = 0.05$, $n = 3$). Right panel, cerebellar granule neurons were transfected as in experiments shown in the left panel. After transfection, neurons were washed and returned to complete medium or to basal medium. After 8 h, neurons were harvested and luciferase activity was determined. Shown are mean \pm S.E. normalized luciferase values of FLAG-NFAT3-expressing neurons relative to control-transfected neurons. FLAG-NFAT3-dependent reporter gene expression was greater in neurons exposed to KCl and serum as compared with neurons deprived of KCl and serum (Student's *t* test; $p < 0.05$; $n = 3$).

FIG. 5. RNAi-mediated knockdown of NFAT3 correlates with increased apoptosis.

A, two RNAi constructs were designed, targeting either the amino terminus (NFAT3-N) or the carboxyl-terminus (NFAT3-C) of NFAT3. **B**, both constructs are efficient in "knocking down" NFAT3. HEK 293T cells were co-transfected with expression plasmids encoding FLAG-14.3.3 protein and either FLAG-tagged NFAT3 (first through fourth lanes) or Rep4-NFAT3 (fifth through ninth lanes) together with NFAT3-N (second lane), NFAT3-C (eighth lane), or cdk2 (ninth lane) RNAi plasmids or empty U6 vector (third and seventh lanes). Protein expression was assessed by Western blotting after 4 days (first through fourth lanes) or 5 days (fifth through ninth lanes). FLAG-14.3.3 expression was used to confirm equal loading (lower panels). **C**, RNAi constructs for NFAT3 induce apoptosis in cerebellar granule neurons. Granule neurons cultured for 2 days *in vitro* were transfected with β -galactosidase expression plasmid together with empty U6 vector (first bar) or RNAi plasmids designed for knockdown of cdk2 (second bar), NFAT3-N (third bar), or NFAT3-C (fourth bar) protein levels. Neurons were cultured for 5 additional days in depolarizing (KCl/serum) media and then fixed. The percentage of β -galactosidase-positive neurons showing apoptotic features is plotted as a percentage of the total number of β -galactosidase-positive neurons.

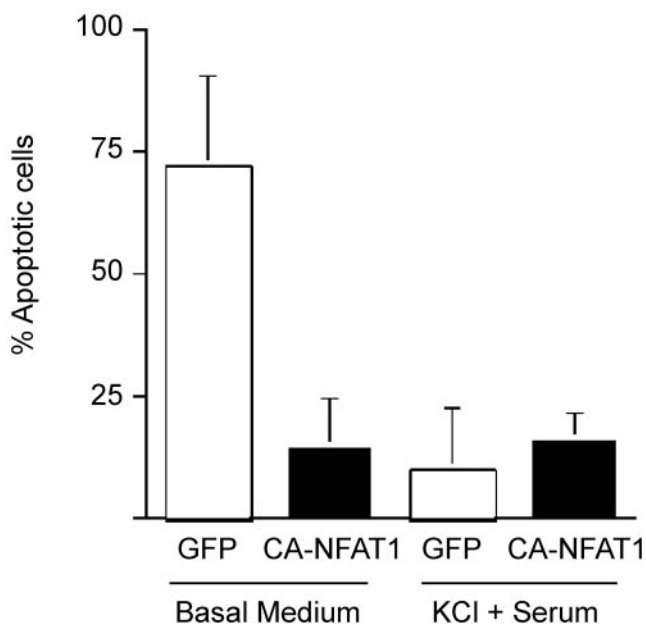
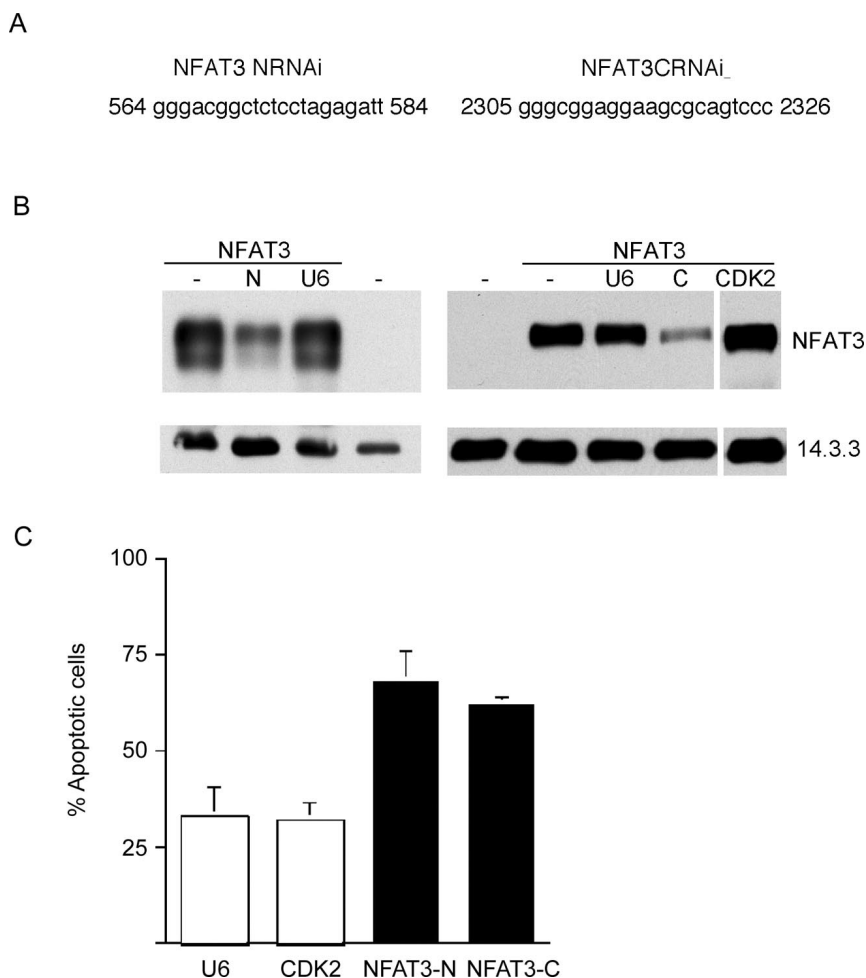


FIG. 6. Constitutively active NFAT inhibits apoptosis induced by KCl deprivation. Cerebellar granule neurons cultured for 4 days *in vitro* were co-transfected with a β -galactosidase expression plasmid and expression plasmids encoding constitutively active NFAT1 (CA-NFAT1, second and fourth bars) or GFP (first and third bars). After 16 h the cells were incubated with basal medium (first and second bars) or depolarizing (KCl/serum) medium (third and fourth bars) for 24 h. The percentage of β -galactosidase-positive neurons showing apoptotic features is plotted as a percentage of the total number of β -galactosidase-positive neurons.

mice (38). Taken together, the NFAT knockout and NFAT3 knockdown results raise the possibility that NFAT3 may have distinct roles in cell survival in different populations of neurons. Alternatively, as shown for retinoblastoma family members, the acute genetic knockdown method might reveal phenotypes that are masked in conventional knock-out mice because of compensatory mechanisms that occur prior to the generation of the cells under study (48). Resolution of the apparent differences in the knock-out mice and the acute RNAi knockdown method will require the generation of time- and tissue-specific *in vivo* knockouts or knockdowns of NFAT3 in different neuronal populations.

We have shown that the localization and function of NFAT3 in granule neurons are tightly controlled by the action of serum growth factors and membrane depolarization. NFAT3 is localized to the nucleus in full survival medium containing serum and high K^+ to depolarize the neurons and promote neuronal activity but is exported to the cytoplasm in the absence of these survival factors. In contrast, NFAT1 immunoreactivity was found in the cytoplasm and not in the nucleus under both conditions of culture, indicating that serum factors and membrane depolarization do not influence NFAT1 localization appreciably. Recent evidence suggests that NFAT proteins play a critical role in axonogenesis in neurons exposed to the neurotrophins brain-derived neurotrophic factor and NT-3 and the secreted protein netrin1 as judged by the phenotype of triple knock-out animals lacking the NFAT proteins NFAT1, -3, and -4 (38). These results emphasize that depending on cellular context and the source and timing of extrinsic signals, distinct NFAT proteins can respond differentially even in the same cell type and therefore mediate different biological outcomes such

as neuronal survival *versus* morphogenesis.

Our study also suggests that GSK3 promotes neuronal apoptosis in part by promoting NFAT3 nuclear export and thereby inhibiting the function of NFAT3 in mediating neuronal survival. This conclusion is based on the use of LiCl; although this compound has a variety of effects on cellular metabolism (49–53), a well documented effect is to inhibit GSK3 (42). GSK3 itself is undoubtedly one of the export kinases for NFAT; nuclear export of NFAT proteins including NFAT3 is enhanced by overexpression of either wild type GSK3 β or a Ser-9 \rightarrow Ala variant of GSK3 β that is resistant to inhibitory phosphorylation by Akt (33, 34, 37). Thus Akt, a known pro-survival kinase in neurons, may enhance neuronal survival by at least two mechanisms that affect the behavior of two different transcription factors. Not only does Akt phosphorylate the pro-apoptotic transcription factor FOXO3 and promote its nuclear export, it may at the same time phosphorylate GSK3, the export kinase for NFAT3, thereby retaining the pro-survival transcription factor NFAT3 in the nucleus. Because GSK3 β has been suggested to play important roles in brain diseases including neurodegeneration (39), it will be interesting to determine whether inhibition of NFAT3 function contributes to the pathogenesis of diseases of the nervous system.

In summary, we have identified a novel function for NFAT3 as a mediator of growth factor and activity-dependent neuronal survival. With these findings, NFAT3 joins CREB and MEF2 as a potentially critical transcriptional mediator of neuronal survival. A major next step is to define the program of gene expression regulated by NFAT3 in granule neurons and to use this information to characterize the mechanisms by which NFAT3 promotes neuronal survival. In this context, the target genes of CREB and MEF2 that promote neuronal survival are just beginning to be identified. A common target of CREB and MEF2 is the gene encoding the neurotrophin NT-3 that turns out to be required for brain-derived neurotrophic factor-induced neuronal survival (14). Microarray analyses of the target genes of NFAT3 will establish whether the target genes of NFAT3 in neurons overlap with those of CREB and MEF2. The use of the constitutively active form of NFAT that promotes neuronal survival should facilitate these studies.

A caveat of the experiments using constitutively active NFAT1 in this study is that NFAT1 and NFAT3 may control distinct sets of genes in neurons. However, in an exhaustive comparison of the ability of constitutively active NFAT1 with another NFAT protein, NFAT2, in regulating gene expression in immune cells, only one substantive difference emerged (54, 55). Because the DNA-binding domains of the four NFAT proteins are very similar (44), the NFAT1-NFAT2 comparison suggests strongly that constitutively active NFAT1 and NFAT3 will also turn on very similar gene expression programs when expressed in a single cell type. In future experiments, it will be important to identify NFAT1- and NFAT3-induced genes in neurons and determine whether an overlapping set of these genes promotes neuronal survival.

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