Signal transducer and activator of transcription 3 (STAT3) degradation by proteasome controls a developmental switch in neurotrophin-dependence*

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*Running title: STAT3 degradation controls a developmental switch

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Keywords: calcineurin, developmental death, neurotrophins, STAT3, p53, Bax, ubiquitin-proteasome pathway.

Background: As neonatal hippocampal neurons mature, STAT3 levels increase via an unknown mechanism, which makes neurons neurotrophin-independent.

Results: STAT3 protein levels are low in neonates because of calcineurin-dependent proteasomal degradation of STAT3.

Conclusion: Calcineurin-dependent STAT3 degradation regulates the survival of neonatal neurons.

Significance: Proteasomal degradation controls the developmental switch of neurotrophin dependence.

SUMMARY

Neonatal brains develop through a program that eliminates about half of neurons. During this period, neurons depend on neurotrophins for their survival. Recently, we reported that, at the conclusion of the naturally occurring death period, neurons become neurotrophin independent, and further, that this developmental switch is achieved by the emergence of a second survival pathway mediated by signal transducer and activator of transcription 3 (STAT3). Here I show that calcineurin plays a key role in controlling the developmental switch in mouse hippocampal neurons.

Calcineurin promotes the degradation of STAT3 via the ubiquitin-proteasome pathway. Inhibition of calcineurin acutely increases total levels of STAT3 as well as its activated forms, resulting in decreased levels of the tumor suppressor p53 and its pro-apoptotic target, Bax. In vivo and in vitro, calcineurin regulates levels of STAT3 and neurotrophin-dependence. TMF/ARA 160, the key mediator of STAT3 ubiquitination, is required for calcineurin-dependent STAT3 degradation. Thus, these results show the ubiquitin-proteasome pathway controls the critical developmental switch of neurotrophin-dependence in the newborn hippocampus.

Protein levels can be regulated not only by transcription and translation, but also by the rate of degradation. The proteasome is a large protease complex ubiquitously found in all types of cells including neurons (1), where proteins tagged with ubiquitin are destined for destruction (2). The proteasomal degradation of misfolded proteins (3) appears to be especially critical in neurons and its failure is thought to underlie a number of neurodegenerative diseases (4). However, besides its role in quality-control, the ubiquitin-proteasome pathway also regulates...
important cellular functions such as cell division (5) and stress response (6). Many signaling pathways including necrosis factor kβ (7), Wnt (8), and hypoxia inducible factor (9), utilize the inhibition of proteasome-dependent degradation as a type of signal transduction. In developing neurons, a precise balance between ubiquitination and deubiquitination is required for the formation of proper neural circuitry (10).

In early postnatal life, the central nervous system (CNS) goes through a specific developmental stage during which approximately half of its neurons are eliminated (11). In rodent hippocampus, this wave of developmental death occurs during the first 10 post-natal days (12,13). During this period, neurons depend on neurotrophin-signaling for their survival (14). Recently, we reported that, after this period of vulnerability, neurons became resistant to the lack of neurotrophin-signaling, and that this developmental switch was achieved by the emergence of a second survival pathway mediated by signal transducer and activator of transcription 3 (STAT3) (15). STAT3 mediates cytokine signaling (16), and plays a critical role in embryonic brain development (17). We found that PSer-747 STAT3 is required for the resistance to the lack of neurotrophin-signaling in hippocampal neurons, and that the developmental switch from the neonatal to adult survival pathway was achieved not simply by changing the phosphorylation state of, but rather the total protein levels of STAT3 (15). However, the mechanism responsible for increasing STAT3 protein during development is unknown.

Calcineurin is a calcium/calmodulin-dependent Ser/Thr phosphatase (18). Although its roles in T-cell activation and cytokine production are well known (19), calcineurin was originally purified from brain tissue (20), and has been implicated in several aspects of neuronal development (21-23) and brain function (24). Here I show that the developmental switch in neurotrophin-dependence in postnatal hippocampal neurons is regulated by calcineurin. In early postnatal hippocampal neurons, calcineurin effectively promotes the proteasomal degradation of STAT3, whose survival signal later allows mature neurons to achieve resistance against neurotrophin deprivation. Thus, this work shows the ubiquitin-proteasome system controls a critical developmental step in newborn hippocampus.

EXPERIMENTAL PROCEDURES

Reagents-- FK506 was purchased from Calbiochem (La Jolla, CA). 4',6'-Diamidino-2-phenylindole dihydrochloride (DAPI), MG-132 and sodium orthovanadate were purchased from Sigma-Aldrich (Bellefonte, PA).

Antibodies-- Antibodies were used at the following dilutions: polyclonal rabbit anti-STAT3 antibody, anti-p53 antibody, p35 antibody and anti-Bax antibody (Santa Cruz Biotechnology, Santa Cruz, CA), 1:500; polyclonal rabbit anti-phospho-Ser727 STAT3 antibody, anti-phospho-Tyr705 STAT3 antibody, anti-cleaved caspase3 (c-cas3) antibody, monoclonal mouse anti-STAT3 antibody (Cell Signaling Technology, Danvers, MA), monoclonal mouse anti-NeuN antibody, anti-ubiquitin antibody (BD Biosciences) 1:500, polyclonal rabbit anti-Fer antibody (Millipore, Ilerica, MA) 1:500, monoclonal mouse anti-β-actin antibody, anti-MAP2 antibody, anti-hypoxia inducible factor 1α (HIF1α), polyclonal rabbit anti-TMF1 antibody (Sigma-Aldrich), 1:10000, 1:1000, 1: 200, respectively. Alexa Fluor488 (and 568)- conjugated goat anti-mouse (and rabbit) IgG antibodies, 1:200, horseradish peroxidase (HRP)- conjugated goat anti-mouse (and rabbit) IgG antibodies (Invitrogen, Eugene, OR), 1:2000. Polyclonal goat anti-TrkB antibody (R&D Systems) was used at 5 µg/ml for function blocking experiments.

Dissociated primary hippocampal culture-- Culture was prepared as described previously (25). Hippocampi from embryonic day 18 (E18) C57BL/6 mouse embryos of either sex were used for both astrocyte (plated at a density of 80,000 cells/ml) and neuron (density: 200,000 cells/ml) cultures. Astrocytes were cultured in Neurobasal (Invitrogen) with 5% fetal bovine serum (FBS) in 5% CO₂ at 37°C for 14 days. Medium was changed completely twice weekly. Neurons were plated on confluent astrocyte beds and cultured in Neurobasal and B27 in 5% CO₂.
STAT3 degradation controls a developmental switch

Western blot—Samples from dissociated culture were collected with 1XSDS loading buffer (60 μl per one 24-well culture dish). Hippocampi were homogenized in 300% (v/w) lysis buffer with protease inhibitor on ice. The homogenates were diluted with 2XSDS loading buffer. The samples were boiled for 5 min, then applied to a 4-10% gradient SDS gel (BioRad, Hercules, CA). The proteins were transferred to a nitrocellulose membrane. The membranes were blocked with 4% skim milk in phosphate buffered saline (PBS) for 30 min. Incubation with antibodies was performed in the blocking solution. Membranes were washed with Tris-buffered saline with 0.05% Tween 20. The proteins were visualized with SuperSignal West Pico System (Pierce), and detected and analyzed with a BioChemi System (UVP BioImaging Systems, Upland, CA). Mean±SEM are plotted.

Immunocytochemistry— Cultures were fixed with 4% paraformaldehyde, permeabilized in 0.5% Triton X-100, and blocked with PBS containing 5% normal goat serum (NGS, Vector Laboratories, CA). Primary and secondary antibodies were diluted with the blocking solution. Samples were incubated with antibodies for 2 hr with antibodies. For the in vivo injection analyses, pups were perfused with 4% PFA two days after the injection. Consecutive coronal slices 50 μm thick were made by a Leica VT100S vibrating microtome (Leica, Allendale, NJ) and were immunostained with a neuronal marker, NeuN, and an apoptotic marker, c-cas3. Slices were compared with respect to distance from the injection site. The analysis was done blind with respect to the content of the injections.

Cell quantification— Fluorescent images were taken with a Zeiss confocal microscope (LSM-510) equipped with a 10x, 25x, or 40x lens. Z-stacked images from eight sections (1 μm intervals) were used for the analyses. Images were analyzed with ImageJ. For cell survival assays, images were taken from 5 fields: one from the center of the coverslip, and two vertically and two horizontally 400-3000 μm from the center. Because the densities of neurons were higher near the rim of coverslips than in other regions, we avoided sampling the coverslips’ edges. The mean number of neurons of 5 fields was then calculated. Each coverslip was defined as an individual culture. Numbers given represent mean±SEM. For immunofluorescence intensity analyses, ROI manager in ImageJ was used to select soma areas of Z-stacked images and mean intensities were measured. TMF/ARA 160 distribution assay followed the Golgi distribution assay (26); the position where the apical dendrite emerges was defined as θ=0°, and quadrants 1 to 4 were defined as 315° - 44°, 45° - 134°, 135° - 224° and 225° - 315°. The background was subtracted in each image. All analyses were done blind.

Transfection— Transfection was performed using Lipofectamine 2000 (Invitrogen). Cells were transfected with 1.6 μg/ml of pEGFPC1 vector (Clontech, Mountain View, CA), and/or 8 μg/ml constitutively activated calcineurin, ΔCaN/pSRa vector (gift from Dr. U. Siebenlist, NAID/NIH), and/or 30 pM mouse ARA 160 siRNA or mouse TMF/ARA 160 siRNA (Santa Cruz) and/or wild-type STAT3 IRES EGFP/pMX in OPTI-MEM (Invitrogen) for 15 min, then the medium was replaced with NeuroBasal Medium. Cells were cultured in NeuroBasal Medium (osmolarity adjusted to 290 Osm with sucrose), before and after the transfection in order to maximize the transfection efficiency.

Reverse transcription (RT)-PCR— Hippocampi were homogenized in 300% (v/w) lysis buffer (150 mM NaCl, 1% NP-40, 50 μM Tris-HCl, pH 8.0) containing a protease inhibitor cocktail (Roche) on ice. Mouse hippocampal culture was incubated with lysis buffer with protease inhibitor cocktail for 20 min on ice (60 μl per one 24-well culture dish). RNA was isolated from the homogenates using TriPure Isolation Reagent (Roche, Welwyn Garden City, UK). RT-PCR was performed using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). Using 5 μg of total RNA, first-strand cDNA synthesis reaction by reverse transcriptase was done using Oligo(dT)12-18 as
primers. PCR was performed using Taq polymerase (Roche). The sequences of the primers are the following: 5’-CAGTCGGGCTCACGCC-3’ and 5’-AGGACATTGGACTCTTGC-3’ for mouse STAT3, 5’-ACCACAGTCCATGCCATCAC-3’ and 5’-TCCACCACCCTGGTCTGTA-3’ for mouse GAPDH, 5’-GATGGTGATGGCCTGGCTCC-3’ and 5’-GGTCGGCGGTTCATGCCCCC-3’ for mouse p53, 5’-GAGCGTGGGTGATGCGCTCT-3’ and 5’-AATTTAAAGAGAAGCCTATA-3’ for rat STAT3, 5’-CCACACTTTCTACAATGAGC-3’ and 5’-CCGTCAGGATCTTCATGAGG-3’ for rat b-actin. Conditions for PCR reactions are: 35 cycles of 95°C (30 sec), 62°C (30 sec), 72°C (30 sec). The PCR products were separated in 2% agarose gel.

Immuno-precipitation— Cultures were incubated for 15 min with 40 µl lysis buffer per well (150 mM NaCl, 1% NP-40 and 50 mM Tris-HCl pH 8.0) containing a protease inhibitor cocktail (Roche), then collected and centrifuged at 12000 g for 10 min. Supernatants were pre-absorbed with 10% (v/v) protein A-conjugated sepharose beads (Amersham Biosciences, Piscataway, NJ) for 1 hr, then centrifuged at 3000 g for 3 min. The supernatant was incubated with 1% (v/v) the STAT3 antibody for 2 hr followed by 10% (v/v) protein A-conjugated sepharose beads for 1 hr. The beads were then washed with the lysis buffer twice. Proteins were eluted with 10 times (v/v) SDS sample buffer. Procedure was done at 4°C.

Chromatin immuno-precipitation (ChIP)— Chromatin immuno-precipitation assays were performed as described by Ballas et al. (27). Cultures were fixed with 4% paraformaldehyde, permeabilized in 0.5% Triton X-100, and collected with 40 ml per well of cell lysis buffer (CLB; 5 mM Hepes pH 8, 85 mM KCl, and 0.5% Triton X-100) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), then centrifuged at 3000 rpm for 2 min at 4°C, and the pellet was resuspended in CLB with PMSF and centrifuged at 3000 rpm for 2 min at 4°C two times. The pellet was then resuspended in nuclear lysis buffer (NLB; 50 mM Tris-HCl pH8, 10 mM EDTA, 1% SDS) with 1 mM PMSF and was sonicated to yield 100 bp to 1000 bp DNA on ice, and was centrifuged at 12000 rpm for 15 min at at 4°C. The nuclear lysate was pre-absorbed with recombinant protein G agarose (rProtein G agarose; Life Technologies, Grand Island, NY) pre-incubated with 200 µg/ml yeast tRNA and 200 µg/ml salmon sperm (Invitrogen) for 1 hr at 4°C. The chromatin suspension was diluted with ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8, 167 mM NaCl), then immuno-precipitated with 5 µg/ml of monoclonal mouse anti-STAT3 overnight at 4°C. The chromatin suspension was incubated with rProtein G agarose pre-treated with 3% BSA and yeast tRNA and salmon sperm for 4 hr at 4°C. Agarose beads were washed with series of solutions as following at room temperature: ChIP dilution buffer, dialysis buffer (2 mM EDTA, 50 mM Tris-HCl pH 8, 0.2% sarkosyl), TSE-500 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl), LiCl detergent (100 mM Tris pH 8, 500 mM LiCl, 1% Triton X-100, 1% deoxycholic acid), and TE (10 mM Tris-HCl pH 8, 1 mM EDTA). To change the solution, the beads were centrifuged at 3000 rpm for 1 min and the supernatant was aspirated. The samples were eluted from the beads with 300 µl of elution buffer (50 mM NaHCO3, 1% SDS). Samples were incubated overnight at 65°C to reverse PFA cross links, following the addition of 20 µl of 5 M NaCl. DNA was then purified from the eluted samples using QiaGen PCR purification kit (Qiagen, Valencia CA). PCR was performed to analyze the STAT3 binding site in mouse p53 promoter using the following DNA primers: 5’-GGGCCCGTFTTGGTTCATCC-3’ and 5’-CCGCGAGACTCCTGGCACAA-3’. Conditions for PCR reactions were: 30 cycles of 94°C (30 sec), 60°C (30 sec), 72°C (1 min). The PCR products were separated in 1.5% agarose gel.

Calcineurin assay— The enzymatic activity of calcineurin was determined using Colorimetric Calcineurin Assay Kit (Calbiochem). Cultures and brain tissue were homogenized with lysis
buffer with protein inhibitor cocktail as described above. Phosphatase assays were performed following the manual provided by the vendor. The activity was measured with 150 μM RII phosphopeptide as a substrate, incubated at 30°C for 30 min. Absorbance at 620 nm was measured using a Beckman spectrophotometer DU600 (Beckman, Brea, CA). The endogenous phosphate concentrations were separately measured in the assay buffer without calmodulin, and the values were subtracted. Protein concentration was determined using Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). For each reaction, 5 μl of sample lysate, which contained approximately 90 μg of total protein, was used.

In vivo injection-- In vivo injection to CA1 was described previously (28). Briefly, Sprague Dawley rat pups (P2) of either sex were anesthetized by hypothermia (in ice for 5 min) prior to the surgery. The anesthetized animal was placed on ice in a stereotaxic instrument. The stereotaxic coordinates from bregma are the following: AP +1.5, ML +/-1.8, VD -1.8 mm. 0.3 μl of reagents were delivered at a rate of 0.1 μl/min using a using a Hamilton needle and syringe attached to a microsyringe pump controller (World Precision Instruments (Sarasota, FL). Concentrations of reagents were: FK506, 30 mM and anti-TrkB antibody, 0.5 mg/ml. PBS was used as control. The incision was closed using a polyglycolic acid suture (CP Medical, Portland, OR). Animals were allowed to recover at 37°C for 1-2 hr.

Statistical analyses--Statistical significance between two groups was determined with a two-tailed paired Student’s t-test. For multiple groups, statistical comparisons were made by ANOVA followed by individual group tests with the Bonferroni correction made for multiple comparisons.

RESULTS
STAT3 increases during postnatal development via post-transcriptional regulation

To examine if STAT3 transcription is regulated during the first 10 days post-natal, mRNA levels in tissue samples were analyzed by RT-PCR. Despite the notable increase in protein level detected by western blot analysis during this period, mRNA levels remained unchanged (Fig. 1A). The in vitro system exhibited the same phenomenon: an increase in protein level while mRNA level stayed constant (Fig. 1B). Immunostaining showed that MAP2+ cells increased STAT3 between 7 days in vitro (DIV7) and DIV14 (Fig. 1C). These results indicate that the increase in neuronal STAT3 during development was caused by post-transcriptional regulation.

Calcineurin is involved in regulating STAT3-p53-Bax pathway

What regulates the STAT3 protein level in developing neurons? Inhibition of calcineurin by FK506 acutely up-regulated STAT3 protein levels (Fig. 2A and B): within 15 min of adding FK506 to young neurons (DIV7), STAT3 protein dramatically increased (5.0 ± 1.3 fold, n=4, p=0.025, Student’s t-test), as did its phosphorylated forms (PSer-STAT3 and PTyr-STAT3). A lesser but notable increase also occurred in more mature neurons (DIV14) that are not dependent on neurotrophin TrkB signaling for survival (15). Phosphorylation of STAT3 induces it to dimerize and translocate to the nucleus, where it binds to its target DNA (29). Immunostaining for STAT3 revealed an accumulation of STAT3 in nuclei (Fig. 2B), consistent with the increase in active forms of STAT3. STAT3 is known to negatively regulate the tumor suppressor gene, p53 (30). To check if inhibition of calcineurin affects the binding of STAT3 to the p53 promoter, chromatin immunoprecipitation (ChIP) analysis was performed. A brief (15 min) exposure to FK506 resulted in an increase in STAT3 binding to the p53 promoter (Fig. 2C). After 2 hr incubation with FK506, both the mRNA level, and the protein level of p53 decreased (assessed by RT-PCR, and western blot with immunostaining, respectively, Fig. 3A and B). Bax, a pro-apoptotic target of p53 (31), was also decreased by FK506 (Fig. 3A). To test if STAT3 is involved in the regulation of p53 levels, STAT3 was knocked down by siRNA. Following a 2 hr treatment with FK506, neurons co-transfected with siRNA for STAT3 showed strong staining for p53 whereas neurons transfected only with GFP-expression vector showed much weaker
staining for p53 (Fig. 3C). These data suggest that calcineurin down-regulates p53 via STAT3. Significant reductions in levels of p53 and Bax in hippocampus were observed during neonatal development in vivo (Fig. 3A), suggesting that these changes may be developmentally relevant.

**Calcineurin regulates neurotrophin-dependence through STAT3**

Next, the effect of calcineurin on developmental neuronal death was examined. We reported previously that neurons during the developmental death period depend on BDNF for survival (14). Consistent with our previous observation, the number of neurons transfected with GFP-expressing vector decreased during the death period (DIV5-DIV8), and the number of transfected neurons further decreased when treated with the function blocking anti-TrkB antibody (Fig. 4A). Treatment with FK506, the inhibitor of calcineurin, blocked the spontaneous neuronal death and the number of surviving neurons did not decrease following co-incubation with the anti-TrkB antibody (Fig. 4A), suggesting that neurons became neurotrophin-independent, and the survival effect of FK506 is not achieved by inhibition of TrkB signal down-regulation. As we previously reported, these young neurons during the death period contain very low levels of STAT3 (15). Concomitantly, STAT3 knock down by siRNA did not increase the amount of death, however, treatment of these neurons with FK506 did not block their death (Fig. 4A), suggesting that the survival effect of FK506 is STAT3-dependent.

Calcineurin becomes calcium-independent and constitutively activated when the C-terminus of its catalytic subunit is deleted (32). When neurons were co-transfected with a vector expressing constitutively-activated calcineurin (ΔCaN), the spontaneous neuronal death and the number of surviving neurons did not decrease following co-incubation with the anti-TrkB antibody (Fig. 4A), suggesting that neurons became neurotrophin-independent, and the survival effect of FK506 is not achieved by inhibition of TrkB signal down-regulation. As we previously reported, these young neurons during the death period contain very low levels of STAT3 (15). Concomitantly, STAT3 knock down by siRNA did not increase the amount of death; however, treatment of these neurons with FK506 did not block their death (Fig. 4A), suggesting that the survival effect of FK506 is STAT3-dependent.

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To examine whether calcineurin activity is developmentally regulated, the phosphatase activity levels of immature (DIV7) and older (DIV14) neurons were compared. A significant decrease in calcineurin activity was observed between primary cultures maintained at DIV7 and DIV14 (Fig. 4B). A similar result was obtained when the phosphatase activity was measured using acutely prepared hippocampal tissue (Fig. 4B). Whether calcineurin is responsible for TrkB-independence in the mature neurons (DIV14) was checked next. As reported previously (15), treatment with anti-TrkB antibody did not cause death in the GPF-expressing mature neurons (Fig. 4C). Neurons co-transfected with a vector expressing ΔCaN only showed a tendency to reduced numbers presumably owing to higher levels of endogenous BNDF, but a significant number of neurons died following addition of the function-blocking anti-TrkB antibody, suggesting that the neurons became TrkB-dependent. The effect of over-expressing ΔCaN was completely reversed by adding FK506, or over-expressing STAT3 (Fig. 4C), suggesting that STAT3 is also responsible for calcineurin-induced TrkB-dependence in the mature neurons. Taken together, these results suggest that calcineurin regulates TrkB-dependency in immature and more mature neurons through STAT3.

**Calcineurin induces STAT3 degradation via the ubiquitin-proteasome pathway**

Next, the post-transcriptional mechanism that allows calcineurin to regulate STAT3 protein level was explored. Pre-incubation with anisomycin, an inhibitor of translation, did not block the effect of FK506 on STAT3 protein levels (Fig. 5A), suggesting that calcineurin regulates the stability of STAT3 protein. Moreover, when neurons were incubated in MG-132, an inhibitor of the proteasome, STAT3 protein increased to a level similar to that in neurons incubated in FK506 (Fig. 5A). To further investigate the role of calcineurin in proteasome-dependent STAT3 degradation, immuno-precipitation analyses were performed (Fig. 5B). Immuno-precipitation using the antibody against STAT3 gave a single band at around 90 kDa, and no bands were observed in the sample treated without the antibody in the immunoblotting with STAT3 antibody, showing the antibody specifically immunoprecipitated STAT3 (Fig. 5B).
STAT3 degradation controls a developmental switch

sample treated with the proteasome inhibitor, MG-132 showed a high smeared signal in addition to an increased level of 90 kDa species (Fig. 5B). Treatment with FK506 increased the level of 90 kDa without the higher molecular species (Fig. 5B). When these samples were analyzed by ubiquitin immuno-blot, a reduction in the 90 kDa band was observed in the FK506-treated sample as compared to the control, whereas the MG-132-treated sample showed a smeared signal both higher and lower than 90 kDa (Fig. 5B). These results suggest that calcineurin controls the ubiquitination of STAT3.

A previous study showed that ubiquitinated PTyr-STAT3 was not detected (33), suggesting that only the inactive form of STAT3 is subjected to degradation. Therefore, if calcineurin changed the phosphorylation state of STAT3 was tested next. In the presence of the proteasome inhibitor MG-132, neither PSer-STAT3 nor PTyr-STAT3 was increased by the addition of FK506, whereas the general Tyr phosphatase inhibitor, orthovanadate, dramatically increased PTyr-STAT3 without affecting PSer-STAT3 levels (Fig. 5C), suggesting that calcineurin does not dephosphorylate PTyr-STAT3 in these neurons. Then, the effect of Tyr phosphorylation on STAT3 stability using orthovanadate was further examined. Exposure to orthovanadate caused only a small increase in the total STAT3 as compared to the increase induced by MG-132 (Fig. 5D). Together, these results suggest that calcineurin affects the stability of STAT3 mainly by promoting proteasome-dependent degradation and that the stabilization effect by dephosphorylating STAT3 is limited.

For a protein to become ubiquitinated, it has to be directed to the E3 ubiquitin ligase complex (34,35). Human immunodeficiency virus 1 TATA element modulatory factor (TMF) (36)/androgen receptor co-activator 160 (ARA160) (37) has been reported to interact with elongin C in the E3 ligase complex through the BC-box motif, and to mediate degradation of STAT3 (38). Therefore, if TMF/ARA160 is involved in the regulation of STAT3 levels in hippocampal neurons was checked next. Immunostaining for TMF/ARA160 confirmed its expression in immature neurons. Applying siRNA for TMF/ARA160 effectively knocked down this expression, and resulted in a large increase in STAT3 (Fig. 6A). Whether or not a similar mechanism operates in more mature neurons was tested next. When older neurons (DIV14) were transfected with ΔCaN-expressing vector, STAT3 levels were significantly reduced (Fig. 6B, p<0.001). However, when neurons were co-transfected with siRNA for TMF/ARA160, STAT3 levels were not affected (Fig. 6B). These results suggest that TMF/ARA160 is involved in regulating STAT3 levels.

To examine if TMF/ARA160 is sensitive to calcineurin activity, the distribution of TMF/ARA160 was analyzed. Immunostaining for TMF/ARA160 in control neurons showed scattered puncta throughout the soma area, whereas in FK506-treated neurons puncta were accumulated on one side of the peri-nuclear region (Fig. 7A). Quadrant distribution analyses showed that TMF/ARA160 in the cell body was evenly distributed in the control condition, whereas FK506-treated neurons showed higher TMF/ARA160 signals on the side of apical dendrites (Fig 7A). These results suggest that the distribution of TMF/ARA160 is sensitive to calcineurin activity.

To further investigate how calcineurin affects STAT3 degradation, the effects of FK506 and MG-132 on other proteins that are degraded by ubiquitin-proteasome system were compared. A tyrosine kinase Fer is known to interact with TMF/ARA160 (39). Treatment with FK506 increased levels of Fer protein to levels similar to that induced by MG-132 treatment (Fig. 7B). In contrast, p35, the activator for cyclin-dependent kinase (Cdk5) and hypoxia inducible factor 1α (HIF1α), whose degradation is regulated by a tumor suppressor protein VHL (40), were sensitive to MG-132 but not to FK506 (Fig. 7B). In fact, no effect on the total ubiquitin levels was observed when neurons were treated with FK506 (Fig. 7B). These results suggest that the effect of calcineurin is more TMF/ARA160 specific than regulating proteasome activity itself. Also, total ubiquitin levels were found not to change during postnatal development (data not shown). Therefore, it is unlikely that the developmental increase in
STAT3 degradation controls a developmental switch

Calcineurin regulates survival of newborn hippocampal neurons in vivo

Next, whether or not the regulation of STAT3 by calcineurin controls the survival of neonatal hippocampal neurons in vivo was examined. When FK506 was injected into CA1 of P2 hippocampus, STAT3 protein levels increased without a concomitant increase in mRNA levels (Fig. 8A), consistent with the results obtained in vitro. Apoptotic hippocampal neurons in these pups (P4) were analyzed by immunostaining for c-cas3 and NeuN (Fig. 8B). c-Cas3+ neurons showed fragmented nuclei revealed by DAPI staining (Fig. 8B), a feature characteristic of apoptotic cells. Inhibiting calcineurin decreased the numbers of apoptotic neurons in the entire hippocampus including CA3 and stratum oriens (SO), as well as CA1 (Fig. 8C). Consistent with experiments performed in vitro, when FK506 and anti-TrkB antibody were co-injected into the pups, the numbers of apoptotic neurons were significantly lower than those injected with vehicle, indicating that treated neurons could survive independent of TrkB signaling (Fig. 8C). These experiments confirm that calcineurin indeed regulates the survival of newborn hippocampal neurons in vivo.

DISCUSSION

Calcineurin promotes proteasome-dependent degradation of STAT3 in neonatal hippocampal neurons.

As we reported previously, as neurons pass through the developmental death period, they lose their early dependence on neurotrophin when a new survival pathway mediated by STAT3 signaling arises to take its place (15). Results presented here, summarized in Fig. 9, show this developmental change is caused by a decrease in the calcineurin activity that promotes degradation of STAT3 through the ubiquitin-proteasome pathway. These results establish: (1) calcineurin inhibitor rapidly increased the amount of STAT3 protein in the presence of anisomycin; (2) proteasome inhibitor increased STAT3 with a similar rate and magnitude as the calcineurin inhibitor; (3) immuno-precipitation of STAT3 revealed the accumulation of ubiquitinated STAT3 by a proteasome inhibitor, an effect that was attenuated by the calcineurin inhibitor. Further, TMF/ARA 160, the protein responsible for translocating STAT3 to the proteasome (38), was present in hippocampal neurons, and knocking down its gene by siRNA effectively up-regulated STAT3 protein levels. Over-expressing ΔCaN caused a striking decrease in STAT3 levels, an effect that was attenuated by co-expressing TMF/ARA 160 siRNA, suggesting that calcineurin affects TMF/ARA 160-mediated degradation. Thus, this study assigns an important new role for calcineurin in regulating the ubiquitin-proteasome dependent STAT3 degradation.

Overall levels of ubiquitinated protein were unchanged during the post-natal period. FK506 did not affect total ubiquitinated protein levels, either. The observation that FK506 affected the distribution of TMF/ARA 160 suggests that the association of the STAT3-E3 ligase complex is calcineurin dependent. Moreover, FK506 increased the levels of another protein, Fer, that also interacts with TMF/ARA 160, but other proteins whose ubiquitination is mediated by different systems were not affected. These results suggest that calcineurin affects TMF/ARA 160 specifically, rather than indirectly by regulating proteasome activity. TMF/ARA 160 was originally identified as a DNA binding protein (36,37); however, it was later shown that TMF/ARA 160 mainly localizes in the Golgi apparatus (41). A scattered distribution of TMF/ARA 160 was also observed in myoblast cells when they are starved of serum (38), and it is thought that dispersion of TMF/ARA 160 enhances accessibility to STAT3 and thus increases the degradation of STAT3. The precise mechanism by which calcineurin causes dispersion of TMF/ARA 160 remains to be determined. Understanding the trafficking of TMF/ARA 160 may provide some clues.

Calcineurin regulates neurotrophin-dependence by promoting STAT3 degradation.

The calcineurin inhibitor increased both PSer- and PTyr- STAT3 species in amounts
proportional to the total amount of STAT3 protein, and, as a result, STAT3 accumulated in neuronal nuclei, and bound to the promoter of p53. Calcineurin inhibitor decreased the levels of p53 and its target gene product, Bax, in a STAT3-dependent manner. Consistent with this decrease in the pro-apoptotic protein Bax, calcineurin inhibitor promoted the survival of immature neurons. This survival effect of calcineurin inhibitor is not achieved by blocking the negative regulation of Trk signaling because these neurons become resistant to anti-TrkB treatment. In a complementary finding, over-expressing constitutively-activated calcineurin caused mature neurons to re-express a regressive sensitivity to the blockade of neurotrophin signaling; mature neurons otherwise do not require neurotrophin to survive. Gain-and-loss of STAT3 function experiments suggested that the effect of calcineurin on survival depends on STAT3. In vivo and in vitro, immature neurons contained low levels of STAT3, high levels of p53 and Bax, and high calcineurin activity as compared to more mature neurons after the death period.

The phosphorylation state of the proteins plays a key role in the induction of proteasome-dependent degradation (7-9). Although calcineurin is a Ser phosphatase (42), calcineurin has been reported to promote dephosphorylation of PTyr-STAT3 (43), and the PTyr-705 STAT3 species has not been detected among ubiquitinated proteins (33) in non-neuronal cells. This raises the possibility that the survival effect of calcineurin inhibitor might be mediated by the inhibition of dephosphorylation of PTyr-STAT3 in young neurons. However, while orthovanadate, the general Tyr phosphatase inhibitor, effectively up-regulated PTyr-STAT3, the calcineurin inhibitor did not change levels of PTyr-STAT3 in the presence of the proteasome inhibitor. Moreover, FK506 increased STAT3 protein levels in a similar manner as the proteasome inhibitor, whereas orthovanadate only slightly increased STAT3 in young neurons. These results establish that, in these neurons, any contribution of phosphorylation state to the stability of STAT3 is minimal; rather, the calcineurin inhibitor promotes survival by reducing STAT3 degradation by proteasome.

Calcineurin responds to neuroactivity and mediates synaptic plasticity (44-46). Therefore, the survival effect of FK506 may be achieved through neuroactivity. In fact, as we previously demonstrated, neuronal activity plays a critical role in the survival of neonatal neurons (14). However, it is unlikely because knockdown of STAT3 completely attenuated the survival effect of FK506. Calcineurin is known to influence cell survival by regulating transcription factors (47-49). However, the results reported here reveal an important new function of calcineurin. Calcineurin also mediates developmental change in synaptic properties (50). Therefore, inhibiting calcineurin in these young neurons may lead to a delay of normal synaptic maturation.

Calcineurin in newborn hippocampus showed much greater enzymatic activity than that in older hippocampus that had already gone through the developmental death period. The mechanism for regulating calcineurin has not yet been determined. When young neurons (DIV7) were briefly (2 min) depolarized with 50 mM KCl in the presence of anisomycin, STAT3 protein levels significantly decreased in 2 hr (52.7±19.1% of the control, n=4, p=0.012), suggesting that neuroactivity induced degradation of STAT3. However, blocking spontaneous activity induced death instead of mimicking the survival effect of calcineurin (14). Moreover, elevation of excitability did not decrease the total levels of STAT3 (15). These results suggest that spontaneous activity may not be the major pathway to the activation of calcineurin. Calcineurin is regulated by anchoring proteins or regulators (51-53). Intriguingly, the spatial and temporal expression patterns of the regulators of calcineurin (RCANs) are dramatically reconfigured during the postnatal period (54). Although calcineurin activity declines during the post-natal period, it nonetheless remains important for normal hippocampal function in the adult (55), suggesting its activity needs to be well titrated. In fact, both dysregulated calcineurin activity (56), and a marked decrease in STAT3 (57) have been observed in the aged brain. Therefore, the connections between calcineurin activity and neuronal survival signaling reported here are
likely to contribute to novel approaches to treat neurodegenerative diseases.
STAT3 degradation controls a developmental switch

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STAT3 degradation controls a developmental switch

FOOTNOTES

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The abbreviations used are: AKAP, A-kinase anchoring protein; ARA 160, androgen receptor co-activator 160; ChIP, chromatin immunoprecipitation; CNS, central nervous system; c-cas3, cleaved caspase3; ΔCaN, constitutively active calcinerin, DIV, days in vitro; FBS, fetal bovine serum; JAK, Janus kinase; NGS, normal goat serum; NFAT, nuclear factor of activated T-cells; PBS, phosphate buffered saline; RCANs, regulators of calcineurin; STAT3, signal transducer and activator of transcription 3; TMF, TATA element modulatory factor.

FIGURE LEGENDS

FIGURE 1. STAT3 protein levels increase through post-transcriptional regulation. (A) RT-PCR and western blot analyses of STAT3 in mouse hippocampi. (B) RT-PCR and western blot analyses of STAT3 in dissociated mouse hippocampal neuronal cultures. (C) Immunostaining of STAT3 (red) in dissociated neurons. MAP2 (green) was used as a neuronal marker.

FIGURE 2. Calcineurin inhibition acutely increases STAT3 protein levels. (A) Western blot analyses of total (panSTAT3) and activated forms (PSer-STAT3 and PTyr-STAT3) of STAT3. The young neurons (DIV7) and older neurons (DIV14) were incubated with 1 mM FK506 for the indicated times. (B) Immunostaining of STAT3 in young neurons (DIV7) with or without incubation with 1 µM FK506 for 15 min. (C) ChIP analysis on the p53 promoter (n=4, Asterisk: p<0.05, Student’s t-test).

FIGURE 3. Calcineurin inhibition causes STAT3-dependent decrease in levels of p53. (A) RT-PCR of p53 and western blot analyses of p53 and Bax. GAPDH and β-actin were used as controls for RT-PCR and western blot, respectively (n=4, Asterisks: p<0.05, Student’s t-test). (B) Immunostaining of p53 and MAP2. (C) Co-transfection of GFP-expressing plasmid and siRNA for STAT3. Cells were immunostained for p53 and GFP (n=10, Asterisk: p<0.001, Student’s t-test). Young neurons (DIV7) were incubated with 1 µM FK506 for 2 hr.

FIGURE 4. Calcineurin controls survival of developing neurons. (A) Left: Representative examples of GFP- (and ΔCaN-) expressing neurons at DIV8. Right: Numbers of GFP-expressing neurons. The cultures were transfected 18 hr prior to the treatments, and treated from DIV5 to DIV8 (n=7, Asterisks: p<0.05, 1-way ANOVA). (B) Calcineurin activity in vitro (left) and in vivo (right) hippocampal tissues (n=5, Asterisks: p<0.05, Student’s t-test). (C) Numbers of GFP-expressing neurons. The cultures were transfected at DIV11, and treated from DIV12 to DIV14. 5 mg/ml of anti-TrkB antibody, 1 µM FK506 were used (n=5, Asterisks: p<0.05, 1-way ANOVA).

FIGURE 5. Calcineurin affects proteasome-dependent STAT3 degradation. (A) Western blot analysis of total STAT3 levels in immature neurons (DIV7). The cultures were pre-incubated with anisomycin for 30 min, then incubated with FK506 or MG-132 for 15 min. (B) Immuno-precipitation analyses of ubiquitinated STAT3 levels. Immature neurons (DIV6) were incubated with the drugs for 15 min. (C) The effects of FK506 (upper panel) and othovanadate (OV, lower panel) on phosphorylated forms of STAT3. The cultures were pre-incubated with MG-132 for 30 min then incubated with FK506 or OV for the indicated times. (D) The effect of OV on the total STAT3 levels. The cultures were incubated...
with MG-132 and/or OV for 30 min. 50 μM anisomycin, 1 μM FK506, 50 μM MG-132, and 1 mM OV were used.

**FIGURE 6. **TMF/ARA 160 regulates STAT3 levels. (A) Immunostaining for TMF/ARA 160. The culture was co-transfected with GFP-expression vector and siRNA against TMF/ARA 160 at DIV6. The cells were fixed at DIV7 (n=4, Asterisks, p<0.05, Student’s T-test). (B) Immunostaining for STAT3. The culture was co-transfected with GFP- and ΔCaN- expression vectors and siRNA for TMF/ARA 160 at DIV13. The cells were fixed at DIV14 (n=4, Asterisks: p<0.05, Student’s t-test).

**FIGURE 7. **Calcineurin regulates TMF/ARA 160-dependent degradation. (A) Immunostaining for TMF/ARA 160 and MAP2 at DIV7. Lower panel: Pseudocolor scale for TMF/ARA 160 immuno-fluorescent signal. Mean intensities for quadrant 1 to 4 are shown (n=10, Asterisks: p<0.05, 1-way ANOVA). (B) Western blot analyses of proteins that are regulated by ubiquitin-proteasome system. The cultures (DIV7) were incubated with 1 μM FK506 for 15 min (n=4, Asterisks: p<0.05, 1-way ANOVA).

**FIGURE 8. **Injection of calcineurin inhibitor in vivo affects survival of developing neurons. (A) RT-PCR and western blot analyses of STAT3. β-Actin was used as a control for RT-PCR and western blot (n=4, Asterisk: p<0.05, Student’s t-test). (B) Immunostaining for c-cas3 and NeuN. SO, stratum oriens; SR, stratum radiatum; CA1 Pyr, CA1 pyramidal layer. (C) Number of apoptotic neurons (n=6). 30 μM FK506 was injected into CA1 at P2 and analyzed at P4 (n=7, Asterisks: p<0.05, 1-way ANOVA).

**FIGURE 9. **Schematic drawing of developmental switch in neurotrophin dependence. In an immature neuron, calcineurin stimulates degradation of STAT3 by the ubiquitin-proteasome pathway. TMF/ARA 160 is required for STAT3 degradation. In a mature neuron, diminished calcineurin activity results in stabilization of STAT3, which effectively represses a tumor suppressor, p53. The STAT3 survival signaling allows mature neurons to achieve resistance against neurotrophin deprivation.
Signal transducer and activator of transcription 3 (STAT3) degradation by proteasome controls a developmental switch in neurotrophin-dependence

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