Nuclear Factor of Activated T Cells 5 Deficiency Increases the Severity of Neuronal Cell Death in Ischemic Injury

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Key Words
NFAT5 • Ischemia • SMIT • Oxidative stress • Knockout

Abstract
Nuclear factor of activated T cells 5 (NFAT5) has been implicated in regulating several genes that are thought to be neuroprotective in ischemic injury. Because of the embryonic lethality of NFAT5 knockout (NFAT5⁻/⁻) mice, the heterozygous (NFAT5⁺/⁻) mice were used to study the in vivo role of NFAT5 in hypoxia/ischemia (H/I) condition. The NFAT5⁺/⁻ mice exhibited more severe neurological deficits, larger infarct area and edema formation associated with increased aquaporin 4 expressions in the brain. Under in vitro H/I condition, increased apoptotic cell death was found in NFAT5⁻/⁻ neurons. Moreover, SMIT, a downstream to NFAT5, was upregulated in NFAT5⁺/⁻ neurons, while the SMIT level could not be upregulated in NFAT5⁻/⁻ neurons under H/I condition. The elevation of reactive oxygen species generation in NFAT5⁻/⁻ neurons under H/I condition further confirmed that NFAT5⁻/⁻ neurons were more susceptible to oxidative stress. The present study demonstrated that activation of NFAT5 and its downstream SMIT induction is important in protecting neurons from ischemia-induced oxidative stress.

Introduction
Maintaining brain cells in ionic and osmotic balance is essential for the proper function of the central nervous system. Brain interstitial and cerebrospinal fluids are in osmotic equilibrium with blood plasma. The blood plasma osmolality is tightly maintained under normal physiological conditions. In addition, a rigid skull limits the expansion of brain tissues, since the changes in total brain cell volume can result in detrimental neurological disorders [1–3]. Changes in brain tissue or CSF osmolality after cerebral ischemia are documented [4, 5]. Similar to the osmotic adaptation in renal epithelial cells, brain cells possess osmoprotective mechanisms and enable them to adapt the changes in cell volume [6–8]. When animals were subjected to systemic hypertonicity, the mRNA levels of osmoprotective genes were induced in brains [9], and the water content of brain tissue restored back to normal level [10]. The transcriptions of osmoprotective genes are thought to be primarily under the control of nuclear factor of activated T cells (NFAT) 5 which is also known as osmotic responsive element binding protein (OREBP) or tonicity-responsive enhancer binding protein (tonEBP) [11].

NFAT5, a transcription factor, belongs to the Rel family. Although NFAT5 has similar protein structures to
other members of the Rel family, the biological functions are quite different from the others. NFAT5 is involved in cellular adaptation to hypertonicity by activating osmoprotective genes transcription, such as transporters and enzyme for accumulating organic osmolytes: sodium/myo-inositol cotransporter (SMIT), the sodium-chloride-betaine cotransporter (BGT1), tauinine transporter (Taut) and aldose reductase (AR) [12]. NFAT5 is also involved in stress-related gene transcription, such as heat shock protein 70 (HSP70), which is known to contain osmotic response element (ORE), and is regulated by NFAT5 under hypertonic stress [13].

In ischemia, a number of genes are actively transcribed and translated. An antiapoptotic protein, HSP70, has been reported to protect the brains from H/I injury [14]. Besides, AR is an enzyme in the polyol pathway and is upregulated during cerebral ischemia, AR deletion protects the brains from ischemic injury [15]. Moreover, the expression of SMIT mRNA increases significantly in the ischemic core after focal cerebral ischemia [16]. The release of taurine, through the taurine transporters, in neurons is neuroprotective during cerebral ischemia [17].

Cerebral ischemia causes extensive osmotic stress in the brain. Similar to renal cells, osmoregulatory mechanism is also found in the brain cells against the osmotic stress [6–8]. NFAT5 expression is primarily found in the neurons of the rat brains [18] and it is, therefore, possible that NFAT5 may play an important role in regulating the transcription of its target genes in response to and cope with the ischemic injury. In the present study, the biological function of NFAT5 in H/I injury was studied by using NFAT5 heterozygous knockout (NFAT5+/−) mice. The NFAT5+/− mice were subjected to transient middle cerebral artery occlusion (tMCAO). They exhibited more severe neurological deficits, larger infarct and cerebral edema with increased blood brain barrier breakdown and aquaporin-4 expression. In the in vitro study using NFAT5 knockout neurons, we have demonstrated that H/I injury led to activation of NFAT5 resulting in increased NFAT5 protein synthesis, nuclear translocation and transcription activity in the neurons. All of these cellular processes were involved in neuronal protection against ischemic injury. The NFAT5-dependent transcription of ischemic-related genes, such as AR, SMIT, TAUT and HSP70 was also studied by real-time PCR. The increased mRNA expression of SMIT after 3-h H/I was observed in the NFAT5+/− neurons but not in the NFAT5+/+ neurons. The NFAT5+/− neurons were under more severe oxidative stress when compared with NFAT5+/+ neurons after H/I insult. Taken together, NFAT5 is not only important in protecting neuronal cells from osmotic stress during the hypertonic condition, but it also plays a protective role by regulating the cytoprotective SMIT expression in response to ischemic environment.

### Materials and Methods

Mice were housed under diurnal lighting condition and allowed free access to food and water. The protocol of this study was reviewed and approved by the Committee on the Use of Live Animals in Teaching and Research in The University of Hong Kong.

#### Generation of NFAT5−/− Mice

The mouse nfas gene was inactivated by partially replacing the region of nuclear localization sequence and DNA-binding domain within the exon 5 and exon 6 with neomycin-resistance cassette in embryonic stem (ES) cells. Homologous recombination integrated the targeting construct into nfas locus. The gene-targeted nfas−/− ES cells were verified by using polymerase chain reaction (PCR) and Southern blot analysis. Injection of gene-targeted ES cells into the blastocytes from C57BL/6N mice generated chimeric founder that transmitted the mutated allele into the germline. To generate NFAT5−/− embryos, mice heterozygous for the NFAT5 alleles (NFAT5+/−) on SVJ129/C57BL/6N background was intercrossed [19], and the morning vaginal plug examination was designated as embryonic day 0.5 (E0.5d). The NFAT5 knockout allele was identified by PCR amplification using PCR primers, p3: 5′-AGGCACA-CAGTCTTGTGACATCTCAC-3′; p3RA: 5′-CC-TCTATGCTCTACCTGAC-3′; p3RA: 5′-GG-TACGAGCCCTCTGTCTGCA-3′, with denaturing temperature 95°C, 1 min; annealing temperature 60°C, 1 min; extension temperature 72°C, 1 min.

#### RT-PCR Analysis

RT-PCR analysis was performed using total RNA isolated from brain tissues from various genotype mice. NFAT5 primers (5′-CACACCGACACCTGAGCTGCTG-3′ and 5′-TCTCACAATCTTGGTTTATT-3′, with denaturing temperature 95°C, 1 min; annealing temperature 60°C, 1 min; extension temperature 72°C, 1 min.

#### Western Blot Analysis

Proteins were isolated brain tissues and homogenized in lysis buffer (50 mmol/l Tris-Cl, pH 6.8, 150 mmol/l NaCl, 5 mmol/l EDTA, 0.5% sodium deoxycholate, 0.5% NP-40 plus protease inhibitor cocktail) [20]. Homogenate was centrifuged at 3,000 g for 5 min at 4°C. Blots were incubated with antibodies against NFAT5 (1:400, a kind gift from Prof. H.M. Kown, University of Maryland) and α-tubulin (1:5,000, sc-5286, Santa Cruz, Calif., USA). Signals were visualized by ECL (Amersham) and quantitated by using Phospholimager (Molecular Dynamics).

### Transient Focal Cerebral Ischemia

Transient focal cerebral ischemia was induced by intraluminal occlusion of the right middle cerebral artery as previously...
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Cell Culture
SH-SY5 neuroblastoma cells (a kind gift from Dr. M.W. Cheung from CUHK) were cultured in MEM and DMEM-F12 supplemented with 10% fetal bovine serum, glutamine, penicillin and streptomycin. To obtain mouse embryonic primary cortical neurons, NFAT5+/− mice male and female were intercrossed. Primary neurons were cultured in neurobasal medium supplemented with B-27, glutamine, penicillin and streptomycin. After 5 days, the neuronal culture was subjected to 100 μM H2O2 or to in vitro H/I for 3 h (anaerobic chamber, Thermofisher).

Luciferase-Activity Measurement
The SH-SY5 cells were co-transfected with 600 ng of ORE-Luc and 300 ng of phRG-TK. For the control, the cells were co-transfected with 600 ng of pGL3 and 300 ng of phRG-TK. Transfection was carried out using FuGENE 6 according to the manufacturer’s instructions. 24 h after transfection, the cells were subjected to in vitro H/I. In vitro H/I was induced in neuronal cultures by subjecting cultures to hypoxia in an anaerobic chamber (Forma Scientific, Ohio, USA) that preconditioned with 5% CO2/85% N2/10% H2 and glucose-free conditions for 0, 1 and 3 h. The O2 content in the chamber was measured by an oxygen electrode (Microelectrodes, Inc., Nashua, N.H., USA), and less than 10 ppm O2 was detected. The ORE activity was measured by luciferase report assay. After H/I treatment, the cells were lysed with 500 μl of passive lysis buffer and luciferase activity was determined by using dual luciferase reporter assay system. 100 μl of luciferase assay reagent II (LAR II) was added into a luminometer tube. Then, 20 μl of cell lysate was added into the luminometer tube and mixed by pipetting. The tube was plated in the luminometer. The firefly luciferase activity was recorded. 100 μl of Stop and Glo Reagent was added and mixed. The Renilla luciferase activity was recorded. The luciferase activity was normalized by Renilla luciferase activity.

Immunocytochemical Analysis of Primary Cultured Neurons
The primary neuronal cells were plated onto a coverslip in a 12-well plate. After 5 days of incubation, the cells were treated with in vitro H/I. The medium was aspirated and the cells were washed twice with 1× PBS. The cells were fixed with 4% paraformaldehyde for 15 min at room temperature and washed three times with 1× PBS. The cells then were laid with methanol for 3 min and washed with 1× PBS three times. The sample was blocked by 3% BSA for 1 h and then incubated with rabbit anti-NFAT5 antibody (1:4,000) for 2 h. Then, the cells were washed three times with 1× PBS with each time for 5 min at room temperature and incubated with FITC-conjugated secondary antibody. After 1 h of incubation, the cells were stained with DAPI for 5 min. Then, the cells were washed three times with 1× PBS for 5 min, air-dried and mounted in Fluorosave reagent.

Lactate Dehydrogenase Measurement
To detect the ischemia-induced cytotoxicity on the primary cultures of NFAT5+/−, NFAT5+/- and NFAT5−/− neuronal cells, lactate dehydrogenase (LDH) assay kit was used. 3 h after H/I treatment, 100 μl of medium was removed from the culture dish and added to 96-well plate. 100 μl of the reaction mixture from the LDH kit was added to each sample. The plate was then incubated for 30 min at room temperature in the dark. The spectrophotometrical absorbance of the samples was measured using a microtiter plate (ELISA) reader. The absorbance wavelength for the

Brain Infarct Analysis
The brain was collected after tMCAO and sectioned into six 2-mm-thick slices coronally. The brain slices were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma, St. Louis, Mo., USA) in the dark at 37 °C for 10–15 min. The posterior surface of each slice was photographed and analyzed by image analysis program (Sigma Scan Pro 5.0, Statistic Package for the Social Sciences: SPSS Inc.). Infarct area and volume percentages were calculated using an indirect method and presented as the percentage of the infarct area of the contralateral hemisphere to eliminate the contribution of edema to the ischemic lesion [20]. The hemispheric brain swelling was assessed as the increase in volume in ipsilateral hemisphere relative to the contralateral volume expressed in a percentage manner [20].

Immunocytochemical (ICC) Analysis of Brain Tissue
After tMCAO, brain samples were collected after 24 h, 7-μm paraffin-embedded tMCAO-treated brain sections were fixed with 4% paraformaldehyde and stained with antibodies against NFAT5 (1:500, a kind gift from Prof. H.M. Kown, University of Maryland), Occludin (1:50, Santa Cruz) and AQP-4 (1:200, Chemicon International). For IgG immunostaining, sections were incubated with biotinylated goat anti-mouse IgG (1:200 dilution; Vector Laboratories, Inc., USA). Signals were visualized by Vectastain ABC kit (Vector Laboratories, Burlingame, Calif., USA) with 3,3'-diaminobenzidine tetrahydrochloride (Zymed, South San Francisco, Calif., USA). Photomicrographs were taken with a Zeiss Axioshot microscope. For controls, primary antibodies were omitted on adjacent sections.

Quantification of Blood-Brain Barrier (BBB) Breakdown
Quantification of the BBB data was performed by quantification of the IgG contra- and ipsilateral of NFAT5+/− and NFAT5+/- brain sections. Photomicrograph (magnified ×20) of brain sections with IgG leakage were analyzed with Image J software, which allowed us to measure the degree of the IgG leakage, and therefore, the integrity of the BBB.

described [20–23]. Age-matched littermates of NFAT5+/+ and NFAT5+/- adult mice (8–12 weeks old) were subjected to tMCAO by the filament method. Briefly, a nylon monofilament (Johnson and Johnsoon, Brussels, Belgium) coated with impression material (3 M; Dental Products, St. Paul, Minn., USA) was inserted into right internal carotid artery to block the origin of right middle cerebral artery. Regional cerebral blood flow (rCBF) was monitored during the whole surgical procedure to confirm appropriate suture placement while rectal temperature was maintained at 37 °C ± 0.5 °C with a temperature control system (FHC, Brunswick, Me., USA). After 2 h of occlusion, the filament was pulled out to allow reperfusion for 22 h and the mouse was kept in an intensive care system (ThermoCare Inc., Incline Village, Nev., USA) at 32 °C for 4 h before transferred back to home cage. After 22 h of reperfusion, neurological deficits evaluations were performed before brains were collected for further analysis. Mice were evaluated for neurologic deficits [20] as follows: (0) no observable neurologic deficits (normal); (1) failure to extend opposite forepaw (mild); (2) circling to the contralateral side (moderate); (3) loss of walking and righting reflex (severe). The person did the rating was naïve to the animal identity and to the treatment protocol.

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formazan product is 492 nm and the background absorbance wavelength is 690 nm. The amount of LDH release was defined as the ratio of LDH activity in the medium to the LDH activity observed after total cell death according to the manufacturer’s protocol and was expressed as percentage of total LDH activity.

**Terminal Deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL) Staining**

The neuronal cells were plated onto a coverslip in a 12-well plate. After 5 days of incubation, the cells were subjected to in vitro H/I. The medium was aspirated and the cells were washed twice with 1× PBS. The cells were fixed with 4% paraformaldehyde for 1 h at room temperature and washed with 1× PBS three times. The cells were incubated with blocking for 10 min at room temperature and washed with 1× PBS three times. The cells then were permeabilized in methanol for 2 min on ice and washed twice with 1× PBS. 50 μl of TUNEL reaction mixture was added on the samples and incubated for 60 min at 37 °C in a humidified atmosphere in the dark. The samples were counterstained with DAPI for 5 min and washed with 1× PBS for 5 min, air-dried and mounted in Fluorsave® reagent.

**Sample Preparation, RNA Isolation, cDNA Preparation and Real-Time PCR for Measurement of Abundance of Specific RNAs**

Total RNA was isolated from primary cortical neurons and brain slices. DNase treatment was performed to minimize contamination by genomic DNA. cDNA was prepared with TaqMan reverse transcription reagents using random hexamers according to the manufacturer’s instructions (Applied Biosystems). cDNA was quantified with ABI Prism 7900HT sequence detection system (Applied Biosystems). The accumulation of the PCR product was monitored in real time by a fluorogenic 5′-nuclease assay, using probes specific for each cDNA being tested. Primers and probes were designed from mouse cDNA sequences. The PCR primers were designed to span a sequence of genes that contains introns, namely SMIT (Mm00444330_s1, Applied Biosystems), TauT (Mm00436909_m1, Applied Biosytems), 18S rRNA primers and 18S probes (Applied Biosystems), AR and HSP70-2 [24].

**Analysis of Real-Time PCR Data**

The results were analyzed using ABI Prism 7900 system software (Applied Biosystems). Relative mRNA abundance was calculated from the real-time PCR data using the following principles. (1) By definition the number of specific cDNA molecules at the threshold (N(t)) is constant for a given cDNA, independent of the number of cycles that it takes to reach it. (2) For a specific cDNA the ratio N(exp)/N(cont), is independent of i, assuming that the efficiency (E) of PCR for a specific template is constant, where i is the cycle number, and N(X), is the number of specific cDNA molecules in a sample (X = control or experimental) at cycle i. (3) The ratio of the number of specific cDNA molecules at a cycle, Ct, to the number at another cycle, i, is Ni/Ni Ct = 1/E(Ct-i). The control and experimental results were compared. The number of specific molecules at an arbitrary cycle was normalized, I, chosen for convenience to be the largest whole number that was less than any of the experimental values of Ct. Then, Ni/Ni Ct for each sample was calculated. Experimental results were presented as the relative amount of the corresponding control value.

**Measurement of Intracellular ROS Generation**

Intracellular oxidative stress was monitored by measuring the changes of fluorescence resulting from intracellular probe oxidation. The probe 2′,7′-dichlorofluorescin (DCFH-DA, 10 μM; Molecular Probes) is a non-polar compound and readily diffuses into cells. It can be oxidized by ROS and yields the fluorescent product. Thus, increases in DCFH to DCF are suggestive of ROS generation. Neuronal cultures after different H/I treatments were incubated with the probe in the dark for 30 min before viewed under fluorescent microscope. Culture dishes were viewed on a Zeiss Axiosvert 135 inverted confocal microscope equipped with a ×20 Neofluor objective and Zeiss LSM 410 confocal attachment (Carl Zeiss, Minneapolis, Minn., USA). DCF fluorescence was measured at an excitation wavelength of 488 nm and emission at 515–540 nm. At least five fields of each dish were randomly selected and the fluorescence intensity value was measured (Karl Zeiss vision system). The mean relative fluorescence intensities were obtained by averaging the five values, and were used for comparisons.

**Statistical Analysis**

Data are presented as mean ± SEM and statistical tests were calculated by using GraphPad Prism software (San Diego, Calif., USA). The neurological score, infarct volume and hemispheric swelling data comparison between the NFAT5+/+ and NFAT5+/- mice were analyzed with the Mann-Whitney test [19]. One-way ANOVA was used in all other measurements.

**Results**

**NFAT5+/– Mice Displayed More Severe Neurological Deficits, Larger Infarct Size, Volume, and Brain Swelling after MCAO-Induced Cerebral Ischemia**

To investigate the role of NFAT5 in cerebral ischemia, NFAT5 knockout mice could be used in an in vivo study. However, due to the embryonic lethality of NFAT5–/– mice, therefore, the NFAT5+/– heterozygous mice were used as a model of NFAT5 deficiency. RT-PCR and Western blot results showed that about a half-fold reduction of NFAT5 mRNA and protein expression were observed in heterozygous brains when compared with that of the wild-type mice, respectively (fig. 1a, b). Next, NFAT5+/+ and NFAT5+/– mice were subjected to tMCAO to induce ischemia and reperfusion injury. No significant differences were observed between the two genotypes in both rCBF and body temperature (data not shown), indicating that these animals had similar physiological state before and after tMCAO. However, after tMCAO, more severe neurological deficits were observed in NFAT5+/– mice (1.62 ± 0.14; n = 13, p < 0.05 by the Mann-Whitney test) when compared with NFAT5+/+ mice (1.11 ± 0.11; n = 9).

TCC staining analysis showed that the percentage of infarct area in brain slices 2 and 3 was significantly larger in NFAT5+/– mice than that in the NFAT5+/+ mice (ta-
NFAT5 Protects Brain against Ischemic Injury

Fig. 1. Expression of NFAT5 in NFAT5-deficient mouse brains at embryonic day 14.5, and analysis of infarct and swelling volume in brains from adult NFAT5+/+ and NFAT5+/– after tMCAO. a Representative figure showing the mRNA expression level of NFAT5 and histogram showing the complete loss of NFAT5 expression in NFAT5+/– brains and significantly reduced expression was detected in NFAT5–/– brains. b Western blot analysis showing the loss of NFAT5 protein expression in NFAT5+/– brains and partial loss of expression in NFAT5–/– brains. c Histograms showing the percentage of infarct volume in brain slices (NFAT5+/+, n = 7; NFAT5+/–, n = 8). d Histograms showing the percentage of hemispheric swelling after tMCAO (NFAT5+/+, n = 7; NFAT5+/–, n = 8). NFAT5+/– brain displayed a significantly larger infarct volume than NFAT5+/+ brain, * p < 0.03 Mann-Whitney test. A statistically higher percentage of swelling was also found in the ipsilateral hemisphere of NFAT5+/– brain after tMCAO, ** p < 0.02 Mann-Whitney test. Data are presented as mean ± SEM.

Blood-Brain Barrier Breakdown Was Exacerbated in NFAT5+/– Brain after Transient MCAO with Downregulation of Occludin Level and Upregulation of Aquaporin-4 Staining in Astrocytic End-Feet

The increased brain swelling in NFAT5+/– mice after tMCAO suggested the loss of integrity of the blood-brain barrier (BBB). To investigate the integrity of the BBB in NFAT5+/+ and NFAT5+/– mice, the leaky endogenous IgG immunoreactivity was used as a marker of BBB breakdown after tMCAO. In the contralateral hemispheres of both NFAT5+/+ and NFAT5+/– brains, low background levels of IgG extravasation were observed (fig. 2ai and iii, respectively). Significant increased staining intensities were observed in the ipsilateral side of both NFAT5+/+ and NFAT5+/– brains (fig. 2aii and iv, respectively), with a significantly increased intensity in the ipsilateral side of NFAT5+/– cerebral vessels, suggesting that BBB leakage was more severe in NFAT5+/– brains. In line with IgG leakage results, occludin, a tight junction protein associating with BBB, was also downregulated in ipsilateral cerebral vessels of NFAT5+/– brains after tMCAO (data not shown). This indicated that the NFAT5+/– mice were more susceptible to BBB breakdown after tMCAO.
ing in NFAT5+/– mice after tMCAO also associated with AQP-4 expression in astrocytic end-feet. Cellular localization of AQP-4, a water channel protein responsible for modulating water transport in the brain [25], was determined by immunocytochemistry. In the contralateral side of both NFAT5+/+ and NFAT5+/– brains, AQP-4 staining was localized in astrocytic end-feet, which were in contact with cerebral vessels, thereby displaying their outline (fig. 2bi and ii). The immunoreactivity of AQP-4 was nearly abolished in the ischemic core in both NFAT5+/+ and NFAT5+/– brains (fig. 2bi and vi, respectively). Near the ipsilateral areas, AQP-4 staining around the cerebral vessels was more diffused when compared with that in the contralateral side. The outlines of cerebral vessels were not distinct. The AQP-4 staining was much higher in the peri-infarct area (fig. 2bi, v, respectively). The intensity of AQP-4 staining in ischemic core was also higher in NFAT5+/– brain than that of NFAT5+/+ brain (fig. 2bi, vi, respectively). Increased AQP-4 staining was more pronounced and diffuse in the NFAT5+/– swollen astrocytic processes, suggesting that after tMCAO, there was an up-regulation of AQP-4 in astrocytic end-feet of the NFAT5+/– brains.

**Increased Expression and Nuclear Translocation of NFAT5 in Neurons after Transient MCAO**

NFAT5+/– mice showed an increase of water content after tMCAO, and this implied that the loss of NFAT5 might lead to exacerbation of ischemia-induced osmotic stress in the brains. To determine the possible involvement of NFAT5 in ischemic injury, the sub-cellular localization of NFAT5 was examined in brain sections after tMCAO. ICC results showed that immunoreactivity of NFAT5 was observed in the neurons and was significantly higher in the neurons at the ischemic region (fig. 2cii) when compared with the contralateral side (fig. 2ci), suggesting that NFAT5 took part in the intracellular osmo-regulation in neurons during ischemic stroke.

**Activation of NFAT5 in Neurons under Hypoxia/Ischemia**

Activation of NFAT5 leads to increased NFAT5 protein synthesis, nuclear translocation or increased transcriptional activity. To investigate whether NFAT5 activation would also induce the above activities in the neurons under hypoxic/ischemic (H/I) injury, in vitro experiments were performed. Primary cortical neurons were isolated from wild-type embryos at E14.5d and challenged the primary cortical neurons with 3-hour H/I condition. In agreement with previous results [12], Western blot analysis showed that significant upregulation of NFAT protein level was observed in NFAT5+/+ neurons after 3-hour H/I condition (fig. 3a, b).

To examine the nuclear translocation of NFAT5, the subcellular localization of NFAT5 was determined on the primary cortical neurons from E14.5d embryos exposed to H/I by using immunocytochemistry staining with antibodies specific to NFAT5. Under normal (N) condition, NFAT5 was localized in both cytosol and nucleus (fig. 3cii). The NFAT5 signals were only found in the nuclei after 1 h (fig. 3civ, v, vi) or 3 h (fig. 3cvii, viii, ix) H/I condition and the intensity of signal was also higher than in N condition, suggesting that the H/I condition insult would induce the expression and nuclear translocation of NFAT5 in neurons.

The transcription factor NFAT5 specifically binds to the ORE that enhances the transcription of its target genes [26]. Therefore, ORE-linked luciferase reporter gene was used to examine the transactivating activity of NFAT5 under H/I condition. For the ORE reporter activity, the construct with luciferase driven by ORE sequence and internal control TK-Ren were co-transfected to neuroblastoma cells (SH-SY5Y). For vector control, pGL3 and TK-Ren were co-transfected to SH-SY5Y. The co-transfected cells were subjected to H/I condition for 0 (control), 1 and 3 h, respectively. The luciferase activity was normalized by internal control (TK-Ren). Results showed that the ORE-luciferase activity was significantly

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**Table 1. Percentage of infarct area in ischemic brain slices**

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<th>n</th>
<th>Brain slice No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<tbody>
<tr>
<td>NFAT5+/+</td>
<td>7</td>
<td>12.7 ± 3.7</td>
<td>49.7 ± 1.6</td>
<td>41.2 ± 2.4</td>
<td>14.3 ± 4.0</td>
<td>-2.3 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>NFAT5+/–</td>
<td>8</td>
<td>18.4 ± 6.6</td>
<td>58.1 ± 3.0*</td>
<td>49.9 ± 3.5**</td>
<td>19.8 ± 3.2</td>
<td>-0.2 ± 0.5</td>
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Data expressed as mean ± SEM. * p < 0.04 and ** p < 0.03, Mann-Whitney test.
Fig. 2. IgG extravasation, aquaporin-4 expression and cellular localization of NFAT5 in the brain after tMCAO. 

a) Representative micrographs with the IgG staining in NFAT5+/+ (i, ii) and NFAT5+/− (iii, iv) brain section after tMCAO. Similar IgG staining was detected in cerebral blood vessels (black arrows) in the contralateral side of the NFAT5+/+ and NFAT5+/− hemisphere (i and iii). IgG staining was induced in the area of ischemic core of the NFAT5+/+ and NFAT5+/− ipsilateral hemisphere (ii and iv). Increased IgG leakage (black arrows) in this area was detected in the NFAT5+/− ipsilateral hemisphere (iv) compared to the NFAT5+/+ ipsilateral hemisphere (ii). Con = Contralateral hemisphere; Ip = ipsilateral hemisphere. Scale bar = 100 µm. n = 5. The histogram on the right shows the quantification of the relative IgG staining intensities in the brain sections; *** p < 0.01 Mann Whitney test.

b) Representative micrographs showing the immunostaining of aquaporin-4 (AQP-4) in NFAT5+/+ (i–iii) and NFAT5+/− (iv–vi) brain after tMCAO. Contralateral hemispheres of NFAT5+/+ brain (i) and NFAT5+/− brain (iv) show low level of AQP-4 expression in astrocytic end-feet in contact with cerebral vessels (black arrows). AQP-4 staining was much higher in the peri-infract area (ii and v; black arrows). AQP-4 staining was nearly abolished in Ip of NFAT5+/+, whereas intensity of AQP-4 staining in the similar area shows still strong expression in the NFAT5+/− hemisphere (iii and vi). Con = Contralateral hemisphere; Ip = ipsilateral hemisphere; Pi = peri-infract (n = 5). Scale bar = 100 µm.

c) Representative photomicrographs from immunocytochemical analysis using antibody against NFAT5. NFAT5 expression was mainly localized in cytoplasm of neurons in the contralateral side (Con) of wild-type brain (i, black arrows), whereas NFAT5 were found in the nuclei of neurons in the ipsilateral side (Ip) of brain after tMCAO (ii, black arrows). n = 4. Scale bar = 50 µm.
increased about 3-fold in H/I condition (fig. 3d). This indicated that the transactivating activity of NFAT5 was significantly increased during H/I treatment. In line with increased protein synthesis and nuclear translocation of NFAT5, a trend of induction of NFAT5 transcriptional activity from 0 to 3 h was also observed. The present results demonstrated that ischemia contributed to the activation of NFAT5 through upregulation of protein synthesis, nuclear translocation and increased transcriptional activity of NFAT5.
NFAT5 Deficiency Increased the Severity of Neuronal Death and Apoptotic Cells in the H/I Condition

Previous results demonstrated that, under H/I, there was an increase in transactivation activity in the neuronal cells. Next, we would like to investigate whether NFAT5 is involved in neuronal death in the H/I condition. Cortical neurons from NFAT5-deficient mice at E14.5d were isolated and challenged with 3-hour H/I condition. The media were collected for LDH measurement, which indicated the neuronal cell death. Under normal condition, no significant difference in mortality rate was observed between the NFAT5+/+ and NFAT5–/– neurons (data not shown). Whereas under the 3-hour H/I challenge, the LDH results demonstrated that NFAT5–/– neurons showed a significantly higher cell death than their wild-type counterparts (fig. 4a). This suggested that under H/I condition, NFAT5 deficiency would be detrimental to neuronal cells, and therefore, NFAT5 is important for the survival of neuronal cells under H/I condition.

Activation of NFAT5 is important for the survival of neurons against ischemic insult. To examine if NFAT5 deficiency neurons are more susceptible to cell death and undergo apoptosis in H/I condition, apoptosis-related cell death in neurons was investigated by TUNEL staining. Under N condition, no positive TUNEL staining was observed in both the NFAT5+/+ or the NFAT5–/– primary cortical neurons (data not shown). After the 3-hour H/I-treated neurons, increased TUNEL-positive staining was observed in both the NFAT5+/+ and the NFAT5–/– neurons (fig. 4biii, v and viii, respectively). Qualitative analysis showed that a significantly larger number of NFAT5–/– neurons had undergone apoptosis (NFAT5+/+, 21.07% ± 2.248%; NFAT5–/–, 38.31% ± 4.046%; p < 0.05, Student’s t-test) than that in the NFAT5+/+ neurons (fig. 4c). This result further confirmed that NFAT5 is important in neuronal survival and deficiency of NFAT5 would be harmful to neurons and enhance neuronal apoptosis during the H/I insult.

Regulation of Ischemia-Induced Genes by NFAT5

The activation of NFAT5 is essential in protecting the neurons against H/I injury. It is possible that NFAT5 protects the neurons through regulating its transcriptional program against the H/I stress. The mRNA abundance of several genes that are known to be transcriptional downstream targets of NFAT5, namely AR, SMIT, TauT and HSP70 were therefore examined in NFAT5+/+, NFAT5–/– and NFAT5–/– neurons under 3-hour N condition or H/I condition by real-time PCR (fig. 5). The AR mRNA level was not significantly different between genotypes, but a trend of decrease in AR mRNA abundance was detected in NFAT5+/+ and NFAT5–/– neurons under the N condition. At 3-hour H/I condition, the AR mRNA level was slightly increased while a significant upregulation was observed in NFAT5–/– neurons detected when compared to its N condition (fig. 5a). Similarly, no significant difference of the HSP70 mRNA level was detected in NFAT5+/+, NFAT5+/+ and NFAT5–/– neurons under N condition, but a significantly increased HSP70 mRNA level was observed in NFAT5+/+ neurons under H/I condition (fig. 5b). In addition, the SMIT mRNA level was significantly downregulated in NFAT5+/+ and NFAT5–/– neurons under N condition (fig. 5c). More importantly, a significant increase of SMIT mRNA level was detected in NFAT5+/+ neurons under H/I condition. However, such an induction was not observed in NFAT5+/+ and NFAT5–/– neurons. The level of TauT mRNA was significantly reduced in NFAT5+/+ and NFAT5–/– neurons when compared to that of NFAT5+/+ and NFAT5–/– neurons under the N condition (fig. 5d). Under H/I condition, TauT mRNA expression was significantly downregulated in NFAT5+/+ neurons while the level of TauT was not altered in NFAT5+/+ and NFAT5–/– neurons. Comparing the TauT mRNA abundance under H/I condition, the NFAT5+/+ neurons still showed a higher mRNA level than the NFAT5+/+ and NFAT5–/– neurons. Generally, there was a trend of decreased expression of the NFAT5 target gene, such as AR and HSP70, and significant decreases of SMIT and TauT expression under N condition in NFAT5+/+ and NFAT5–/– neurons. Significant increases of both AR and HSP 70 expression were observed in NFAT5–/– neurons under H/I condition. However, it is still not clear whether the induction of these genes is activated through the NFAT5 directly under H/I condition. The TauT mRNA level was decreased under ischemia suggesting that the transcription of TauT may not regulated by NFAT5 under ischemia. According to the present finding, it is likely that the expression of SMIT was regulated by NFAT5 under hypoxic/ischemic injury.

NFAT5-Deficient Neurons Were More Susceptible to Oxidative Stress-Induced Neurotoxicity

Stress-induced proteins, AR and HSP70, were upregulated in NFAT5–/– neurons after 3-hour H/I condition. The intracellular oxidative stress was monitored by measuring the changes in fluorescence resulting from oxidation of intracellular probe DHE. The NFAT5 primary cortical neurons were isolated and challenged
with 3-hour H/I condition. The H/I injury caused a rapid and significant accumulation of ROS compared with normoxic condition (data not shown). As shown in figure 6a, a significant increase of ROS was found in NFAT5+/– and NFAT5–/– neurons when compared to the NFAT5+/+ neurons (*p < 0.05 and **p < 0.001, respectively). The result showed that H/I-induced ROS generation is in an NFAT5-dependent manner. To examine

Fig. 4. More LDH release and increased apoptosis were observed in NFAT5–/– primary cortical neurons after 3-hour H/I condition. a The cortical neurons were isolated from the brain of 14.5 days NFAT5+/+, NFAT5+/–, NFAT5–/– embryonic mice. After 5 days, the neuronal culture was subjected to 3 h for H/I, the medium was collected for LDH measurement. The percentage of cell death in the NFAT5+/+ and NFAT5–/– neurons were significantly higher than in the NFAT5+/– neurons under H/I condition. Data are presented as mean ± SEM (NFAT5+/+, 99.8% ± 2.8%; NFAT5+/–, 125.8% ± 5.1%; NFAT5–/–, 137.2% ± 10.9%, **p < 0.01, ***p < 0.001 by one-way ANOVA). b Photomicrograph showing the TUNEL-positive cells (green) (ii, v, viii). DAPI stains the nucleus (blue) (i, iv, vii). Scale bar = 20 μm. c Histogram showing the percentage of apoptotic cells in NFAT5+/+, NFAT5+/– and NFAT5–/– neurons (TUNEL-positive cells (apoptotic cells)/DAPI-positive cells (total cells) × 100). Data are presented as mean ± SEM (NFAT5+/+, 21.07% ± 2.25%; NFAT5+/–, 31.62% ± 2.53; NFAT5–/–, 38.31% ± 4.05%; *p < 0.05, Student’s t test).
NFAT5 Protects Brain against Ischemic Injury

**Fig. 5.** Real-time PCR analysis of genes downstream to NFAT5 in neurons. The RNA was extracted from primary cortical neurons isolated from brain of E14.5d embryos from NFAT5+/+, NFAT5+/– and NFAT5–/– mice and subjected to 3 h for N or H/I. Representative histogram showing the relative mRNA abundance of AR (a), HSP70 (b), SMIT (c), and TauT (d) in NFAT5+/+, NFAT5+/– and NFAT5–/– neurons with N or H/I condition. A significant increase of AR mRNA level was detected in NFAT5–/– neurons under H/I condition (N vs. H/I, 0.63 ± 0.33 vs. 2.18 ± 0.45). A significant increase of HSP70 mRNA level was observed in NFAT5–/– neurons under H/I condition (N vs. H/I condition, 0.72 ± 0.33 vs. 2.64 ± 0.65). A significant decrease in SMIT mRNA level was found in NFAT5+/– and NFAT5–/– neurons under N condition (NFAT5+/+ vs. NFAT5+/– and NFAT5–/–, 1.00 ± 0.004 vs. 0.3 ± 0.1 and 0.42 ± 0.18). A significant upregulation of SMIT mRNA level was detected in NFAT5+/+ neurons under H/I condition (N vs. H/I condition, 1.000 ± 0.004 vs. 2.9 ± 0.7). No induction of SMIT mRNA was observed in NFAT5+/+ and NFAT5–/– neurons under H/I. A significant decrease in TauT mRNA level was significantly downregulated in NFAT5+/+ and NFAT5–/– neurons compared to NFAT5+/+ neurons under N condition (NFAT5+/+ vs. NFAT5+/– and NFAT5–/–, 1.000 ± 0.004 vs. 0.43 ± 0.12 and 0.51 ± 0.21). A significant decrease in TauT mRNA level was detected in NFAT5+/+ neurons under H/I condition (N vs. H/I condition, 1.000 ± 0.004 vs. 0.57 ± 0.17). Data are presented as mean ± SEM. * p < 0.05, ** p < 0.01, by one-way ANOVA, n = 4.

the protective role of NFAT5 against oxidative stress-induced neurotoxicity in H/I condition, NFAT5 primary cortical neurons were treated with 100 μM H$_2$O$_2$ for 3 h. The media were collected for LDH measurement, which indicates the neuronal cell viability. Figure 6b showed that a significantly higher LDH was released in NFAT5+/+ and NFAT5–/– neurons than that of NFAT5+/+ neurons. It implied that NFAT5-deficient neurons were more susceptible to H$_2$O$_2$-induced neuronal cell death.
Fig. 6. Increased ROS generation during 3 h H/I condition and more LDH released were observed after 3 h H2O2 treatment in NFAT5+/– primary cortical neurons. a The cortical neurons were isolated from the NFAT5+/+, NFAT5+/–, NFAT5–/– mice at embryonic day E14.5. After 5 days, the neuronal culture was subjected to 3-hour H/I condition, the ROS production was measured by the DHE fluorescence intensity. The percentage of ROS generation in the NFAT5+/+ and NFAT5+/– neurons was significantly higher than in the NFAT5–/– neurons under H/I condition. Data are presented as mean ± SEM (NFAT5+/+, 100% ± 18.15%; NFAT5+/–, 177.6% ± 18.4%; NFAT5–/–, 356.9% ± 24.01%; *p < 0.05; ***p < 0.001 by one-way ANOVA). b The cortical neurons were isolated from the NFAT5+/+, NFAT5+/–, NFAT5–/– mice at embryonic day E14.5. After 5 days, the neuronal culture was subjected to 3 h for 100 μM H2O2, the medium was collected for LDH measurement. The percentage of cell death in the NFAT5+/+ and NFAT5+/– neurons was significantly higher than in the NFAT5–/– neurons under H/I condition. Data are presented as mean ± SEM (NFAT5+/+, 99.97% ± 5.316%; NFAT5+/–, 122.6% ± 6.057%; NFAT5–/–, 148.7% ± 15.23%; *p < 0.05; **p < 0.01 by one-way ANOVA).

Discussion

Edema causes a substantial brain swelling and damage [27]. In line with the present study, NFAT5+/– mice showed more severe neurological deficits and more BBB breakdown in their brains associated with increased brain swelling and larger infarct size after tMCAO.

AQP-4, a water channel protein in the brain, is important in brain volume homeostasis, and involves in BBB breakdown, hemispheric swelling and brain edema formation. AQP-4 is highly expressed in astrocytes especially in astrocytic processes that are in contact with capillaries [28]. Under ischemic condition, cerebral swelling correlates with AQP-4 upregulation [29]. Besides, AQP-4 expression in astrocytic end-feet contributes to BBB disruption and brain swelling during brain injury [19, 30]. In AQP-4 knockout mice, they showed a reduced brain swelling after acute water intoxication and ischemic stroke [31]. Primary astrocytes, which are isolated from AQP-4-deficient mice, show decreased water permeability, suggesting the role of astrocytic AQP-4 in water transport in the brain [32]. The increased AQP-4 expression in the astrocytic end-feet in NFAT5+/– brains after tMCAO contributed to the increase of hemispheric brain swelling and water accumulation, and this is in line with the elevated BBB disruption and astrocytic hypertrophy in other studies [19, 30]. However, AQP-4 also plays a role in eliminating excess water in vasogenic edema. AQP-4 is therefore required for the compensatory response to fluid accumulation and edema formation [33]. In the renal system, TonEBP/NFAT5 involves in water homeostasis by regulating the expression of water channel protein AQPI [34]. In the present study, NFAT5+/– brains showed an increase of water content after tMCAO, the upregulation of AQP-4 expression may responsible for removing excess fluid in the brain parenchyma and restore the water homeostasis back to the normal. NFAT5 may play a neuroprotective role against cerebral edema.

During cerebral ischemia, there is a change of cellular osmolarity in the brain [35]. Neurons undergo intracellular hyperosmotic shock due to the failure of Na+/K+ ATPase pump [35]. This subsequently leads to influx of ions into the cells and increase the intracellular ionic strength, resulting in a rapid swelling and cytotoxic edema formation [35]. The change of intracellular ionic strength triggers the activation of NFAT5 [36], thus increases the compatible osmolytes [38–40] and induces the molecular chaperone [13] to normalize the intracellular volume and the ionic strength, thus to maintain the proper protein folding and optimize the intracellular environment. NFAT5 may function in monitoring and adjusting the intracellular environment by biosynthesis of macromolecules in response to...
the stress [37]. In the in vitro H/I study, the neurons were cultured in isotonic medium. The transcriptional activity of NFAT5, therefore, was due to the intracellular hypertonic shock induced by ischemia rather than the change of external tonicity. Since the downregulation of NFAT5 would lead to a reduced transcription of its target genes [36, 41], AR, TauT and SMIT mRNA levels, these downstream targets are possibly regulated by NFAT5.

The SMIT is induced after cerebral ischemia and neuronal injury [16, 42]. Under H/I injury, SMIT is likely regulated by NFAT5. SMIT mRNA was induced in NFAT5+/+, but not in NFAT5−/−, neurons after 3-hour H/I condition, suggesting NFAT5-dependent transcription of SMIT under ischemia. Ischemia-induced influx of Na+ and Ca2+ together with water into the neurons results in altering the solute and water transport. This markedly disrupts cellular ionic strength and osmotic homeostasis. Myo-inositol (MI) may play an important role to protect the cells against cytotoxicity induced by high intracellular electrolyte concentrations. Previous study showed that veratridine, a neurotoxin that activates voltage-gated Na+ channels, causes cytotoxicity by accumulating Na+ ion and also induces SMIT mRNA level in neurons. Induction of MI plays a protective role against veratridine-induced neuronal cytotoxicity [43]. Therefore, it is speculated that disturbance of ionic strength after ischemia induces activation of NFAT5 and increases the transcription of SMIT. SMIT transports MI in exchange of sodium against a high intracellular ionic strength. Since MI plays a role in osmoregulation, it prevents the neuronal swelling from high intracellular Na+ and Ca2+ ions. The upregulation of SMIT protects neurons against cellular toxicity induced by high electrolyte concentration.

Hypertonicity induces ROS generation in renal epithelial cells [44]. The failure of Na+/K+ ATPase pump after ischemia leads to increase in intracellular ionic strength. It is plausible that intracellular electrolyte concentration contributes to ROS production. The increase of AR mRNA in NFAT5−/− neurons under H/I condition suggests the AR expression is not regulated by NFAT5-dependent transcription. Since a promoter region of the AR gene contains not only the osmotic response element (ORE), but also the activator protein-1 (AP-1) site and two antioxidant response element (ARE)s [45], AR may, therefore, respond to multiple stresses. AR is transcriptionally regulated and activated by a variety of stimuli including ROS generated in ischemia [46–48], AR can be designated as an oxidative stress-inducible protein. This is in line with the increased expression of AR in NFAT5−/− neurons under H/I condition. Also, increased AR activity contributes to oxidative stress in the pathogenesis of cerebral ischemia [15, 47]. The level of AR acts as an indicator of oxidative stress; therefore, the upregulation of AR is associated with a significant increase of ROS in NFAT5+/+ and NFAT5−/− neurons in H/I condition. This suggests that neuronal cells with NFAT5 deficiency will suffer from greater oxidative damage, and therefore NFAT5+/+ and NFAT5−/− neurons showed higher mortality than that of NFAT5+/+ in H2O2-induced neurotoxicity. This may be due to increased AR expression and intracellular ROS as observed in NFAT5+/+ and NFAT5−/− neurons in H/I injury. The present results are also in agreement with other studies that increased ROS activates the osmoregulatory transcription factor NFAT5/TonEBP/OREBP [44, 48] and deficiency of NFAT5 transcription would exacerbate the neuronal cells under pathological conditions. NFAT5 may protect neurons against oxidative stress under ischemic condition.

HSP70 functions as a molecular chaperone or antiapoptotic molecule which is substantially induced in cells under stresses [49]. It has been shown that HSP70 is regulated by NFAT5 [50]. In the present study, the HSP70 mRNA was induced in neurons under H/I condition. When compared with the NFAT5+/+ neurons, higher HSP70 expressions were observed in NFAT5+/− and NFAT5−/− neurons, suggesting that the transcription of HSP70 under H/I is independent of NFAT5 in neuronal cells which is different from other cell types [51, 52]. Since the stress-induced heat shock factor can also mediate the transcription of HSP70 [53], therefore, the stress-inducible molecular chaperone could be regulated by transcription factor other than NFAT5 under H/I injury. NFAT5+/− and NFAT5−/− neurons were more susceptible to ischemic injury with a higher HSP70 expression level which might be resulted from biosynthesis of macromolecules in response to the ischemic stress. It is speculated that increased neuronal damage leads to more molecular chaperones for proper protein folding in NFAT5−/− neurons to cope with neurodegeneration.

The TauT mRNA was downregulated in neurons under in vitro H/I condition, which is in agreement with the observation of in vivo experimental stroke model (data not shown). The immunostaining signals of TauT were abolished in the region of ischemic core. During ischemia, cellular swelling may trigger the efflux of amino acids. Studies showed that ischemia-evoked release of amino acid is mediated by Na+-dependent transporters. A depolarization-induced reversal of the Na+-dependent amino acid plasma membrane transporter makes a substantial contribution to the efflux of amino acids [54–56].
Ischemia causes a rapid decline in ATP levels and fails the Na⁺/K⁺ pump. This leads to the increase of K⁺ extracellularly and decrease of Na⁺, the cells become depolarized. The reduced Na⁺ gradient would be expected to facilitate ischemia-evoked transporter-mediated release of amino acids. Taurine uptake into cells is also mediated by a Na⁺-dependent transporter [57], which operates in a reversed manner when the normal transmembrane ionic gradients is disturbed under H/I conditions. Like in taurine, which has a higher intracellular to extracellular concentration gradient in a normal state, the transporter-mediated efflux can cause a dramatic increase of extracellular taurine during ischemic condition. And this ischemia-evoked taurine release has been suggested to be neuroprotective [58]. The release of taurine, due to an excess of excitatory amino acids, may prevent excitotoxicity in neurons and a higher level of taurine allows the cells to have a greater tolerance to hypoxia [59]. Taurine also enhances Cl⁻ conductivity which reduces cell excitability [60]. Moreover, taurine inhibits the intracellular Ca²⁺ uptake elicited by NMDA [61]. Therefore, the increased taurine level is required for a proper homeostasis in neurons upon hyperexcitation. The downregulated-TauT expression in NFAT5⁻/⁻ neurons under normal and H/I condition may lead to a reduced TauT-mediated taurine efflux and to more susceptibility to H/I injury.

In conclusion, NFAT5⁻/⁻ and NFAT5⁻/⁻ neurons showed more severe damages under H/I condition resulting from the downregulation of neuroprotective genes (SMIT and TauT) and upregulation of cytotoxic gene (AR) transcriptions. Taken together, NFAT5 is not only essential for neuronal adaptation to the hypertonic condition but also plays an important role in regulating the transcription of genes that restore the neuronal cells back to a normal environment, and therefore protect the cells from ischemic injury.

Acknowledgement

This work was supported by the Research Grant Council Grants General Research Fund HKU to Professor S.K. Chung 7504/06M and the Area of Excellence from University Grants Council of Hong Kong on Molecular Neuroscience (AoE/B-15/01).

Disclosure Statement

The authors declare no conflict of interest.

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